

**POSITIVE REGULATION OF PKB/AKT KINASE ACTIVITY BY THE
VACUOLAR (H⁺)-ATPASE IN THE CANONICAL INSULIN SIGNALING
PATHWAY: IMPLICATIONS FOR THE TARGETED PHARMOCATHERAPY
OF CANCER.**

By

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**A thesis submitted in conformity with the requirements for the degree of
Doctor of Philosophy
Medical Biophysics Graduate Department
University of Toronto**

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Positive regulation of PKB/Akt kinase activity by the vacuolar (H⁺)-ATPase in the canonical insulin signaling pathway: implications for the targeted pharmacotherapy of cancer.

Sevag Kaladchibachi, Doctor of Philosophy, 2014

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Abstract

The canonical PI3K/Akt pathway is activated downstream of numerous receptor tyrosine kinases, including the insulin and insulin-like growth factor receptors, and is a crucial regulator of growth and survival in metazoans. The deregulation of Akt is implicated in the pathogenesis of numerous diseases including cancer, making the identification of modifiers of its activity of high chemotherapeutic interest. In a transheterozygous genetic screen for modifiers of embryonic Akt function in *Drosophila*, in which the PI3K/Akt signaling pathway is conserved, we identified the A subunit of the vacuolar ATPase (Vha68-2) as a positive regulator of Dakt function. Our characterization of this genetic interaction in the larval stage of development revealed that *Vha68-2* mutant phenotypes stereotypically mimicked the growth defects observed in mutants of the *Drosophila* insulin signaling pathway (ISP). The loss of *Vha68-2* function, like *Dakt*-deficiency, was found to result in organismal and cell-autonomous growth defects, and consistent with its putative role as a positive regulator of Dakt function, both the mutational and pharmacological inhibition of its activity were found to downregulate Akt activation. Genetic epistasis experiments in somatic clones of Vha68-2/dPTEN double mutants demonstrated that the loss of Vha68-2 function suppressed the growth defects

associated with dPTEN-deficiency, placing Vha68-2 activity downstream of dPTEN in the ISP, while the examination of PI3K activity and PH domain-dependent membrane recruitment in pharmacologically inhibited larval tissues further placed Vha68-2 function downstream of PI3K. These findings were recapitulated in cultured NIH-3T3 cells, whose treatment with bafilomycin A1, a potent and specific inhibitor of V-ATPase, resulted in the downregulation of Akt phosphorylation, particularly in non-cytoplasmic intracellular compartments. Furthermore, cellular subfractionation of bafilomycin-treated NIH-3T3 cells demonstrated a decrease in the localization of Akt to early endocytic structures, and a downregulation in the localization and activation of Akt in the nuclei of both *Drosophila* and mammalian cells. Finally, the pharmacotherapeutic relevance of V-ATPase inhibition was addressed in two tumor models – multiple myeloma and glioblastoma – and our preliminary findings in these cancers, which are often associated with ectopic PI3K/Akt signaling, showed significant cytotoxic efficacy *in vitro*, warranting its consideration as a tractable pharmacological option in the treatment of cancer.

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List of Abbreviations

AA: amino acid
ADP: adenosine diphosphate
AMP: adenosine monophosphate
AMPK: AMP-activated protein kinase
ATP: adenosine triphosphate
BAD: Bcl-2 associated death promoter
Bcl-2: B cell lymphoma 2
BDNF: brain-derived neurotrophic factor
BH3: Bcl-2 homology 3
cAMP: cyclic adenosine monophosphate
CCV: clathrin-coated vesicles
CIE: clathrin-independent endocytosis
CME: clathrin-mediated endocytosis
CREB: cAMP response element-binding protein
CSR: class switch recombination
DIR: *Drosophila* insulin receptor
EEA1: early endosome antigen 1
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
ER: endoplasmic reticulum
ERT: endoreplicating tissue
FBS: foetal bovine serum
FGF: fibroblast growth factor
FGFR: fibroblast growth factor receptor
FoxO: forkhead box subgroup O
GAP: GTPase activating protein
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GBM: glioblastoma multiforme
GEF: guanine nucleotide exchange factor

GF: growth factor
GFP: green fluorescent protein
GLC: germline clone
GPCR: G protein coupled receptor
GSK3: glycogen synthase kinase 3
GTP: guanosine triphosphate
HA: hemagglutinin
HM: hydrophobic motif
IGF: insulin-like growth factor
IGFR: insulin-like growth factor receptor
IGH: immunoglobulin heavy chain
IR: insulin receptor
IRS: insulin receptor substrate
ISP: insulin signaling pathway
JNK: c-Jun N-terminal kinase
MAPK: mitogen-activated protein kinase
MDM2: murine double minute 2
MEF: mouse embryonic fibroblast
MM: multiple myeloma
MNC: mononuclear cell
MOMP: mitochondrial outer membrane permeabilization
mTORC: mechanistic target of rapamycin complex
MVB: multivesicular body
NGF: nerve growth factor
PDGF: platelet-derived growth factor
PDGFR: platelet-derived growth factor receptor
PDK1: phosphoinositide-dependent kinase 1
PH: pleckstrin homology
PI: phosphatidylinositol
PI3K: phosphatidylinositol-3-kinase
PIP: phosphatidylinositol phosphate

PKA: protein kinase A
PKB: protein kinase B/Akt
PKC: protein kinase C
PM: plasma membrane
PP2A: protein phosphatase 2A
PTEN: phosphatase and tensin homolog
pY: phosphotyrosine
RTK: receptor tyrosine kinase
SAPK: stress-activated protein kinase
S6K: S6 kinase
SG: salivary gland
SGK: serum-and glucocorticoid-induced protein kinase
SH2: Src homology 2
TNF: tumor necrosis factor
TOR: target of rapamycin
TSC: tuberous sclerosis complex
V-ATPase: vacuolar ATPase

List of publications

***Kaladchibachi SA*, Doble B, Anthopoulos N, Woodgett JR, Manoukian AS: **Glycogen synthase kinase 3, circadian rhythms, and bipolar disorder: a molecular link in the therapeutic action of lithium.** J Circadian Rhythms 2007, 5:3.**

***Kaladchibachi SA*, Scanga SE, Manoukian AS: **Genetic characterization of Vha68-2 as a cell-autonomously acting positive regulator of Akt signaling in *Drosophila*.** Manuscript in preparation.**

***Kaladchibachi SA*, Scanga SE, Manoukian AS: **Bafilomycin downregulates Akt activation and endomembrane localization in cultured NIH-3T3 fibroblasts.** Manuscript in preparation.**

CHAPTER 1

GENERAL INTRODUCTION

Prologue

Over the course of 3.5 billion years, the evolutionary timeline of life on Earth has been characterized by remarkable increases in the size, complexity, and diversity of organisms. The prokaryotic progenitors of subsequently evolved unicellular eukaryotes (protozoans) and primitive multicellular metazoans possessed relatively simple body plans and metabolic networks. The occurrence of major transitions in form, function, and organization, resulting in the appearance the simple ancestral protozoan eukaryotes ~1.5 billions years ago, and thereafter, that of the earliest multicellular metazoans approximately 1 billion years ago, produced the diverse contemporary array of organisms, ranging from microscopic and relatively simple unicellular prokaryotes to large and comparatively complex animals and plants consisting of multiple specialized subcellular organelles, cells, tissues, and organs (**Conway Morris 1993, Maynard Smith and Szathmáry 1997**).

The diverse range of evolutionary novelties and the increasing complexity of morphologies and physiologies resulting from the transition to multicellularity also posed commensurate novel challenges to the earliest organisms bearing this trait with respect to cell-cell adhesion, communication, differentiation and specialized function. Most of the crucial regulatory genes and signaling molecules deployed during the development of multicellular animals are widely conserved across metazoans, and the current hypothesis of early metazoan origins posits that these multicellular organisms already possessed a substantial part of the genetic “tool-kit” required for the establishment and maintenance of a multicellular body plan (**King 2004, Nichols et al 2006, Ruiz-Trillo et al 2007**), including ancestral homologues of various receptor tyrosine kinase classes (**Diagram 1-1**), whose ligand-dependent activation is largely responsible for the coordination of multicellular function in metazoans. Whereas the fossil record documents the abrupt appearance of fully diversified eumetazoan body plans during the burst in diversification known as the “Cambrian explosion” occurring between 570 and 540 Mya, the fossil evidence of diploblastic sponges (Porifera), one of the earliest branching and morphologically simplest animal phyla, date further back to the Precambrian (between

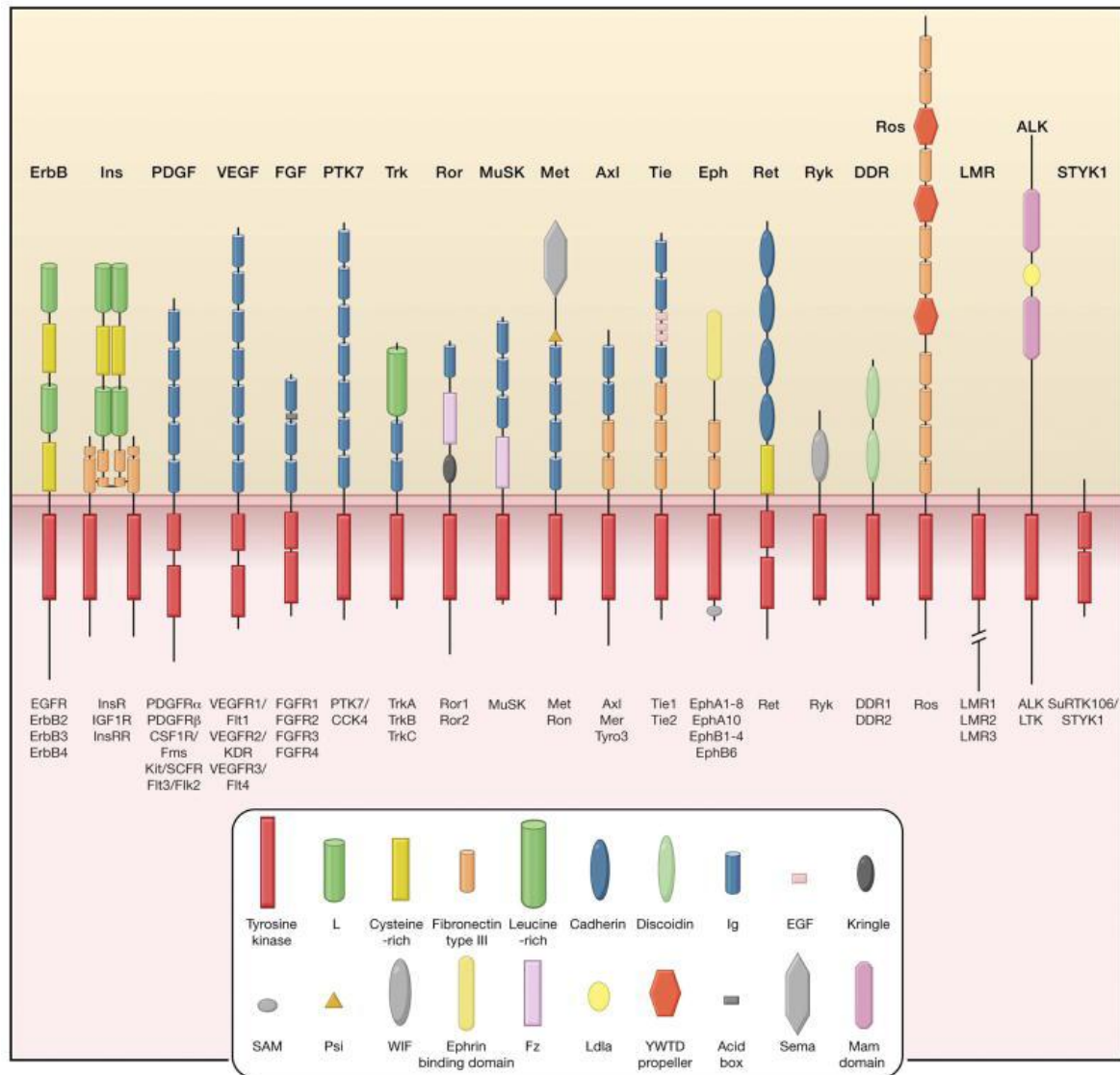


Diagram 1-1. Receptor tyrosine kinase families. (A) Reproduced from **Lemmon and Schlessinger (2010)**. Human receptor tyrosine kinases (RTKs) contain 20 subfamilies, shown here schematically with the family members listed beneath each receptor. The intracellular (tyrosine kinase) domains are shown as red rectangles, while the various extracellular domains that contribute to ligand specificity are graphically depicted for each of the subfamilies.

580-600 Mya) and reveal a relatively simple body plan that has since remained nearly unchanged (**Valentine et al 1996, Li et al 1998a**). Porifera were suggested to share a common ancestor with early metazoan phyla based on the conservation of numerous peptide sequences coding for crucial cell surface molecules required for multicellularity, such as transmembrane tyrosine kinase receptors (**Müller and Schäcke 1996**), transmembrane adhesion molecules including integrins (**Pancer et al 1997**), and G-protein-linked transmembrane molecules such as the glutamate/GABA-like receptor (**Perovic et al 1999**). In the poriferan marine sponge *Geodia cydonium*, a gene encoding a receptor tyrosine kinase (RTK) homologous to class II RTKs (the insulin receptor family) was identified (**Schäcke et al 1994**), and more recently, expressed sequence tag (EST) analysis of the marine sponge *Oscarella carmela* has revealed the presence of genes encoding core components of most of the main animal cell signaling pathways traditionally thought to be exclusive to metazoans - Wnt, transforming growth factor β (TGF- β), numerous receptor tyrosine kinases (including epidermal, insulin-like, and fibroblast growth factor receptors, see [Diagram 1-1](#)), Notch, Hedgehog, and Jak/Stat signaling pathways; as well as homologs of nearly all eumetazoan cell adhesion gene families, including those encoding cell surface receptors, cytoplasmic linkers, and extracellular-matrix proteins (**Nichols et al 2006**). Consistent with these observations made in Poriferans, the diploblastic phylum Cnidaria, which includes Anthozoan (sea anemones and corals) and Hydrazoan (hydra) classes, are also living fossils that, like Porifera, branch deeply within Metazoa in the Precambrian, approximately 600 Mya. Interestingly, among Cnidarians, genes encoding an insulin receptor-like RTK (*HTK7* in *Hydra vulgaris*) and a fibroblast growth factor receptor-like RTK (*kringelchen* in *Hydra vulgaris*; *NvFGFRa* and *NvFGFRb* in the sea anemone *Nematostella vectensis*) have also been identified (**Steele et al 1996, Sudhop et al 2004, Matus et al 2007, Rentzsch et al 2008**), as well as coding sequences for a set of Hox-like genes (*anthox2* and *anthox6*) in *Nematostella vectensis* that regulate early axial patterning (**Finnerty and Martindale 1999**), consistent with a model for RTK-mediated signaling that precedes metazoan evolution.

Proceeding further back in eukaryotic evolution, estimates for the divergence between fungi and metazoans range from 1,000-1,600 Mya (**Douzery et al 2004**,

Heckman et al 2001), and premetazoan model organisms such as the budding yeast *Saccharomyces cerevisiae*, the filamentous fungus *Neurospora crassa*, the fission yeast *Schizosaccharomyces pombe*, and the slime mold *Dictyostelium discoideum* encode protein kinase complements (kinomes) that mediate what are essentially unicellular-specific functions (**Pincus et al 2008**), with a limited capacity for intercellular communication and coordination that only permits an aggregative form of multicellularity. As shown in [Diagram 1-2](#), these premetazoan organisms typically lack the metazoan-specific arsenal of tyrosine kinases, phosphatases, and phosphotyrosine-binding proteins required for signaling efficiency in, and sophisticated control of, the development, differentiation, and intercellular communication of multicellular organisms (**Darnell 1997, Manning et al 2002a**). Interestingly, numerous genes closely associated with multicellularity were found to be present in the nearest known relatives of animals to date – choanoflagellates, which are unicellular, colonial, flagellated protozoans whose divergence from animals is estimated at ~900 Mya. EST studies in the marine choanoflagellate *Monosiga brevicollis* demonstrated the presence of a full complement of non-metazoan homologs of animal genes involved in cell signaling and cell adhesion, including protein tyrosine kinases, their signaling components, and cadherins (**King and Carroll 2001, King et al 2003, King et al 2008, Pincus et al 2008**). The possession in choanoflagellates of a tyrosine signaling network as elaborate and diverse as those found in any known metazoans (**Manning et al 2008**) placed the evolution of this protozoan system closest to the advent of true (non-aggregative) metazoan multicellularity, temporally preceding the subsequent onset of cell-surface RTKs as the dominant mechanism of relaying extracellular signals to the cellular interior, and as a mode of intercellular communication ubiquitously conserved throughout the animal kingdom for the coordination of cell behavior (reviewed in **Mayer 2008**).

In animals, protein tyrosine kinases are generally activated in response to endogenously produced and humorally circulated extracellular growth factors, mediating the regulation of physiological responses including developmental, metabolic, and proliferative processes (reviewed in **Lemmon and Schlessinger 2010**). Although our knowledge of the signal transduction cascades initiated downstream of RTK activation had progressed considerably in the 35 years since the initial discovery of tyrosine

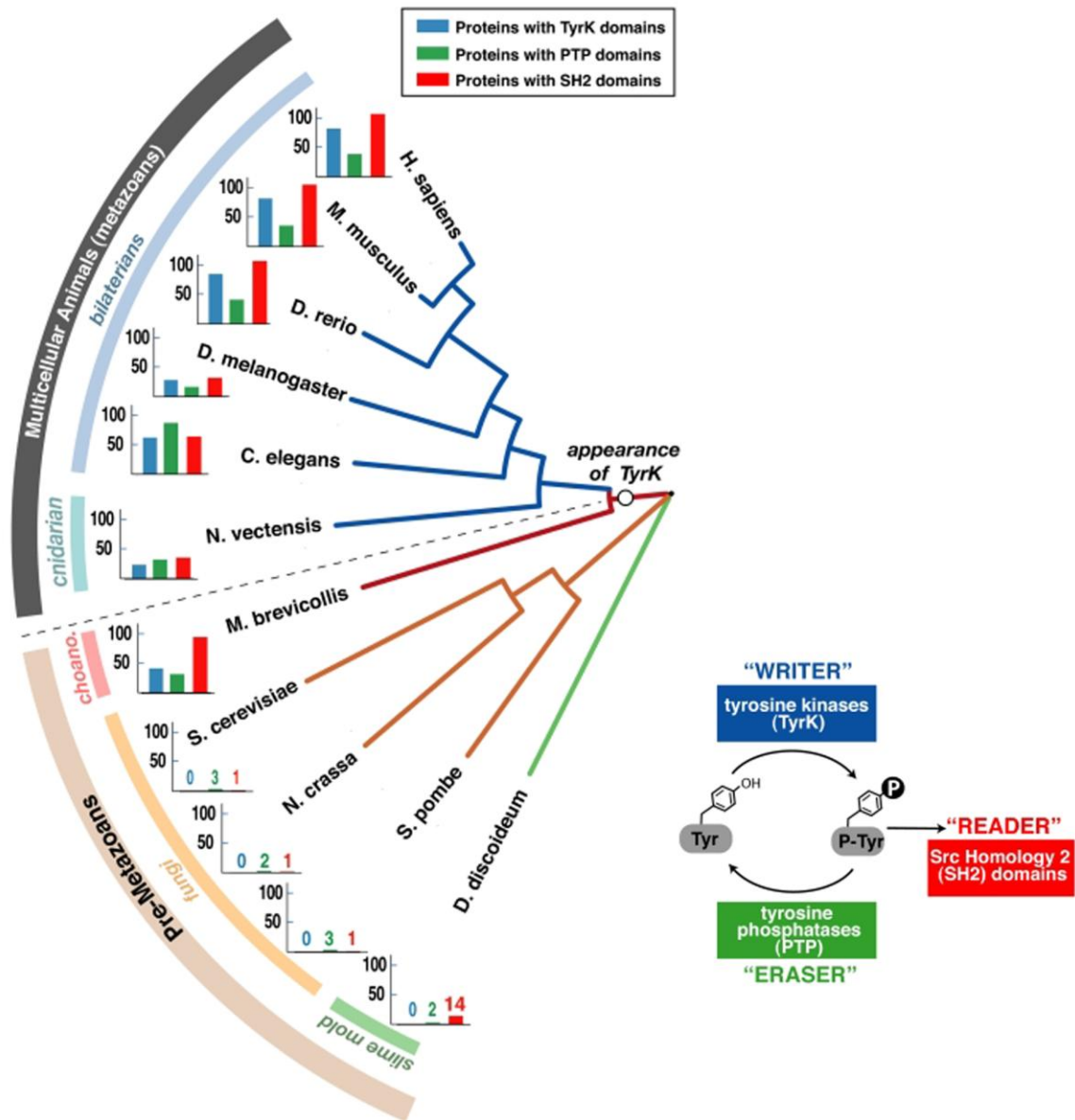


Diagram 1-2. Genomic representation of the phospho-tyrosine signaling machinery in different eukaryotic lineages. Reproduced from Pincus et al (2008). As graphically depicted in the lower right panel, phospho-tyrosine (P-Tyr) signaling systems are built from a three-component system comprised of tyrosine kinase (TyrK, writer), tyrosine phosphatase (PTP, eraser) and Src homology 2 (SH2, reader) domains. The number of proteins containing TyrK, PTP, or SH2 domains throughout the various branches of the metazoan phylogenetic tree are graphically depicted in the panel on the right. Only choanoflagellates and metazoans have high numbers of all three domains. All other premetazoans only have small numbers of PTP and SH2 domain proteins (no TyrK). These data imply an early evolution of PTP and SH2 domains, followed by an expansion in all domains only after invention of the TyrK domain (white circle). Protein numbers are lower-bound estimates as predicted by the SMART domain identification resource.

phosphorylation (**Eckhart et al 1979**), which was followed in 1980 by the characterization of Src as a tyrosine kinase (**Hunter and Sefton 1980**), and subsequently, over the next decade, by the identification of numerous growth factor receptors such as IR and EGFR as molecules possessing this novel catalytic activity (**Avruch et al 1982**, **Yarden and Schlessinger 1987**), much of the signaling events and molecular interactions taking place downstream of RTK activation that elicited the associated cellular response(s) remained, despite important advances, poorly understood by the close of the 1980s. The autophosphorylation of specific tyrosine residues of RTKs, which is the primary biochemical consequence of receptor activation for most RTKs, had however been demonstrated to result in (and be required for) an increase in the phosphorylation of various catalytic molecules either already shown or suspected to be components of RTK-mediated signaling pathways (**Cantley et al 1991** and references therein). This group of catalytically diverse putative RTK signal transducers notably included Src and Src-like non-receptor tyrosine kinases, phospholipase C γ (PLC γ), the regulatory subunit (p85) of phosphatidylinositol-3-kinase, as well as the small GTPase Ras, and members of what would eventually be referred to as the Ras-dependent MAPK^{ERK} signaling cascade, such as the mammalian Ras GTPase-activating protein (p120 RasGAP), a negative regulator of Ras signaling, and the serine/threonine kinase Raf, a positive effector acting directly downstream of Ras..

The 1990s saw a flourish of studies aimed at deciphering these signaling events, including the identification of signaling components within the various pathways activated downstream of RTKs, their regulation and functions in normal cellular physiology, and the contribution of their deregulation in the pathogenesis of various diseases including cancer. The remainder of this introductory chapter is a detailed description of the evolutionarily conserved PI3K-dependent branch of RTK-borne signal transduction, its function as a crucial modulator of cellular growth and survival, and the central role of the Akt kinase in the intracellular propagation of these PI3K-dependent signals; followed by a brief review of vacuolar ATPase (V-ATPase) function as a backdrop to my experiments investigating the positive genetic interaction initially detected by my supervisor, Dr. Armen Manoukian, between V-ATPase and Akt function in *Drosophila*.

1-1 – PIP₃-dependent stimulation of class I PI3K effectors

As a response to cell-surface receptor activation, multiple catalytic activities amplify the physiological signal to be transduced by triggering the production of second messengers such as cyclic AMP (cAMP) and various lipid derivatives; or alternatively, by modulating intracellular levels of signaling ions such as calcium (Ca²⁺). The phosphatidylinositol (PI) lipid molecule and many of its phosphorylated derivatives are present in prokaryotes and primarily involved in the regulation of intracellular traffic, but it is only in eukaryotes that they appear as integral membrane-bound components of receptor-dependent *signal transduction* processes (reviewed in **Di Paolo and De Camilli 2006, Haucke and Di Paolo 2007, Michell 2008**). As shown in [Diagram 1-3](#), PI is the precursor of all its phosphorylated derivatives, and constitutes ~10% of all lipids in eukaryotic membranes (**Rameh and Cantley 1999**). The lipid kinases responsible for the generation of the various phosphorylated derivatives can be broadly classified into three major types (PIPK, PI4K, and PI3K) based the position of the inositol ring that they phosphorylate, and each major class can be further divided into subtypes based on substrate preference (reviewed in **Sasaki et al 2009, Vanhaesebroeck et al 2010**). As the most abundant phosphorylated forms in all eukaryotes, nearly 5% of the cellular PI pool is phosphorylated at the 4-position (D4) of the inositol ring (PI-4-P), with another 5% additionally phosphorylated at D5 (PI-4,5-P₂, henceforth referred to as PIP₂). The PI(4)P lipid moiety, which is involved in the regulation of vesicular traffic, is generated by PI4K activity, and critical for the synthesis of other PI derivatives with greater degrees of phosphorylation, including PIP₂ and PI(3,4,5)P₃ (henceforth referred to as PIP₃). The PIP₂ molecule can be generated by the activity of type I PIPKs, which phosphorylate PI(4)P; and type II PIPKs, which can phosphorylate PI(5)P, the far less abundant catalytic product of the type III PIPK. The PIP₂ molecule is diffusely present in the plasma membrane under basal conditions, and its hydrolysis by PLC in response to GPCR activation likely represents the first primarily non-intracellular traffic-related signaling mechanism involving PIs to appear in eukaryotes, whereby its hydrolysis into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃) leads respectively to PKC

activation, and the initiation of Ca^{2+} -dependent signaling processes (reviewed in **Suh et al 2008**).

In contrast to the relatively high basal abundance of PI(4)P and PIP₂, PIs phosphorylated at D3 make up less than 0.25% of the cellular PI pool, and are generated exclusively through the activity of PI3Ks (**Rameh and Cantley 1999**). The identification of PI3K lipid products by Lewis Cantley and others in the late 1980s (**Whitman et al 1988, Traynor-Kaplan et al 1988**) was followed by their demonstration shortly thereafter that the stimulation of diverse receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) resulted in acute increases of PI3K activity and the rapid synthesis of PIP₃ (which as shown in [Diagram 1-4](#), is the exclusive product of class I PI3Ks) from membrane-bound pools of PIP₂ (**Ruderman et al 1990, Stephens et al 1991, Hawkins et al 1992**); and furthermore, the association of PI3K with oncogenic forms of Ras was shown to stimulate its catalytic activation (**Sjölander et al 1991, Rodriguez-Viciana et al 1994, Kodaki et al 1994, Rodriguez-Viciana et al 1996**). Importantly, these landmark studies focused attention on the role of this novel lipid product in the transduction of extracellular signals, and provided a salient link between PI3K activation and the regulation of cell growth and proliferation in both normal and pathological signaling contexts. Meanwhile, studies published between 1993 and 1994 reported that wortmannin, a metabolite of *Penicillium wortmannin* with anti-inflammatory properties first described in 1974 (**Wiesinger et al 1974**), was a potent, selective, and cell-permeable inhibitor of PI3K (**Arcaro et al 1993, Yano et al 1993, Okada et al 1994, Powis et al 1994, Thelen et al 1994**); and moreover, the contemporaneously reported availability of LY294002 (Eli Lilly), the first synthetic inhibitor of PI3K activity (**Vlahos et al 1994**), provided the initial pharmacological tools with which PI3K function could be investigated. Despite limited applicability *in vivo* due to considerable toxicity in animals (reviewed in **Knight and Shokat 2007**), the use of wortmannin and LY294002 in conjunction with genetic and immunological approaches targeting PI3K activity in cultured cells would allow the investigation of PI3K-dependent cellular functions *in vitro*. These studies would implicate PI3K activation in diverse cellular processes including mitogenesis, glucose uptake, cytoskeletal rearrangement and membrane ruffling, as well as chemotaxis and secretion, through the PI3K effectors

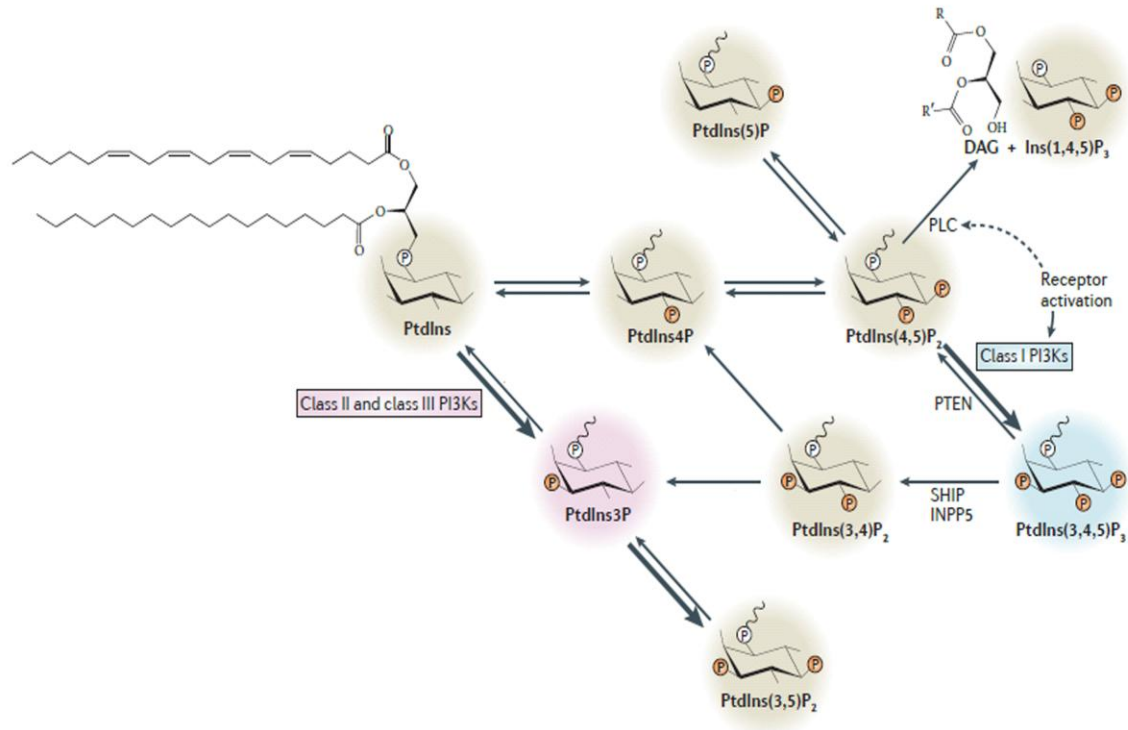
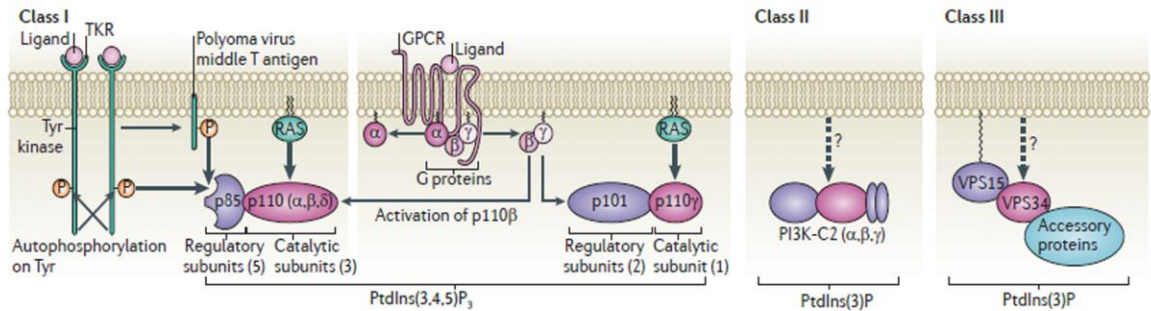


Diagram 1-3. Lipid kinase reactions catalyzed by PI3Ks. Reproduced (with modifications) from Vanhaesebroeck et al (2012). Class I PI3Ks are activated by cell-surface receptors, and upon activation, phosphorylate PIP₂ to generate PIP₃. Class II and III PI3Ks phosphorylate PI to generate PI(3)P, and are integral regulatory components of vesicular traffic and endocytosis.

A



B

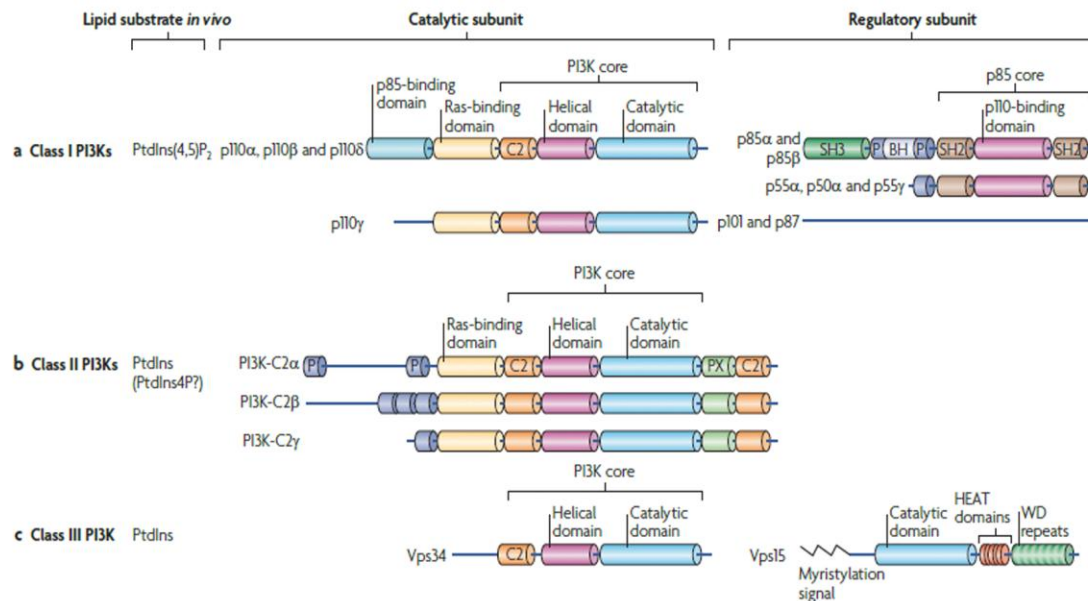


Diagram 1-4. PI3K structure and function. (A) Reproduced from **Vanhaesebroeck et al (2012)**. Class I PI3K activation downstream of RTKs, Ras, and GPCRs. (B)

Reproduced from **Vanhaesebroeck et al (2010)**. Classification and domain structure of mammalian PI3Ks, which are divided into three classes based on their structural and biochemical features. All PI3K catalytic subunits have a core structure consisting of a C2 domain, a helical domain and a catalytic domain. Class I PI3Ks exist in complex with a regulatory subunit, either a p85 isoform (for p110α, p110α and p110δ) or p101 or p87 (for p110γ). All p85 isoforms have two Src homology 2 (SH2) domains, which are essential for their interaction with activated RTK complexes. Conversely, p101 and p87 lack SH2 domains, and do not have homology to other identifiable domains. Class III PI3K has one catalytic member, vacuolar protein sorting 34 (Vps34), whose regulatory subunit (Vps15) contains WD repeats thought to be essential for its interaction with effectors such as Rab5. BH (BCR homology domain), P (Proline-rich region); SH2 and SH3 (Src-homology 2 and 3 domains).

responsible for the execution of these cellular responses remained obscure at the time (reviewed in **Vanhaesebroeck et al 2012**). In the early 1990s, just as the identification and characterization of SH2 domains by Tony Pawson and his colleagues had shed light on the mechanistic aspects of phosphotyrosine (pY)-based effector recruitment to activated RTKs (reviewed in **Koch et al 1991, Pawson 1992, Pawson and Gish 1992, Pawson 2004**), bioinformatics-based approaches had also identified several other conserved modular protein domains in diverse signaling molecules. One such module initially identified as a duplicated region in the pleckstrin molecule (**Tyers et al 1988**), and determined to be a loosely conserved SH2-like module of approximately 120 amino acids, was the pleckstrin homology (PH) domain (**Haslam et al 1993, Mayer et al 1993**), which was determined to be a common structural feature of class I PI3K effectors such as Akt, whose PH domain was subsequently shown to possess a high PIP₃/PIP₂ preferential binding ratio (reviewed in **Lemmon and Ferguson 2000, Scheffzek and Welte 2012**).

Soon after its characterization as a distinct protein module, the PH domain was first demonstrated to bind PIP₂ (**Harlan et al 1994**), raising the possibility that Akt and other PH domain-containing signaling molecules could be direct effectors of PIs. In 1995, several independent groups reported the important discovery that Akt is rapidly activated by growth factors such as insulin in a PI3K-dependent fashion (**Burgering and Coffey 1995, Franke et al 1995, Kohn et al 1995**), leading to the subsequent demonstration that the PH domain of Akt could directly bind PIP₃ with high affinity *in vitro* [PIP₃>>PIP₂] (**James et al 1996, Franke et al 1997, Frech et al 1997**). This high affinity PI3K-dependent interaction was demonstrated to recruit Akt to the membrane (**Stokoe et al 1997, Andjelković et al 1997**), thereby promoting its phosphorylation and activation by phosphoinositide-dependent kinase 1 (PDK1), which can itself be recruited to the membrane through its own lipid-binding PH domain (see **Section 1-3.2**). Importantly, the defective or deregulated PH domain-mediated membrane recruitment of PI3K effectors was demonstrated to have pathological consequences. For example, certain mutations in the PH domain of Bruton's tyrosine kinase (Btk), which normally has a strong binding preference for PIP₃ (**Rameh et al 1997, Várnai et al 1999**), were found to cause X-linked agammaglobulinaemia (XLA), and to correlate with a diminished capacity for the Btk PH domain to bind PIP₃ (**Salim et al 1996**); while more recently, a rare

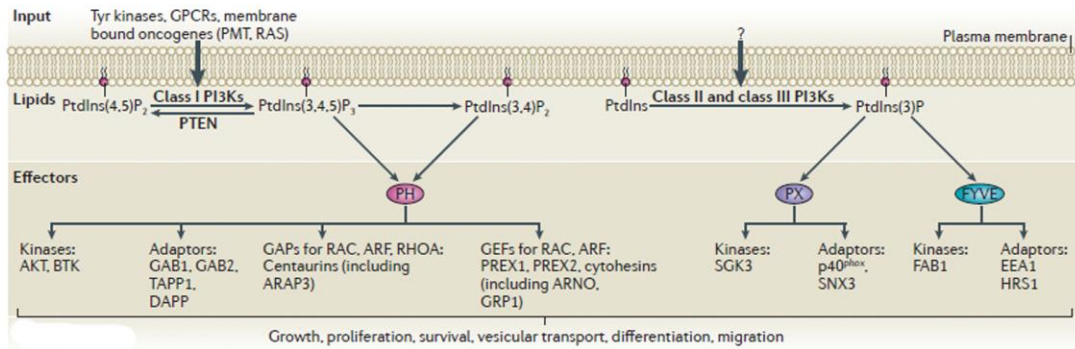
transforming mutation in the phospholipid-binding pocket of the Akt PH domain (E17K) has been clinically detected in human breast, colorectal, ovarian, lung, as well as melanoma tumor samples, and shown to induce the ectopic membrane localization of the protein (**Carpten et al 2007, Do et al 2008, Bleeker et al 2008, Davies et al 2008, Malanga et al 2008, Shoji et al 2009**).

A large number of PH domain-containing proteins have now been identified (1,482 sequences in primates according to the Sanger Institute Pfam database, or ~10% of all proteins expressed in primates), and as shown in [Diagram 1-5A](#), notably include various GEFs and GAPs for small GTPases of the Rho, Rac, Ras, and Arf families; as well as several non-catalytic signaling adaptors such as IRS and Gab, and protein kinases such as Akt and PDK1 (reviewed in **Lemmon and Ferguson 2000, Scheffzek and Welte 2012**). However, only a small fraction of characterized PH domains bind PIPs, and moreover, only a minor subset of this small fraction specifically interacts with D3-phosphorylated PIs, and even then with varying degrees of selectivity. The preferential affinity for PIP₃ demonstrated not only for the PH domains of kinases activated downstream of PI3K such as Akt (**James et al 1996, Franke et al 1997, Frech et al 1997**) and PDK1 (**Currie et al 1999**), but also for those of IRS1 (**Dhe-Paganon et al 1999, Kaburagi et al 2001**) and Gab1 (**Maroun et al 1999, Rodrigues et al 2000**), which act upstream of PI3K as docking proteins for the recruitment of PI3K regulatory subunits to activated RTKs (such as IR/IGFR in the case of IRS1, and EGFRs in the case of Gab1), established the PIP₃-dependent PH domain-mediated recruitment of signaling components as the central mechanism for the activation of signal transduction in the evolutionarily conserved PI3K/Akt signaling pathway ([Diagram 1-5B](#)).

1-2 – PTEN-mediated downregulation of PIP₃-dependent signaling

As a byproduct of transient RTK-dependent PI3K activation, the relative abundance of the PIP₃ membrane moiety, like that of pY peptide motifs, is tightly regulated in order to ensure (1) the appropriate extent and duration of PI3K-dependent signaling, and (2) the re-establishment of sensitivity to PI3K-activating signals following prior activation and PIP₃ accumulation. The dynamic stimulation of PI3K-dependent PIP₃

A



B

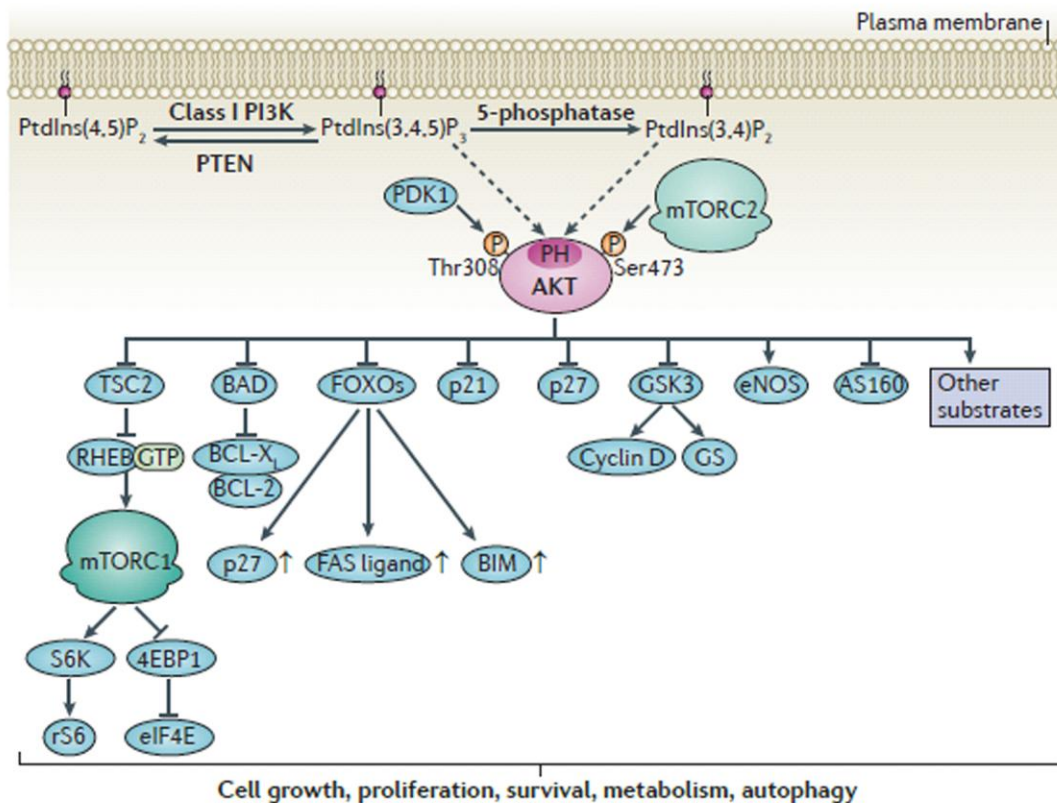


Diagram 1-5. PH domain-mediated activation of Akt and other PIP₃ effectors.

Reproduced from **Vanhaesebroeck et al (2012)**. (A) Examples of PI(3,4,5)P₃- and PI(3)P- binding effectors involved in growth, proliferation, and endocytic traffic. (B) The generation of PIP₃ induces the translocation of Akt to the membrane, and promotes the activating phosphorylation of Akt by PDK1 (at T308) and mTORC2 (at S473). Substrates of Akt catalytic activity include a number of molecules with important roles in growth, proliferation, and survival.

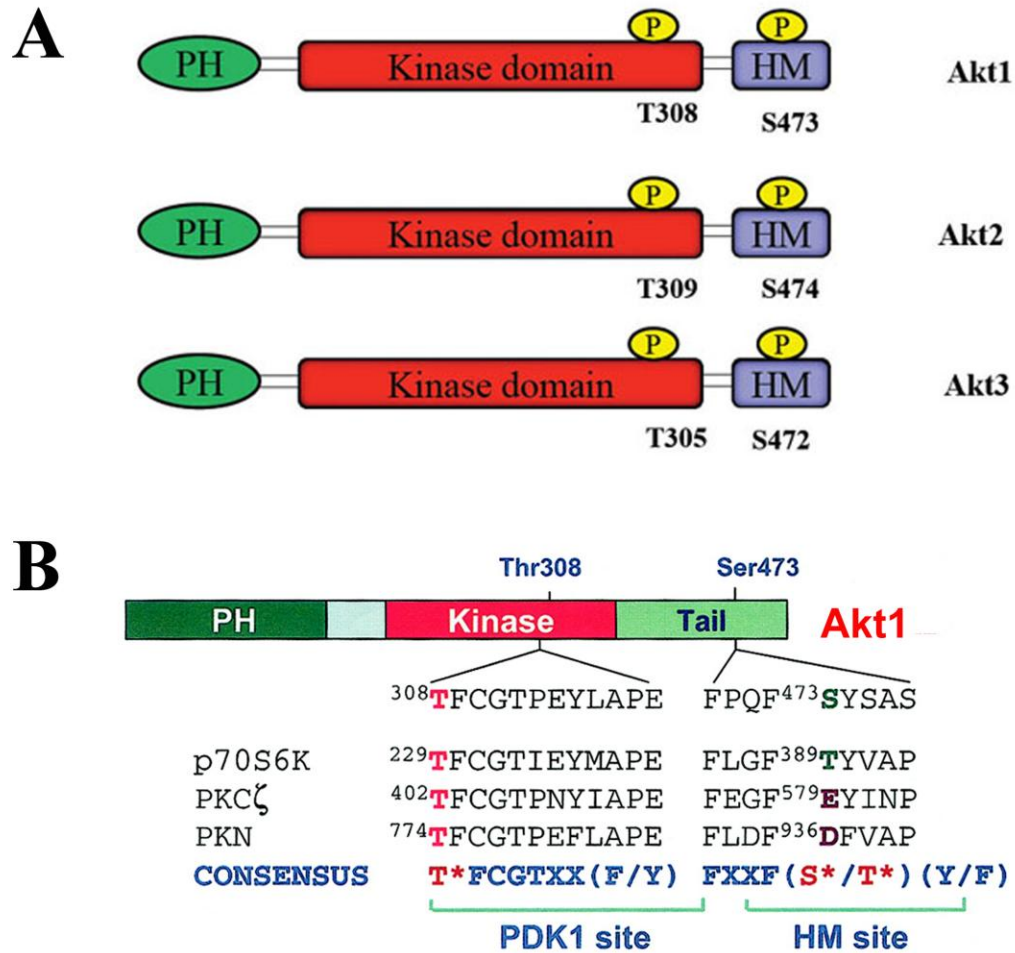


Diagram 1-6. Akt structural organization. (A) Reproduced from **Viglietto et al (2011)**. In mammals, Akt is represented by three highly homologous members with >80% protein sequence identity. All three Akt isoforms share the same structural organization, and consist of an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal regulatory region. The PH domain of Akt can bind specifically to D3-phosphorylated phosphoinositides with high affinity and mediates Akt activation. Full Akt activation is achieved through the phosphorylation of two critical residues: the first within the kinase domain's T loop (T308 in Akt1) and the second in the C-terminal hydrophobic motif (S473 in Akt1). (B) Reproduced from **Dong and Liu (2005)**. PDK1 phosphorylates Akt and other AGC (cAMP dependent, cGMP dependent, and protein kinase C) family kinases on a conserved threonine or serine residue in the activation loop nine residues upstream of the APE motif. In contrast to the T-loop site commonly targeted by PDK1, the C-terminal hydrophobic motif (HM) phosphorylation site is not conserved in some of these AGC family kinases (such as PKCζ and PKN), while the in the case of Akt1 and S6K1, which both possess an S/T residue in the HM, the surrounding sequence shows considerable variance, a fact reflected by their phosphorylation by the functionally distinct mTORC2 and mTORC1 complexes, respectively. See text for further details.

production is opposed by the catalytic activity of phosphoinositide 3-phosphatases that specifically dephosphorylate 3-phosphorylated PIPs (reviewed in **Sasaki et al 2009**). With the exception of the TPIP molecule (TPTE and PTEN homologous inositol lipid phosphatase) which is mainly expressed in the testes, brain, and stomach (**Walker et al 2001**), the dephosphorylation of PIP₃ at D3 is predominantly carried out by the ubiquitously expressed PTEN molecule (reviewed in **Song et al 2012**). As described herein, the loss of PTEN function, like constitutively activating mutations of PI3K, results in unrestrained PIP₃-dependent signaling, and as discussed in **Chapter 4**, can result in cellular transformation and contribute to tumorigenesis. In fact, cytogenetic studies carried out in the 1980s had initially revealed the frequent partial or complete loss of chromosome 10 in cases of brain, prostate, and bladder cancer (**Bigner et al 1984, Atkin and Baker 1985**); and the eventual characterization of PTEN as the preeminent cellular PIP₃ phosphatase stems from the initial identification of the *PTEN* gene (or *MMAC1*, mutated in multiple advanced cancers 1) at the 10q23 locus in 1997 by two independent groups who had sought to identify potential tumor suppressors located on chromosome 10. In their preliminary screens of cultured cancer cells, the group led by Ramon Parsons (**Li et al 1997**) detected mutations of *PTEN* in 31% of glioblastoma cell lines (13/42), all four examined prostate cancer cell lines, 6% of breast cancer cell lines (4/65), and 17% of primary glioblastomas (3/18). Similarly, Peter Steck and his colleagues (**Steck et al 1997**) recapitulated these observations in advanced stage gliomas, as well as prostate cancer and breast cancer cell lines, and added kidney cancer cell lines to the list of *PTEN* mutation-bearing advanced carcinomas. Furthermore, in addition to the detection of *PTEN* mutations in these sporadic tumor types, *PTEN* was also subsequently demonstrated to be mutated in the germline of 60-80% of patients with cancer predisposition syndromes such as Cowden's disease, Bannayan-Riley-Ruvalcaba syndrome, Lhermitte-Ducros disease, Proteus syndrome, and Proteus-like syndrome (**Marsh et al 1998, Zhou et al 2003**). Such hereditary disorders that result from germline mutations in *PTEN* are all characterized by the development of multiple hamartomas (benign neoplasms), an increased risk of tumorigenesis, and are collectively classified as types of PHTS, or PTEN hamartoma tumor syndrome.

1-2.1 – Structure and biochemical function of the PTEN phosphatase

The PTEN molecule (~55-kDa) is a dual specificity phosphatase (DUSP) that can act on both peptide and lipid substrates, and contains the HCxxGxxR active site consensus motif common to all PTPases (reviewed in **Tonks 2006**). Consistent with its initial characterization as an important tumor suppressor, its activity has been implicated in the regulation of diverse cellular processes that are subverted during tumorigenesis, including metabolism, growth, survival, senescence, stem cell renewal, polarity, motility, and the maintenance of genomic integrity (reviewed in **Chalhoub and Baker 2009, Song et al 2012**). Its catalytic domain is most similar in sequence to other DUSPs such as Cdc14 (**Li et al 1997, Steck et al 1997**), and can dephosphorylate highly negatively-charged peptide substrates at pS/pT and pY residues *in vitro* (**Li and Sun 1997, Myers et al 1997**); but is a poor catalyst for this reaction in comparison to its high affinity for the D3 phosphate of the PIP₃ molecule (**Maehama and Dixon 1998**), suggesting that the principle *in vivo* catalytic function of the PTEN molecule is to oppose PI3K activity by dephosphorylating PIP₃ at D3, thereby converting PIP₃ back to PIP₂, depleting pools of PIP₃ at the membrane (**Diagram 1-5**), and terminating the stimulation of PIP₃-dependent signaling (reviewed in **Rameh and Cantley 1999**). The consensus catalytic HCKAGKGR sequence of human PTEN is located between residues 123 and 130, and the analysis of its crystal structure revealed that its catalytic cleft is wider than those of other PTPases, allowing key positively charged catalytic residues access to the relatively bulky negatively charged PIP₃ head group (**Lee et al 1999a**). Many PTEN mutations found in cancer map to exon 5 in this stretch of the catalytic domain (**Rasheed et al 1997, Marsh et al 1998, Ali et al 1999**), and like C124S, inactivate both protein- and lipid-directed phosphatase activities (**Myers et al 1997**). In support of the notion that its unique role in the dephosphorylation of PIP₃ is its central contribution as a signaling molecule and tumor suppressor, the lipid phosphatase activity of PTEN (rather than its putative protein phosphatase activity) has been demonstrated to be the major driving force of its tumor suppressing function in normal cells (reviewed in **Song et al 2012**). Accordingly, in contrast to the absence of PTEN mutations that selectively disrupt its protein phosphatase activity in human cancers, cultured cells expressing the cancer-derived G129E mutant PTEN molecule, which abrogates lipid phosphatase activity while

sparing protein phosphatase activity, was also demonstrated to result in the loss of PTEN-mediated tumor suppression *in vitro* (Myers et al 1998).

Whereas the substrate-binding catalytic motif of PTEN is evolutionarily conserved amongst PTPs, the N-terminal portion of PTEN (the first ~200 amino acids of the 403 residue human protein) that encompasses the catalytic motif also displays homology, as its nomenclature implies, to molecules that are unrelated to PTP sequences, namely the actin-binding molecule tensin 1 (TNS1), which is associated with the actin cytoskeleton at focal adhesions (Lo et al 1994). Consistent with this structural homology, overexpressed PTEN was demonstrated to interact directly with focal adhesion kinase (Fak), to reduce Fak tyrosine phosphorylation, and to inhibit cell migration, integrin-mediated cell spreading, and focal adhesion formation (Tamura et al 1998), suggesting a role in cytoskeletal rearrangement and cell migration in addition to its role in the regulation of cellular growth. The C-terminal portion of the PTEN molecule mostly consists of the C2 domain which is thought to be essential for its stability and membrane localization (Lee et al 1999a). Other notable domains include a short N-terminal-most PIP₂-binding domain (PBD), as well as a C-terminal tail containing PEST (proline, glutamine, serine, and threonine) sequences that enhance proteolytic sensitivity; and a C-terminal-most PDZ domain that is also thought to contribute to protein stability, and to mediate protein-protein interactions (reviewed in Song et al 2012).

1-2.2 – Genetic characterization of PTEN function

As discussed in further detail in **Chapter 2**, the characterization of various components of the evolutionarily conserved insulin signaling pathway in genetic model organisms like *Drosophila* has uncovered a stereotypical growth phenotype for positive regulators of this pathway including the Dilp (*Drosophila* insulin-like peptide 1-7) insulin homologs (Rulifson et al 2002, Zhang et al 2009), the *Drosophila* insulin receptor DIR (Brogiolo et al 2001, Oldham et al 2002), the IRS1-4 homolog chico (Böhni et al 1999), the fly homologs of the catalytic and regulatory PI3K subunits Dp110 and p60 (Weinkove et al 1999); as well as Dakt (Verdu et al 1999, Scanga et al 2000), and various other downstream-or parallel-acting components. Their respective loss-of-function or hypomorphic phenotypes invariably include diminished organismal size in

comparison to wildtype throughout development, reflecting cell-autonomous defects in the regulation of cell size and/or cell number. Consistent with its role as an *antagonist* of PI3K-mediated signaling, *Drosophila* PTEN (*dPTEN*) loss-of-function mutants display increased cell and organ size, whereas *dPTEN* overexpression result in the opposite phenotype (**Huang et al 1999, Goberdhan et al 1999, Gao et al 2000**). Importantly, the *dPTEN* molecule was also ascribed a negative role in the Dp110/Dakt-mediated promotion of survival during embryonic development (**Scanga et al 2000**); and furthermore, the loss of Dakt function or membrane-recruitment was demonstrated to fully suppress the cell size phenotype caused by the loss of *dPTEN* function (**Gao et al 2000, Scanga et al 2000, Stocker et al 2002**), firmly placing Dakt downstream of *dPTEN*, and suggesting that ectopic Dakt activation lies at the heart of the *PTEN* mutant phenotype in multiple signaling contexts. In contrast, although certain combinations of *DIR* loss-of-function alleles result in decreased cell size, they do not suppress the *dPTEN* phenotype, consistent with a role for *dPTEN* function downstream of *DIR*, in parallel to Dp110, and upstream of Dakt (**Gao et al 2000**).

Homozygosity for a null mutation of *PTEN* in mice results in mid-gestational lethality (E9.5), demonstrating its requirement for mammalian embryonic development (**Di Cristofano et al 1998, Suzuki et al 1998**). The analysis of haploinsufficient *PTEN* heterozygotes, however, additionally revealed its postulated tumor suppressive role as evidenced by the increased incidence of various types of tumors despite the retention of a single wildtype copy of *PTEN* (**Di Cristofano et al 1998, Suzuki et al 1998, Podsypanina et al 1999, Kwabi-Addo et al 2001**). In addition to these studies with haploinsufficient heterozygotes, the analysis of a series of hypomorphic *PTEN* mutant mice with subtle differences in *PTEN* levels demonstrated that even a partial loss of *PTEN* activity provided a growth/survival advantage, and that susceptibility to cancer correlated with *PTEN* dosage over a continuous range – a “continuum” model of *PTEN* activity, as opposed to a “two-hit” model predicated on *PTEN* copy number (**Alimonti et al 2010**). Importantly, *PTEN* was demonstrated to negatively regulate cellular growth and Akt-dependent cell survival (**Stambolic et al 1998, Wu et al 1998, Li et al 1998b, Sun et al 1999, Backman et al 2001, Kwon et al 2001, Groszer et al 2001, Stiles et al 2002**), an observation that was recapitulated in *Drosophila* (**Gao et al 2000, Scanga et al**

2000, Stocker et al 2002) as mentioned above. Furthermore, in keeping with its frequent inactivation in various types of tumors, and the crucial role attributed to PTEN in restraining the PI3K-dependent activation of Akt (reviewed in Cantley and Neel 1999), the loss of PTEN function was correlated with elevated levels of Akt activity in a number of malignancies *in vitro*, including glioblastoma (Haas-Kogan et al 1998), multiple myeloma (Hyun et al 2000), as well as prostate (Davies et al 1999), endometrial (Kanamori et al 2001), and ovarian carcinomas (Kurose et al 2001). In order to circumvent the lethality of homozygous *PTEN* deficiency, mouse models of conditional gene-targeted *PTEN* inactivation have been developed to examine the tissue-specific physiological functions of PTEN, collectively revealing a broad array of additional tissue-specific defects in its absence (reviewed in Knobbe et al 2008, Suzuki et al 2008), including heart failure (Crackower et al 2002) and defective immunoglobulin class switch recombination (Suzuki et al 2003). With respect to the role of PI3K-dependent signaling in insulin-sensitive tissues, muscle-specific conditional inactivation of PTEN was demonstrated to result in insulin hypersensitivity, and to protect against insulin resistance and diabetes (Wijesekara et al 2005), consistent with its previously demonstrated role *in vitro* as a negative regulator of insulin-stimulated PI3K/Akt-dependent metabolic signaling processes (Nakashima et al 2000, Ono et al 2001).

1-3 – The Akt kinases

In 1977, the AKR murine strain was reported by Stephen Staal to exhibit a high incidence of leukemia and lymphoma from spontaneous thymomas (Staal et al 1977), and an acutely transforming retrovirus termed AKT8 was isolated from virological studies of tumor cell lines established from said thymomas. The AKT8 provirus was subsequently cloned, and the putatively oncogenic genetic component, which lacked sequence homology with other known oncogenes, was designated v-*Akt* (Staal 1987), and subsequently demonstrated to be tumorigenic both *in vitro*, transforming cultured mink lung cells, and *in vivo*, resulting in thymic lymphomas after its inoculation in susceptible mouse strains (Staal and Hartley 1988). Two cellular homologs of the v-*Akt* murine oncogene, *Akt1* (located at 14q32) and *Akt2* (located at 19q13) were identified in

humans (*Akt3* at 1q44 would be cloned and characterized in 1995), and the detection of a 20-fold amplification of *Akt1* in one of five gastric adenocarcinomas tested as part of a survey of 225 human tumors screened for changes in *Akt1* expression (**Staal 1987**) suggested that this novel oncogene may be involved in the pathogenesis of *human* malignancies as well. Similar surveys would also thereafter reveal the *Akt2* gene (**Jones et al 1991b**) to be amplified or overexpressed in several ovarian and breast carcinomas, as well as pancreatic cancer cell lines (**Cheng et al 1992, Bellacosa et al 1995, Miwa et al 1996, Cheng et al 1996**). Independently using different strategies, three groups initially cloned and characterized the cellular homolog of v-*Akt* in 1991 as a novel cellular protein kinase (**Bellacosa et al 1991, Jones et al 1991a, Coffey and Woodgett 1991**). Philip Tsichlis and his colleagues cloned AKT8 proviral DNA from transformed mink lung cells, and sequence analysis showed that the v-*Akt* gene product consists of the viral *Gag* sequence (p12, p15, Δp30) N-terminally fused to the transduced cellular oncogene, which they called c-*Akt* (**Bellacosa et al 1991**).

The examination of c-*Akt* structure (reviewed in **Hanada et al 2004**) revealed the molecule (480 amino acids, ~56-kDa) to consist of three highly conserved regions whose contributions to catalytic activation and function, as described below, would begin to be uncovered in the latter half of the 1990s. These three regions consist of: (1) an N-terminal (~100 residues) SH2-like motif which was later redefined as a PH domain (**Haslam et al 1993, Mayer et al 1993**); (2) the central catalytic domain (~250 residues) that houses the activation loop (T-loop), whose phosphorylation is necessary for activation; and (3) a C-terminal regulatory domain (~50 residues) containing the hydrophobic motif (HM), whose phosphorylation is required for full catalytic activity (**Diagram 1-6A**). The central catalytic domain and C-terminal regulatory domain sequences of *Akt* were determined at the time to be most closely related to members of the PKC family of serine-threonine kinases, and the biochemical characterization of its *in vitro* enzymatic activity confirmed its capacity to phosphorylate serine and threonine residues (**Bellacosa et al 1991, Bellacosa et al 1993**). Whereas Tsichlis' group identified cellular *Akt* with the viral oncogene as their starting point, Brian Hemmings and colleagues identified *Akt* (which they called RAC – related to A and C kinases) by using a cDNA probe for the α catalytic subunit of porcine *PKA* (also called *cAMP-PK*) in a low-stringency cDNA library screen

for novel kinases related in sequence to *PKA* (**Jones et al 1991a**); while James Woodgett and Paul Coffe identified Akt (which they termed PKB based on its similarity to PKA and PKC) by employing degenerate oligonucleotide primers based on conserved regions between S/T kinases to amplify human epithelial cDNA from HeLa cells by PCR with the aim of identifying cDNAs of novel putative kinases (**Coffe and Woodgett 1991**). Subsequent analyses would refine the structural relation of the Akt catalytic domains with those of related classes of S/T kinases (reviewed in **Scheid and Woodgett 2003**, **Woodgett 2005**), confirming high level of similarity with members of the AGC family of kinases, including PKA, PKC, and S6K, but with highest similarity to serum and glucocorticoid-regulated kinases (SGKs) in the catalytic domain, while the C-terminal regulatory domain seemed most similar to corresponding domains encoded by members of the PKC family.

1-3.1 – Mechanisms of RTK-dependent Akt activation

Prior to its redesignation as a phospholipid-binding domain with significant preference for PIP₃, the classification of the c-Akt PH domain as an N-terminal “SH2-like” motif (**Bellacosa et al 1991**) had come on the heels of the aforementioned implication of SH2 domains in the establishment of pY-directed protein-protein interactions during RTK-mediated signal transduction. This association fuelled speculation that the cytoplasmic serine/threonine kinase c-Akt may form a functional link between cell-surface RTKs and the intracellular responses they activation elicits. The postulated importance of Akt membrane translocation for its efficient activation, though subsequently demonstrated to be PIP₃-dependent and not, as initially speculated, pY-directed, was nonetheless consistent with the biochemical mechanism thought to underlie the oncogenicity of the Gag antigen. The viral Gag epitope was known to be N-terminally myristoylated in retroviruses such as Moloney murine leukemia virus (MMLV) and human immunodeficiency virus type 1 (HIV1), and this myristoylation was shown to be required for proper replication, assembly, and maturation of the viruses (**Schultz and Rein 1989**, **Göttlinger et al 1989**, **Bryant and Ratner 1990**). As the sole viral contribution to the v-Akt molecule (**Bellacosa et al 1991**), the viral (Gag) sequence’s myristoylation was proposed to constitutively target the v-Akt molecule to the plasma

membrane *independently* of RTK-mediated membrane-recruiting cues, resulting in the gradual accumulation of activated molecules, and leading to ectopic and potentially oncogenic activation. In fact, endogenous c-Akt was subsequently demonstrated to reside primarily in the cytoplasm, while the oncogenic v-Akt molecule was myristoylated and dispersed throughout various intracellular compartments, including significant pools at the plasma membrane and in the nucleus, with dramatic consequences with respect to *in vivo* oncogenicity in murine models (**Ahmed et al 1993**). Furthermore, whereas mutations in the PH domain of Akt (R25C substitution or Δ 11-35 deletion) abrogated their RTK-dependent activation *in vitro* (**Franke et al 1995**), the expression of a deletion construct of Akt (Δ 4-129) lacking the PH domain, and whose protein product is synthetically targeted to the membrane by the N-terminal addition of either the 14-amino acid Src myristoylation motif, or alternatively, the p85 SH2 domain, was subsequently demonstrated to increase the basal level of Akt phosphorylation, which was also found to correlate with the activation of S6K (**Kohn et al 1996**), itself (then recently) shown to be activated downstream of Akt (**Burgering and Coffe 1995**). Combined, these studies would establish the importance of membrane localization in the process of Akt activation, though due to conflicting findings (**Kohn et al 1996**, **Datta et al 1996**), the requirement of the PH domain *per se* remained controversial until its role in the phosphorylation-dependent mechanism of Akt activation was gradually clarified.

Following the cloning and structural analysis of c-Akt in 1991, its identification as an effector of RTK-dependent PI3K-mediated signaling in the mid-1990s was predicated on: (1) the observation that Akt could be rapidly and transiently activated downstream of various growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin or IGF1 (**Burgering and Coffe 1995**, **Franke et al 1995**, **Alessi et al 1996a**); (2) the demonstrated correlation between Akt activation and its phosphorylation status (**Burgering and Coffe 1995**, **Kohn et al 1995**, **Kohn et al 1996**); and (3) the demonstration that RTK-dependent activation of Akt could be completely blocked by the pharmacological or genetic inhibition of PI3K activity (**Burgering and Coffe 1995**, **Kohn et al 1995**), or alternatively, further upstream through point mutations in the p85 SH2 domain-binding motifs of receptors that precluded PI3K recruitment and activation

in response to ligand binding (**Franke et al 1995**); whereas (4) the expression of a constitutively *active* form of the p110 catalytic subunit in cultured cells was found to be sufficient for the ectopic activation of endogenous Akt (**Didichenko et al 1996, Klippel et al 1996**). Consistent with its demonstration as a central mediator of the cellular responses induced downstream of multiple RTK-activating extracellular factors operating in a variety of cell types, Akt was also subsequently shown to be PI3K-dependently activated by numerous additional stimuli including not only other circulating extracellular factors such as nerve growth factor (NGF), and various GPCR-activating hematopoietic cytokines (**Andjelković et al 1998, Ahmed et al 1997, Reif et al 1997, Songyang et al 1997, Coffe et al 1998b**) or chemokines (**Tilton et al 1997**), but also in the cellular response to physiological stresses such as hypoxia (**Mazure et al 1997**).

The observed correlation between RTK-dependent Akt activation and its concomitant phosphorylation suggested the latter to be an important determinant of Akt catalytic activation (**Kohn et al 1995, Burgering and Coffe 1995, Kohn et al 1996, Andjelković et al 1996**). Consistent with this correlation, epitope-tagged Akt immunoprecipitates from insulin treated CHO-IR (*IR*-overexpressing) cells, when treated with phosphatase inhibitors, were found to be inhibited in their capacity to phosphorylate peptide substrates *in vitro* in comparison to non-phosphatase treated immunoprecipitates, which readily did so (**Kohn et al 1995**). In these studies, the phosphorylation of Akt could be distinguished by gel electrophoresis as a slower-migrating (higher molecular weight) species of Akt; and furthermore, its detection was (1) dependent on RTK stimulation; (2) vulnerable to pharmacological PI3K inhibition; and (3) eliminated by phosphatase treatment. Two-dimensional (2-D) chromatography suggested a serine residue as the major site of activating phosphorylation (**Burgering and Coffe 1995**), and was subsequently determined by Hemmings and colleagues (**Alessi et al 1996a**) to be located in the C-terminal regulatory domain (S473 in human Akt1, S474 in Akt2, and S472 in Akt3). The comparatively modest 2-D chromatography signal detected for threonine phosphorylation (**Burgering and Coffe 1995**) was proposed to correspond to a residue (T308 in human Akt1, T309 in Akt2, and T305 in Akt3) in subdomain VIII of the catalytic domain (**Alessi et al 1996a**), nine residues upstream of the conserved alanine-proline-glutamate (APE) motif – a position within subdomain VIII (7-10

positions immediately upstream of APE) conserved for the activating phosphorylation site of numerous other kinases ([Diagram 1-6B](#), reviewed in **Hanks and Hunter 1995**). The aforementioned controversy surrounding the functional role of the Akt PH domain was addressed by David Stokoe, Dario Alessi, and colleagues in 1997 with their demonstration that the binding of the PH domain to PIP₃ was required for the phosphorylation of Akt at T308 by an upstream acting kinase (**Stokoe et al 1997**), which as described below, was shortly thereafter identified as PDK1.

The mutational analysis of Akt1 phosphorylation at T308 and S473 by Alessi et al (**1996a**) had suggested that these two crucial sites were the major residues phosphorylated in response to insulin or IGF1, and though synergistic in their contribution to enzyme activation, were likely to be independently regulated. Their *in vitro* experiments with S/T-to-alanine substitution mutants – a T308A construct, which could not be activated by insulin or IGF1, or an S473A construct, which could only be modestly activated by insulin (3-fold) or IGF1 (5-fold), respectively; suggested that catalytic domain phosphorylation at T308 was required for insulin-mediated Akt activation, and that maximal Akt activation in response to insulin (20-fold) or IGF1 (50-fold) required further phosphorylation in the regulatory domain at S473. Notably, whereas T308D/S473D Akt double mutants bearing serine/threonine-to-aspartate mutations at both phosphorylation sites (which ionically mimics the effect of phosphorylation) were shown to be significantly (40-fold) activated in unstimulated cells, could not be further activated by insulin stimulation, and were resistant to wortmannin; the S473D single substitution mutant Akt molecule, which like the T308D mutant, exhibited a 5-fold increase in activity, was found to be wortmannin-sensitive, suggesting that T308 phosphorylation was PI3K-dependent (**Alessi et al 1996a**). Moreover, the T308 and S473 residues were shown to be phosphorylated independently, with no requirement for the prior phosphorylation of either site for the phosphorylation of the other; and furthermore, the retained capacity of a kinase-dead form of Akt to become phosphorylated at T308 and/or S473 suggested that these residues were targeted by the autonomous activities of distinct cellular kinases rather than the result of Akt autophosphorylation (**Alessi et al 1996a**).

1-3.2 – PDK1-dependent phosphorylation of the Akt catalytic domain T-loop

The proposal, based on mutational analysis and sequence context, that the phosphorylation of Akt1 at T308 and S473 is independently regulated by distinct kinase activities (**Alessi et al 1996a**) was supported by Stokoe's study (**Stokoe et al 1997**), which found that Akt-phosphorylating activity from cytosolic extracts resolved into two distinct peaks, one of which eluted at high concentrations of NaCl (475 mM), and phosphorylated Akt both in the presence or absence of PIP₃, but did not increase Akt activity *in vitro*; whereas the other, which eluted at lower concentrations of NaCl (150 mM), only phosphorylated Akt in the presence of PIP₃, and strongly stimulated Akt activity. Furthermore, this second PIP₃-dependent peak of activity was determined by phospho-amino acid analysis to exclusively occur at the threonine residue, and was narrowed down to a single peptide containing T308 in subdomain VIII, suggesting that the threonine phosphorylation occurred at a single site. Meanwhile, a similar low NaCl concentration (200nM) QAE-Sephadex affinity column eluate was isolated and serially purified from the cytosolic fraction of rabbit skeletal muscle by Alessi and his colleagues (**Alessi et al 1997a**), and following the final purification step, the active fraction was found to be comprised of three major protein bands with apparent molecular masses of 85, 67, and 45-kDa. The 67-kDa band was the only species to become phosphorylated in the presence of Mg-ATP (which was greatly increased in the presence of PIP₃), and tryptic peptides from this molecule were distinct, but highly homologous to regions found in the catalytic domains of known kinases (**Alessi et al 1997a**). *In vitro*, the incubation of the newly identified 67-kDa kinase with Akt1 was demonstrated (1) to phosphorylate Akt1 in the presence of Mg-ATP and phospholipids vesicles containing PIP₃; (2) to phosphorylate Akt1 exclusively at T308, activating it by 30-fold *in vitro*; and (3) to do so in a manner that was completely dependent on the presence of PIP₃ or PI(3,4)P₂, hence its designation as PIP₃-dependent protein kinase 1 (**Alessi et al 1997a**). The human PDK1 gene, located on 16p13, was cloned and its product found to be widely expressed as a soluble (cytoplasmic) protein of 556 amino acids (**Alessi et al 1997b**, **Stephens et al 1998**) notably consisting of two important domains – an N-terminal S/T kinase domain typical of AGC family kinases, and a C-terminal PH domain with high affinity for PIP₃ and PI(3,4)P₂ (**Alessi et al 1997b**, **Currie et al 1999**).

Many of the physiological effects elicited by increases in membrane PIP₃ abundance including those regulating growth, survival, and the metabolic responses to insulin, are mediated by a subset of AGC kinase family members such as Akt, whose roles in growth, viability, and glucose homeostasis are thoroughly documented herein, and include: S6K1, which is involved in the regulation of protein synthesis and growth (reviewed in **Magnuson et al 2012**); SGK isoforms, which regulate ion transport, hormone release, neuroexcitability, proliferation, and apoptosis (reviewed in **Tessier and Woodgett 2006**); as well as the p90 ribosomal S6 kinases (p90^{RSK}, or RSK) and the various PKC isoforms, which regulate survival, growth, and motility (reviewed in **Anjum and Blenis 2008, Newton 2010**). Members of the AGC kinase family share structural similarity and a common general mechanism of activation involving dual phosphorylation at two residues each of which is required for full activation, and located in a highly conserved motif – the T-loop in the catalytic domain, and the regulatory HM positioned C-terminal to the kinase domain (**Pearce et al 2010**). Following the identification of PDK1 as the Akt T-loop kinase, the high degree of homology displayed within the activation loop between different members of the AGC kinase family (which now also included PDK1) led to speculation that PDK1 may similarly regulate the T-loop S/T phosphorylation of other AGC kinases in mammalian cells. The flurry of studies investigating this possibility in the late 1990s demonstrated it to be true in the case of S6K (**Alessi et al 1998, Pullen et al 1998**), RSK (**Richards et al 1999, Jensen et al 1999**), SGK (**Park et al 1999, Kobayashi and Cohen 1999**), and various PKC isoforms (**Dutil et al 1998, Le Good et al 1998, Chou et al 1998**), with the definitive genetic evidence provided in PDK1-deficient embryonic stem cells, in which agonist stimulation failed to activate Akt, S6K, and RSK (**Williams et al 2000**), as well as SGK1 (**Collins et al 2003**), thereby establishing PDK1 as a so-called “master regulator” of AGC kinase activation *in vivo* (reviewed in **Mora et al 2004, Bayascas 2010**). Similarly, the genetic analysis of PDK1 function in *Drosophila* has demonstrated that DSTPK61, the functional fruit fly homolog of PDK1 (**Alessi et al 1997b**), regulates the activation loop phosphorylation of Dakt, dS6K, dRSK (**Rintelen et al 2001**).

In unstimulated cells, the PDK1 molecule is detectable both in the cytosol and at the plasma membrane (**Stephens et al 1998, Currie et al 1999**), and has also been shown

to be actively shuttled to the nucleus in response to mitogen stimulation (**Lim et al 2003, Scheid et al 2005**). Surprisingly however, despite its demonstrated capacity to phosphorylate and activate the numerous agonist-stimulated AGC kinases listed above, PDK1 catalytic activity itself is not agonist-dependent *per se*, as suggested by the fact that PDK1 immunoprecipitates from unstimulated cells possessed the same high catalytic activity as those from growth factor-stimulated cells (**Alessi et al 1997b**), and furthermore, contrary to its initial designation as a PIP₃-dependent kinase, its PH domain-mediated interaction with either PIP₃ or PI(3,4)P₂ was not found to alter its catalytic activity (**Alessi et al 1997b, Stephens et al 1998, Currie et al 1999**). Although various regulatory mechanisms and phosphorylation sites have been suggested as modulators of PDK1 activity (**Casamayor et al 1999, Wick et al 2003, Scheid et al 2005, Riojas et al. 2006, Yang et al. 2008, Moon et al 2008**), the original observation by Alessi's group that even bacterially expressed PDK1 was fully active and stoichiometrically autophosphorylated at its own T-loop S241 residue (**Casamayor et al 1999**) – a reaction that was shown to occur in *trans* through an intermolecular interaction (**Wick et al 2003**) – suggested that its likeliest mode of regulation was through its subcellular localization, through which its access to AGC substrates could be limited or directed. As previously mentioned, the N-terminal Akt PH domain preferentially binds PIP₃ with higher affinity than PIP₂. The C-terminal PDK1 PH domain, on the other hand, binds PIP₃ and PIP₂ without preference, but with a higher affinity for either PI than that of the Akt PH domain towards PIP₃ (**Alessi et al 1997b, Currie et al 1999**), consistent with the agonist-independent presence of PDK1 at endomembranes (**Currie et al 1999**), and in stark contrast to the PIP₃-mediated agonist-dependent recruitment of cytoplasmic Akt (**Andjelković et al 1997**), or Akt/PDK1 complexes (**Anderson et al 1998, Filippa et al 2000**). Among all the PDK1 substrates characterized to date, the three Akt isoforms are the only PDK1 effectors to possess a PI-binding PH domain, and this distinction is central to its unique subcellular localization-dependent PDK1-mediated T-loop phosphorylation mechanism (see **Section 1-3.4**).

1-3.3 – mTORC2-dependent phosphorylation of the Akt hydrophobic motif

Though the PDK1 molecule could phosphorylate the T-loop of several agonist-stimulated AGC kinases (including its own in *trans*), the likelihood that a similar “master regulator” was responsible for the HM phosphorylation of the same set of agonist-stimulated AGC kinases, including the S473 residue in Akt1, was deemed to be very low based on a number of observations, including the fact that the agonist-mediated phosphorylation of the HM site on S6K was rapamycin-sensitive (**Chung et al 1992, Price et al 1992, Kuo et al 1992, Pearson et al 1995**), in contrast to the insulin-mediated HM phosphorylation and activation of Akt, which (like SGK, RSK, and most PKC isoforms) was rapamycin insensitive (**Cross et al 1995, Alessi et al 1996a**), while the PKC HM had been suggested to be constitutively and growth factor-independently phosphorylated (**Tsutakawa et al 1995**). The kinase(s) responsible for Akt HM phosphorylation remained elusive, and a number of candidate kinases were proposed to function as the so-called “PDK2”, including autophosphorylation by the Akt molecule itself (**Toker and Newton 2000**); MK2, the mitogen-activated protein kinase-activated protein (MAPKAP) kinase-2 (**Alessi et al 1996a**); ILK, the integrin-linked kinase (**Delcommenne et al 1998**); as well as various PKC isoforms (**Kroner et al 2000, Partovian and Simons 2004, Kawakami et al 2004**), among others (reviewed in **Dong and Liu 2005, Woodgett 2005, Bayascas and Alessi 2005**). Considering the broad expression of Akt, and the requirement of HM phosphorylation for its full activity, a number of observations argued against any of the proposed candidates as a physiologically prominent kinase for the HM residue *in vivo*.

The PDK1 molecule itself could be discounted based on the demonstration that the immunodepletion of PDK1 from cellular extracts lost the capacity to phosphorylate the Akt T-loop, but retained the ability to phosphorylate the HM residue in a PI3K-dependent manner (**Hresko et al 2003**); and the insensitivity of HM phosphorylation to staurosporine, a potent PDK1 inhibitor (**Hill et al 2002**). Autophosphorylation of Akt at the HM could be eliminated as a possibility since HM phosphorylation could still be induced in PDK1-null cells despite the absence of T-loop phosphorylation, which severely restricts Akt catalytic activity, and further argues against the likelihood of PDK1-mediated HM phosphorylation (**Williams et al 2000, Hill et al 2001**). In contrast to the report of ILK-mediated Akt HM phosphorylation (**Delcommenne et al 1998**),

other studies found that ILK could not phosphorylate the Akt HM *in vitro* (**Balendran et al 1999**, **Lynch et al 1999**, **Hill et al 2002**), and furthermore, Akt was demonstrated to be normally phosphorylated in ILK-deficient fibroblasts (**Sakai et al 2003**) and chondrocytes (**Grashoff et al 2003**), suggesting that ILK activity is not essential for Akt HM phosphorylation, and that its effect may be indirect. Similarly, MAPKAP kinase-2, which was originally shown by Alessi and colleagues (**1996a**) to phosphorylate the Akt HM *in vitro*, was deemed unlikely to be a viable candidate as a broadly-acting physiological HM kinase for Akt, since agents that induce its activity, such as TNF α and interleukin-1, do not induce HM phosphorylation or Akt activation, and furthermore, a number of Akt agonists including insulin, IGF1, and PDGF do not stimulate MAPKAP kinase-2 activity, and therefore engage the activity of another kinase(s) for the HM phosphorylation in response to the agonists (**Coffer et al 1998a**).

Rapamycin is a lipophilic macrolide produced as a secondary metabolite by the soil bacterium *Streptomyces hygroscopicus*, and acts as a potent immunosuppressant with antifungal and anticancer properties (reviewed in **Lorberg and Hall 2004**). The initial observation that rapamycin, in its capacity as an antifungal agent, could induce G1 arrest in *S. cerevisiae* (**Heitman et al 1991a**), and inhibit proliferation of helper T cells (**Dumont et al 1990**) in its capacity as an immunosuppressant, suggested that the cellular target of the compound, and by extension, its mode of action, were highly conserved in eukaryotes, and that yeast could be used as a model for its study. Putative cellular target(s) of rapamycin in yeast were identified from eighteen spontaneous rapamycin-resistant yeast mutants (**Heitman et al 1991a**), fifteen of which were found to contain a loss-of-function mutation in the previously identified FK506-binding proline rotamase (FPR1), which encodes the highly conserved cytoplasmic FKBP12 (FK506-binding protein 12), a proline isomerase and *in vitro* binding partner for both rapamycin and FK506, a potent immunosuppressant structurally related to rapamycin (reviewed in **Schreiber et al 1991**). The dispensability of FKBP12 for survival demonstrated by its targeted disruption in yeast (**Heitman et al 1991b**), which was inconsistent with the demonstrated antiproliferative capacity of rapamycin treatment, suggested that FKBP12 inhibition is unlikely to be the direct mechanism through which rapamycin inhibits growth (reviewed in **Heitman et al 1992**). Significantly however, the other three mutants

isolated through rapamycin resistance selection led to the identification of two genes to which the mutants were mapped, called target of rapamycin (TOR) 1 and 2 (**Heitman et al 1991a**), whose cellular functions, as described below, *could* account for the antiproliferative properties of rapamycin.

Shortly after the identification of TOR in yeast, the mammalian homolog of the molecule, mTOR (289-kDa, also initially referred to as FRAP, RAFT, or RAPT) was isolated by virtue of its capacity to bind FKBP12-rapamycin (**Brown et al 1994, Chiu et al 1994, Sabatini et al 1994, Sabers et al 1995**), and was subsequently identified across the eukaryotic evolutionary spectrum, including orthologs in *S. pombe* (**Kawai et al 2001**), *C. elegans* (**Long et al 2002**), *Drosophila* (**Oldham et al 2000, Zhang et al 2000**), and plants such as *Arabidopsis thaliana* (**Menand et al 2002**). Interestingly, the presence of two distinct TOR genes appears to be unique to the protozoan yeast and fungal classes, while higher eukaryotes, including metazoans, encode a single TOR gene. All TOR orthologs share a similar domain structure, and are classified as members of the PI3K-like kinase (PIKK) family, which also includes large protein kinases such as DNA-PK, ATM (ataxia telangiectasia mutated), and ATR (ataxia telangiectasia mutated and Rad3-related), that enable cells to cope with metabolic, environmental, and genetic stresses (reviewed in **Lempiäinen and Halazonetis 2009**). The genetic characterization of TOR function throughout the 1990s attributed an important role for its activity in the coordination of cellular growth and proliferation in response to both circulating growth factors as well as the regulation of the cellular response to changes in nutrient availability (reviewed in **Lorberg and Hall 2004**), as evidenced by the demonstration that rapamycin-treated, or TOR-deficient yeast cells were phenotypically indistinguishable from starved cells (**Barbet et al 1996**).

Since the stimulation of phosphorylation at the Akt HM was known to be rapamycin-insensitive, mTOR was not seriously considered as a possible PDK2 candidate until TOR was demonstrated to exist in one of two complexes in yeast (**Loewith et al 2002**), the first of which was acutely rapamycin-sensitive, and distinguished by the presence of a protein called TORC1, which is homologous to the mammalian Raptor protein; and a second rapamycin-*insensitive* complex, that is distinguished by the presence of TORC2, which is highly similar to the mammalian

Rictor protein, also called mAVO3 (mammalian adheres voraciously 3). The mammalian mTORC1 complex ([Diagram 1-7](#)) was the first AGC family HM motif-kinase to be characterized, and in a complex with Raptor (**Hara et al 2002, Kim et al 2002**), and mLST8, the mammalian homolog of lethal with Sec13 protein 8 (**Kim et al 2003**), was shown to phosphorylate the S6K1 HM at T389, thereby contributing to its activation. Meanwhile, the mTORC2 complex ([Diagram 1-7](#)) was shown to mediate the so-called rapamycin-insensitive functions of mTOR in the process of actin cytoskeletal organization (**Jacinto et al 2004**), leading to speculation that it may also function as the sought-after rapamycin-insensitive cellular Akt HM kinase. Soon thereafter, a seminal study by David Sabatini and his colleagues (**Sarbassov et al 2005**) convincingly reported that the mTORC2 complex consisting of mTOR, mLST8, and rapamycin-insensitive companion of mTOR (Rictor) was in fact the Akt HM kinase, a finding that has since been recapitulated in other systems, and has generally become accepted in the field as the preeminent PDK2 (reviewed in **Reiling and Sabatini 2006, Guertin and Sabatini 2007**). This mTORC2 complex, which also consisted of mammalian stress-activated protein kinase-interacting protein (mSin1, also called MAPKAP1) variants (**Frias et al 2006, Jacinto et al 2006, Yang et al 2006**), and PROTOR (**Pearce et al 2007**), was demonstrated: (1) to phosphorylate the Akt HM in vitro; (2) to enhance subsequent PDK1-mediated T-loop phosphorylation; and (3) to be sensitive to PI3K inhibitors, but insensitive to the PDK1 inhibitor staurosporine (**Sarbassov et al 2005**), both being standard pharmacological properties that must be applicable to any potential PDK2 candidates (reviewed in **Dong and Liu 2005**). Notably, despite its initial characterization as a rapamycin-insensitive complex in response to acute treatment under conditions that strongly inhibited mTORC1, mTORC2 and its role in the activation of Akt were nonetheless demonstrated to be vulnerable to rapamycin following *prolonged* exposure in certain cell types (**Sarbassov et al 2006**). Rapamycin-associated FKBP12 binds and inhibits Raptor-bound (but not Rictor-bound) mTOR (**Loewith et al 2002, Sarbassov et al 2004, Jacinto et al 2004**), and in doing so, is thought to promote the dissociation of Raptor, which acts as a scaffold for mTORC1-interacting molecules, thus preventing access to its substrates (**Kim et al 2002, Yip et al 2010**). Its effect on mTORC2, detected following chronic exposure, has been proposed to result from the sequestration of the

cellular pool of mTOR into a complex with FKBP12-rapamycin, thereby progressively reducing the availability of mTOR for assembly into either mTOR complex, though inhibiting mTORC1 most severely (**Sarbassov et al 2006**).

The siRNA-mediated knockdown of any of the primary mTORC2 components – mTOR, Rictor, mLST8, or mSin1 – was found to result in the inhibition of Akt HM phosphorylation both in response to insulin stimulation in cultured cells, and *in vivo* in genetically modified mice (**Frias et al 2006, Guertin et al 2006**). Nonetheless, the growth-promoting signaling mediated by Akt has been shown to be dependent on mTORC2 in a tissue-, cell type-, and developmental stage-specific manner. Although the activation of Akt does not require mTORC2 activity in skeletal muscle (**Bentzinger et al 2008**), it is the main Akt HM kinase in several human cancer cell lines (**Sarbassov et al 2005**); in agonist-stimulated 3T3-L1 adipocytes (**Hresko and Mueckler 2005**), in which the role of Akt in glucose uptake is well documented; and in murine embryos, both at the one-cell stage (**Zhang et al 2012**), as well as throughout embryogenesis, as demonstrated by its requirement for fetal growth and viability in Rictor-deficient mutant mice (**Shiota et al 2006**). Interestingly, although the loss of Rictor in *C. elegans*, *Drosophila*, murine, and human cells results in the loss of Akt HM phosphorylation (**Sarbassov et al 2005, Yang et al 2006, Guertin et al 2006, Soukas et al 2009**), its effect on Akt substrates is not uniform, as the Akt-mediated phosphorylation of TSC2 (see **Section 1-4.3**) or GSK3 (see **Section 1-4.2**) was unaffected in Rictor-deficient mutants, while the Akt-mediated phosphorylation of the forkhead box subgroup O transcription factors FoxO1 and FoxO3 (see **Sections 1-4.1** and **1-4.7**) was suppressed, suggesting a differential requirement for levels of Akt activation among its substrates, and a potential role for HM phosphorylation as a threshold determinant in the differential regulation of Akt targets (**Guertin et al 2006**).

The role of TORC2-mediated Akt HM phosphorylation in the regulation of growth was also investigated *in vivo* in *C. elegans* and *Drosophila*. Whereas mTORC2 activity is essential for development and required for viability in mice (**Shiota et al 2006**), TORC2 activity was found to be dispensable for viability in both *C. elegans* and *Drosophila* (**Hietekangas and Cohen 2007, Jones et al 2009, Soukas et al 2009**). In *C. elegans*, in which the loss of Rictor results in mild developmental delay, slight decrease

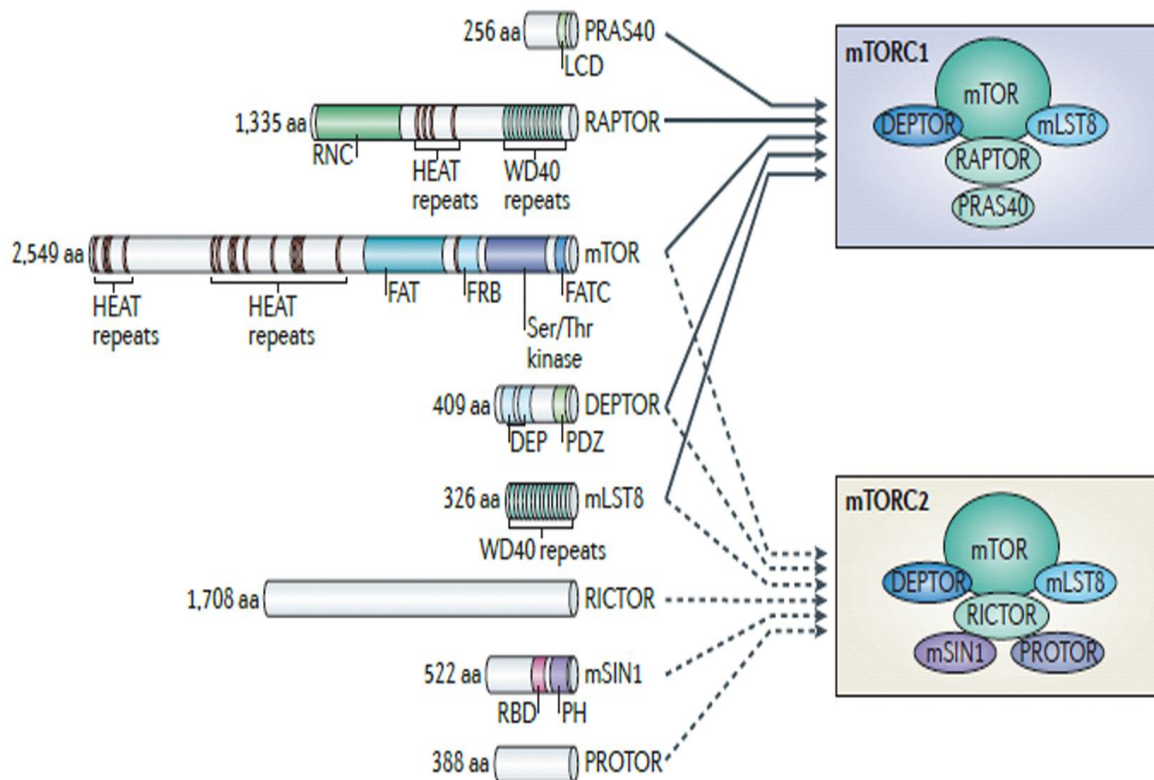


Diagram 1-7. The molecular composition of mTORC1 and mTORC2. Reproduced from Zoncu et al (2011b). mTORC1 and mTORC2 have both shared and unique components. They both contain DEPTOR and mLST8. RAPTOR and PRAS40 are unique to mTORC1, while RICTOR, mSin1 and PROTOR are specific to mTORC2. The domain organization of mTOR resembles that of other PI3K-related protein kinase (PIKK) family members. At the N-terminus, there is a cluster of HEAT (huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR1) repeats, which mediate protein-protein interactions. These are followed by a FAT domain (FRAP, ATM and TRRAP); the FKBP12-rapamycin binding (FRB) domain, which mediates the inhibitory action of rapamycin on RAPTOR-bound mTOR; the S/T kinase catalytic domain; and the C-terminal FATC domain. PRAS40 has a conserved leucine charged domain (LCD), at which phosphorylation by Akt occurs. The scaffolding function of RAPTOR is reflected by its composition of protein-binding domains including several HEAT repeats, which are followed by seven WD40 domains arranged in a β -propeller. DEPTOR consists of tandem DEP domains (Dishevelled, EGL-10 and pleckstrin) that are followed by a single PDZ domain (postsynaptic density of 95-kDa, Discs large, and zonula occludens 1). mLST8 is highly conserved, and its seven WD40 domains form a β -propeller that mediates protein-protein interactions. RICTOR and PROTOR have no clearly identifiable domains or motifs. mSin1 contains a Ras binding domain (RBD), and a pleckstrin homology (PH) domain that is likely to interact with phospholipids. RAPTOR N-terminal conserved (RNC).

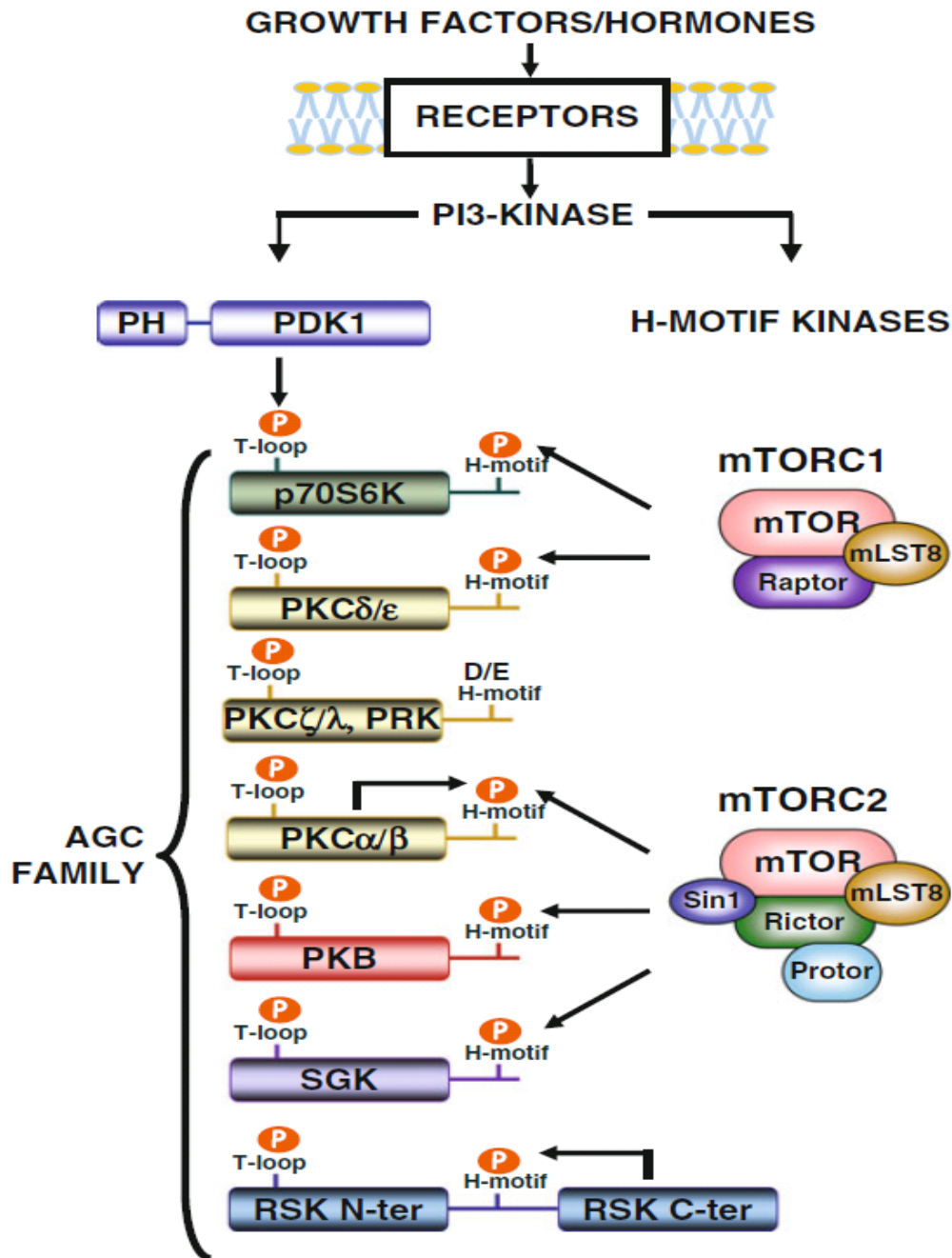


Diagram 1-8. Mechanisms of agonist-stimulated AGC kinase activation by dual T-loop and HM phosphorylation. Reproduced from Bayascas (2010). Following RTK activation and PI3K activation, mTORC1 and mTORC2 play distinct roles in the HM phosphorylation of specific AGC kinases, while PDK1 is a master regulator of AGC kinase T-loop phosphorylation. The HM of PKC β II is autophosphorylated following T-loop phosphorylation by PDK1, whereas the PKC α HM is targeted by mTORC2. The C-terminal RSK kinase domain is activated by ERK1/2, and results in its HM autophosphorylation. See text for details.

in body size, and increased lipid storage, TORC2 was shown to regulate reproduction, life span, lipid metabolism and growth (**Jones et al 2009, Soukas et al 2009**), but with the exception of lipid metabolism which was attributed to the activity of both Akt (Akt-1 and Akt-2) and SGK (Sgk-1), the effects on reproduction, lifespan, and growth were attributed to its regulation of SGK (and not Akt), which as described below, is also activated by mTORC2-mediated HM phosphorylation in mammals (**García-Martínez and Alessi 2008, Yan et al 2008**). In fruit flies, which lack an ortholog of SGK, and therefore represent a more insulated physiological setting for the investigation of the relevance of TORC2-mediated Akt HM phosphorylation *in vivo*, the functional deficiency for either dRictor or dSin1 was found to diminish the HM (S505) phosphorylation of Dakt (**Hietakangas and Cohen 2007**). However, whereas Dakt, the sole ortholog of Akt in *Drosophila*, is required for embryonic viability and development (**Staveley et al 1998**), and the loss of its function results in a cell-autonomous growth phenotype characteristic of positive regulators of PI3K/Akt signaling mutants in *Drosophila* (**Verdu et al 1999, Scanga et al 2000**), the loss of TORC2-mediated S505 phosphorylation (either through TORC2-deficiency or S505A mutation) was found to be dispensable for development, not required for viability, and surprisingly, only resulted in minor growth impairment (**Hietakangas and Cohen 2007**). Similarly, HM phosphorylation at S505 was also shown to be dispensable for growth, while the importance of T-loop phosphorylation at T342 was elegantly demonstrated in experiments examining the growth phenotype of the eye-specific loss of *Dakt*, which led to a severe reduction in eye size. Whereas transgenic expression of a wildtype *Dakt* transgene restored growth in the mutant background, *Dakt* transgenes bearing a single mutation in either the PH domain (R45A substitution), or in the T-loop (T342A) did not show any rescue; whereas in contrast, transgenic expression of a *Dakt* mutant that cannot be phosphorylated at the HM (S505A) still managed to rescue the Dakt mutant phenotype, indicating a considerable retention of *in vivo* biological activity despite the absence of S505 phosphorylation (**Hietakangas and Cohen 2007**).

Interestingly however, in contrast to the mild growth phenotype associated with the loss of S505 phosphorylation in an otherwise wildtype physiological context, the loss of TORC2-mediated Akt phosphorylation was shown to significantly suppress the

hyperplastic growth phenotype observed in mutant backgrounds with ectopically elevated levels of Dakt activation such as dPTEN-deficiency (**Goberdhan et al 1999, Gao et al 2000**), suggesting that in *Drosophila* at least, HM phosphorylation is an important determinant of Akt-mediated growth in specific signaling contexts such as the achievement of maximal size, which requires near-maximal levels of Akt activation (**Hietakangas and Cohen 2007**). The demonstration that Rictor, and by extension, mTORC2 activity, are required for the development of prostate cancer induced by the loss of PTEN in mice (**Guertin et al 2009**), and other recent studies demonstrating a requirement for mTORC2 activity in the growth of several human tumor types including gliomas as well as breast and prostate cancer cells (**Masri et al 2007, Hietakangas and Cohen 2008**) effectively recapitulate this pathological correlation *in vivo*. Considering (1) the frequent elevation of basal Akt activity in human cancers, which is often caused by oncogenic mutations of upstream regulators such as PI3K and PTEN, or by the ectopic activation of PI3K by oncogenic mutations of Ras (reviewed in **Yuan and Cantley 2008**); and (2) the aforementioned evidence of the rapamycin-sensitivity of mTORC2 activity in cases of chronic exposure to rapamycin or its derivatives, and the relevance of its chronic effects on mTORC2 in a clinical setting (see **Chapter 4**); the importance of HM phosphorylation in such pathological signaling contexts (and perhaps just as importantly, the apparent relative insensitivity of some cellular functions to phosphorylation at this site) has profound implications in the context of tumorigenesis and cancer therapy, and underlies the development of numerous mTORC2-targeting pharmacological agents aimed at preventing mTORC2-mediated Akt activation.

Incidentally, the original assertion that the HM phosphorylation of agonist-dependent AGC family members, unlike the PDK1-mediated phosphorylation of the T-loop, was unlikely to be carried out by a “master regulator” was determined to be partially, but not completely true. The RSK molecule is unusual for an AGC kinase in that it is a S/T kinase with two distinct (N-terminal and C-terminal) catalytic domains linked by a central HM. The N-terminal kinase domain is typical of AGC kinases, while the C-terminal catalytic domain is most similar to the CAMK (Ca²⁺/calmodulin-dependent protein kinase) family, and serves a regulatory role (reviewed in **Anjum and Blenis 2008**). In response to mitogen stimulation and MAPK pathway activation,

ERK1/2 phosphorylates and activates the C-terminal kinase domain, resulting in the autophosphorylation of the central HM residue (**Dalby et al 1998**), which is essential for the aforementioned PDK1-mediated activation of the N-terminal catalytic domain (**Frödin et al 2000**). Unlike RSK however, the HM phosphorylation of the remaining members of the agonist-stimulated AGC members (S6K, SGK, PKC, and Akt) seems to involve the mTOR molecule, but true to the original assertion, depends on one of the two distinct mTOR-associated complexes. Like the mTORC1-dependent phosphorylation of the S6K1 HM (**Burnett et al 1998, Isotani et al 1999**), the phosphorylation of the corresponding HM residue in PKC δ and PKC ϵ was shown to be both nutrient and rapamycin-sensitive, suggesting an mTORC1-dependent mechanism of HM phosphorylation (**Parekh et al 1999**). On the other hand, the ablation of mTORC2 components in mice has revealed that mTORC2, in addition to being the Akt HM kinase (**Sarbassov et al 2005**), is also the HM kinase for both PKC α (**Sarbassov et al 2004, Guertin et al 2006**), and SGK (**García-Martínez and Alessi 2008, Yan et al 2008**), completing the identification of the HM kinases of the agonist-stimulated PDK1-regulated AGC family members (**Diagram 1-8**). Importantly however, the characterization of mTORC2 as the predominant physiological PDK2 molecule for Akt does not preclude the capacity of some of the other previous PDK2 candidates (or yet undiscovered kinase activities) to target the Akt HM for phosphorylation in response to stimuli other than growth factor-dependent PI3K activation. The conceptual paradigm in which signaling context allows for the input and integration of multiple and distinct HM-phosphorylating kinase activities is supported by the suggested existence of yet uncharacterized mTOR complexes distinct from mTORC1 and mTORC2 with roles in the regulation of cellular growth (**García-Martínez et al 2009**), and the reported capacity of another PIKK family member, the DNA-dependent protein kinase (DNA-PK), to act as a major Akt HM kinase in the nucleus for the induction of Akt-dependent survival-promoting signals in response to DNA damage (**Feng et al 2004,Dragoi et al 2005, Boehme et al 2008, Bozulic et al 2008**).

The dependence of PDK1-mediated Akt T-loop phosphorylation on PIP₃, consistent with the induction of its activation downstream of agonists like insulin and IGF, as well as the presence of a PH domain in both PDK1 and Akt, initially led to

speculation that the PI3K-mediated agonist-induced phosphorylation of the Akt HM residue may also be regulated by a similarly phospholipid binding-dependent mechanism (**Coffer et al 1998a**). In the mTORC2 complex, the mTOR molecule is the catalytic subunit responsible for the phosphorylation of the Akt HM, but itself lacks a PI-interacting module such as a PH domain, though the complex has been found to localize to the membrane proper, as well as various endomembranes including vesicular structures and the ER in both yeast and mammals (**Kunz et al 2000, Wedaman et al 2003, Boulbés et al 2011**). With respect to the mTOR molecule itself (*Diagram 1-7*), all TOR orthologs share a similar domain structure, consisting of a C-terminal catalytic domain related to that of PI3K but lacking lipid kinase activity in favor of S/T-specific protein kinase activity (**Cafferkey et al 1993, Kunz et al 1993**). Upstream of the kinase domain is the FKBP12-rapamycin binding domain (FRB), which as its name suggests, is the direct binding target of FKBP12-rapamycin to which mutations conferring rapamycin resistance are mapped (**Helliwell et al 1994, Stan et al 1994, Chen et al 1995, Choi et al 1996**), and is followed further upstream by the so-called FAT domain, which is found in all PIKKs, and is itself always accompanied by a C-terminal-most FATC domain (**Alarcon et al 1999, Bosotti et al 2000**), and thought to mediate protein-protein interaction by serving as a binding scaffold. Lastly, the N-terminus of the TOR molecule encodes two large blocks of HEAT repeats, named after the molecules in which they were initially identified (Huntington, elongation factor 3, the A subunit of PP2A, and TOR1), which are also commonly thought to mediate protein-protein interactions (reviewed in **Pawson and Nash 2003**). Subcellular fractionation and immunofluorescence studies in yeast demonstrated that both TOR1 and TOR2 are membrane-associated (though with distinct distribution), and deletion analysis showed that the N-terminal blocks of HEAT repeats were independently responsible for the localization of TOR to membrane compartments (**Kunz et al 2000**), though whether this localization was achieved through the direct interaction of the HEAT repeats with membrane compartments, or alternatively, through their interaction with other membrane associated molecules remained unclear. A common mechanism of subcellular localization is unlikely however, since TORC1 and TORC2 have both overlapping, as well as distinct distribution patterns, with TORC1 predominantly located at vacuolar, late-endosomal, and lysosomal membranes, whereas

TORC2 can be detected at various endomembranes, including early endosomes, vesicular structures, and the ER (**Wedaman et al 2003, Berchtold and Walther 2009, Flinn et al 2010, Boulbés et al 2011, Zoncu et al 2011a**).

Subcellular localization can also, however, be dictated by the mTORC1- and mTORC2-specific companion molecules, which function as scaffolds for complex assembly and substrate/regulator binding (reviewed in **Zoncu et al 2011b**). In the case of mTORC2, the Rictor molecule, which is the main determinant of mTORC2 identity, and the PROTOR1 and PROTOR2 (protein observed with Rictor 1 and 2) molecules, which likely aid complex assembly, all lack clearly identifiable domains or motifs ([Diagram 1-7](#)). The mLST8 molecule is shared by both mTORC1 and mTORC2 as a positive regulator of mTOR function (**Loewith et al 2002**), but lacks a specific membrane interaction motif, and is composed entirely of WD40 repeats implicated in the establishment of protein-protein interactions. The recently identified DEP domain-containing mTOR-interacting protein (DEPTOR), which is also shared by both complexes, acts as a negative regulator of mTORC activity (**Peterson et al 2009**), and most notably encodes a C-terminal PDZ domain that likely directs its protein-protein interactions, as well as an N-terminal DEP domain named after the molecules in which it was first identified - Dishevelled, EGL-10, and pleckstrin domains, and which has been implicated in the regulation of membrane localization (reviewed in **Chen and Hamm 2006**), though a similar role in the case of mTORC subcellular distribution remains to be demonstrated. Last but not least, the mSin1 molecule represents the likeliest candidate (outside of the mTOR molecule's HEAT repeats) to target mTORC2 to membrane compartments (**Frias et al 2006, Yang et al 2006, Pearce et al 2007**), and has been proposed to do so through its C-terminal RBD and PH domains, consistent with the membrane localization of TORC2 in yeast, which was demonstrated to be (1) essential for viability, and (2) mediated by lipid binding of the C-terminal domain of AVO1, the yeast homolog of Sin1 (**Berchtold and Walther 2009**).

1-3.4 – Current mechanistic model of phosphorylation-dependent Akt activation

As shown in [Diagram 1-9](#), the activation of Akt is a crucial event in the stimulation of proliferation-, growth-, and survival-promoting processes downstream of

RTK/PI3K activation. Although a number of constitutively maintained post-translational modifications that stabilize the nascent Akt molecule precede its availability for catalytic activation (**Bellacosa et al 1998**, reviewed in **Liao and Hung 2010**), two key phosphorylation events, one in the activation loop, and the other in the regulatory domain, are necessary and sufficient for the catalytic activation of Akt. The phosphorylation of Akt at the T-loop (T308 in Akt1) by PDK1 is required for catalytic activation, and cooperative phosphorylation of the HM (S473 in Akt1) by mTORC2 is necessary for full catalytic efficiency. The agonist-stimulated phosphorylation of these two sites has been proposed to occur independently, with no absolute requirement of the prior phosphorylation of either site for the phosphorylation of the other. The presumed independence of the phosphorylation at these two crucial sites was initially predicated on the demonstration *in vitro* that the T308A Akt1 mutant construct overexpressed in IGF1-stimulated cells could still be phosphorylated at S473, and similarly, the reciprocal S473A Akt1 mutant could itself still be phosphorylated at T308 (**Alessi et al 1996a**). Following the identification of PDK1 and mTORC2 as the upstream kinases responsible for the phosphorylation of these sites, subsequent studies would mostly recapitulate these findings both in cultured cells and *in vivo*. As mentioned above, mice lacking the mTORC2 components Rictor, mLST8, and mSin1, could still efficiently phosphorylate T308 despite the loss of phosphorylation at S473 (**Jacinto et al 2006**, **Guertin et al 2006**, **Shiota et al 2006**); while in PDK1-deficient cultured cells or in cases of conditional deficiency in insulin-sensitive tissues (the liver and cardiac muscle), the phosphorylation of Akt at S473 was found to be maintained even in the absence of T308 phosphorylation (**Williams et al 2000**, **Mora et al 2003**, **Mora et al 2005**). However, the RNAi-mediated knockdown of Rictor, which presumably disrupted the formation of mTORC2, or treatment with potent small molecule inhibitors of mTOR such as Ku-00637954 and Torin1, were both found to reduce *both* S473 and (though to a lesser extent, and with variance from study to study), T308 phosphorylation levels as well (**Hresko et al 2005**, **Sarbassov et al 2005**, **García-Martínez et al 2009**, **Thoreen et al 2009**), suggesting that S473 phosphorylation may itself potentially promote the Akt-PDK1 interaction, or T-loop phosphorylation. Both possibilities are supported by the finding that Akt HM phosphorylation induces a disorder-to-order transition in the kinase

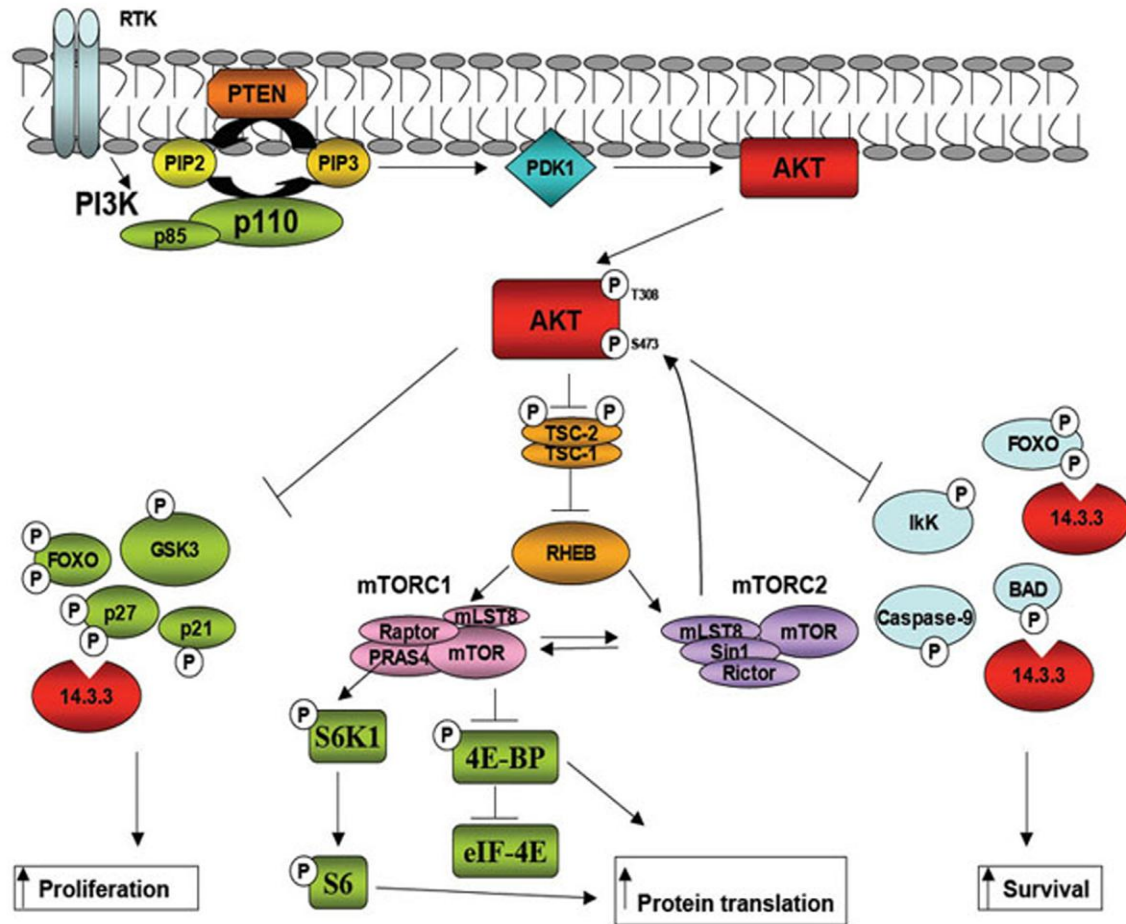


Diagram 1-9. Substrates and signaling processes regulated by Akt activation.

Reproduced from **Viglietto et al (2011)**. In RTK/PI3K/Akt-mediated cellular processes such as the promotion of proliferation, protein translation, and survival, Akt effectors include GSK3 α and β , TSC2, the pro-apoptotic Bcl-2 family member BAD, and the forkhead family of transcription factors (FoxO). See text for details.

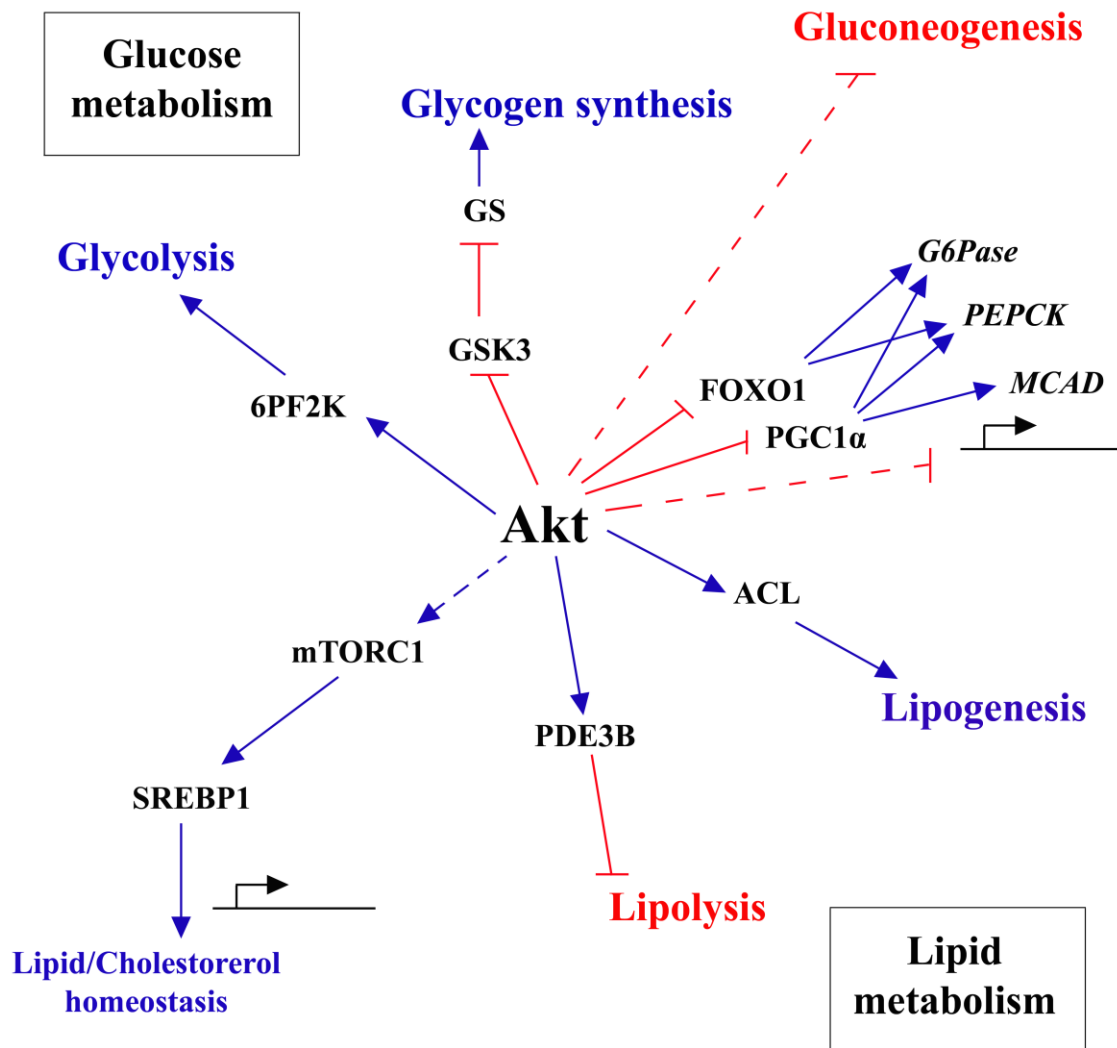


Diagram 1-10. Direct and indirect Akt effectors in glucose and lipid metabolism. In the regulation of glucose metabolism, Akt directly phosphorylates: 6-phosphofructo-2-kinase, activating the enzyme and stimulating glycolysis; GSK3, inhibiting the enzyme, and promoting glycogen synthesis; and the transcription factors PGC1 α and FoxO1, preventing their activation of *G6Pase*, *PEPCK*, and *MCAD* transcription, thereby indirectly inhibiting gluconeogenesis. In the regulation of lipid metabolism, Akt directly phosphorylates: ACL, activating the enzyme and stimulating lipogenesis; and PDE3B, activating the enzyme and inhibiting lipolysis. Furthermore, Akt signaling indirectly activates mTORC1 downstream of Akt-mediated TSC1/2 inhibition, resulting in the transcriptional activation of SREBP1 target genes involved in the maintenance of lipid and cholesterol homeostasis. Processes in blue indicate positive Akt-dependent regulation, while processes in red indicate Akt-dependent inhibition. See text for details.

domain and a concomitant restructuring of the T-loop (**Frödin et al 2002, Yang et al 2002a, Yang et al 2002b**); and by the demonstration that the interaction of PIP₃ with the Akt PH domain itself induces a conformational change thought to expose the T-loop for PDK1-dependent phosphorylation (**Thomas et al 2002, Milburn et al 2003**).

The agonist-stimulated phosphorylation of Akt by PDK1 is PIP₃-dependent (**Alessi et al 1997a, Stokoe et al 1997**), and as mentioned previously, both molecules encode a PH domain with high affinity for PIP₃. In the case of Akt, the PIP₃ molecule serves two purposes in its interaction with the Akt PH domain: first, it promotes the enrichment of Akt at the membrane in response to PI3K activation (**Andjelković et al 1997**), and second, as described below, it redirects the PH domain away from its autoinhibitory intramolecular conformation, allowing the Akt-activating kinases access to their targeted T-loop and HM phosphorylation sites. In contrast to the Akt PH domain, the determination of the PDK1 PH domain crystal structure (**Komander et al 2004a**) suggests that the molecule does not undergo a conformational change in response to PI-binding (**Komander et al 2004b**), nor does this binding alter its catalytic activity (**Alessi et al 1997b, Stephens et al 1998, Currie et al 1999**). However, a number of studies suggest that the PDK1 PH domain is an important contributor to the efficient colocalization of PDK1 and Akt at the membrane in response to increases in PIP₃ abundance. The disruption of the interaction between the PDK1 PH domain and PIP₃ by various PH domain “knockin” substitution mutations such as K465E (positively charged lysine to negatively charged glutamate) and R474A (positively charged arginine to hydrophobic non-polar alanine) have been shown to result in PDK1 cytosolic retention and loss of membrane localization (**Anderson et al 1998**), as well as PDK1 hypophosphorylation (**Scheid et al 2005**); while severely affecting the activation of Akt in ES cells and mutant mice *in vivo* (**McManus et al 2004**), and doing so without altering intrinsic PDK1 catalytic activity (**Bayascas et al 2008**). Nonetheless, homozygous K465E mutant mice (**Bayascas et al 2008**), like hypomorphic mutants that express significantly lower levels of the wildtype PDK1 protein (**Lawlor et al 2002**), are viable, though insulin resistant and of smaller size (~50% of wildtype), and stand in stark contrast with global loss of PDK1 function in knockout animals, which results in embryonic lethality (**Lawlor et al 2002**).

Unlike other AGC kinases, the PDK1 molecule does not itself possess an HM, but instead encodes an HM-binding pocket (PIF) that recognizes its docking site on substrates (agonist-stimulated AGC kinases), binding those with a phosphorylated HM, and catalyzing their T-loop phosphorylation (reviewed in **Bayascas 2010**). Accordingly, in ES cells expressing PIF pocket PDK1 mutants incapable of recognizing the phosphorylated HM of their substrates, the activation of S6K, RSK, and SGK by growth factors was severely impaired *in vivo*, as was the PDK1-dependent stabilization of a number of PKC isoforms, whereas by contrast, Akt activation was unencumbered (**Collins et al 2003, Collins et al 2005**), suggesting that the PDK1-Akt interaction occurred independently of the former's PIF pocket, and therefore, unlike its interaction with other AGC kinases, did not require prior Akt HM phosphorylation – a finding that is consistent with the aforementioned independence of the S473 and T308 phosphorylating activities. Furthermore, the T-loop autophosphorylation of PDK1, though suggested to contribute to catalytic activation upon substrate-binding, was also shown not to be required for the structural integrity of the PIF pocket, suggesting that the interaction of PDK1 with its substrates could occur even in the absence of PDK1 autophosphorylation at S241 (**Komander et al 2005**). Furthermore, an interaction between PDK1 and the scaffolding protein 14-3-3 has been shown to depend on the S241 residue of the PDK1 T-loop, and to negatively regulate the autophosphorylation of PDK1 both *in vitro* and *in vivo*, suggesting a mechanism through which PDK1 autophosphorylation could be prevented in the absence of substrate binding (**Sato et al 2002**).

Subsequent studies using fluorescent reporters have further refined our understanding of the cooperativity between HM and T-loop phosphorylation, and suggest that PDK1 exists as a complex with Akt *in vivo*, in which Akt is maintained in an inactive state through intramolecular interactions between its PH and kinase domains in a so-called “PH-in” conformation, preventing PDK1-mediated T-loop phosphorylation at T308, while a similar interaction between the HM and the kinase domain precludes ectopic HM phosphorylation (**Calleja et al 2007, Calleja et al 2009**). In the inactive conformation, a PH-domain-induced cavity in the kinase domain was described, whose formation was dependent on a tryptophan residue (W80) in the PH domain, and whose apex mediated the inhibitory interaction with the C-terminal HM (**Calleja et al 2009**),

suggesting cooperativity in the establishment of the PH domain's and HM's respective inhibitory conformations. Interestingly, a recent study (**Warfel et al 2011**) has demonstrated that constructs of Akt in which the PH domain is constitutively excluded from its inhibitory interaction with the catalytic domain (either by constitutive membrane targeting or by PH domain truncation) are promiscuously phosphorylated at the HM S473 residue in mSin1-deficient MEFs (which therefore lack the capacity for mTORC2-mediated S473 phosphorylation), suggesting that the primacy of mTORC2 as the preferred cellular Akt HM kinase, rather than reflecting a uniquely high affinity for the Akt HM as a substrate, may partially lie in its putatively distinctive capacity (in relation to other potential HM kinases) to aid the membrane translocation-dependent relief of the autoinhibitory conformation adopted by the PH domain in the inactive PH-in state, thereby exposing its target, the HM residue, for subsequent phosphorylation.

Cumulatively, the emerging model of Akt activation posits that the agonist-induced increase in membrane PIP₃ promotes the colocalization of Akt and PDK1 at the membrane. This membrane recruitment is promoted by the displacement of the Akt PH domain from its autoinhibitory interaction with its kinase domain (the PH-in conformer), and this conformational transition may be aided or stabilized by the coordinated interaction of Akt with mTORC2, resulting in the relief of autoinhibition (the PH-out conformer). The displacement of the PH domain from the kinase domain also eliminates the PH domain-induced cavity in the kinase domain, liberating the HM, and making it available for subsequent mTORC2-dependent HM phosphorylation. The phosphorylation of the freed Akt1 HM only weakly activates catalytic activity (3-5 fold in response to insulin/IGF), but favors a T-loop conformation that is suitable for PDK1-mediated T308 phosphorylation, resulting in a fully active Akt molecule (20-50 fold activation) which can then dissociate from PDK1 and the membrane, and transduce the appropriate downstream signals.

1-4 – The role of Akt signaling in the mediation of cellular growth and survival

In addition to the PI3K/Akt-mediated regulation of glucose uptake in insulin-sensitive tissues (reviewed in **Schultze et al 2011**), the context-dependent cellular

responses to Akt activation also direct diverse cellular processes including: (1) metabolic functions such the promotion of glycogen synthesis, protein synthesis, glycolysis, and lipogenesis, as well as the suppression of gluconeogenesis and lipolysis; (2) growth promoting processes including proliferation and cell cycle progression; as well as (3) the promotion of cellular survival and resistance to programmed cell death (reviewed in **Brazil et al 2002, Manning and Cantley 2007, Franke et al 2008, Hers et al 2011**). Substrates of Akt catalytic activity are distinguished by the presence of a minimal consensus recognition sequence defined as RXRXX(S/T) Ψ , with a preference for a bulky hydrophobic residue (Ψ) immediately downstream of the phospho-acceptor S/T residue (**Alessi et al 1996b**). The consensus recognition sequence is similar to that targeted by two other AGC kinases, RSK and S6K, with the exception of Akt's specific requirement for arginine residues upstream of the S/T phospho-acceptor (positions -2 and -5), which lies in contrast to the preference for a lysine at the corresponding residues in the substrate recognition of RSK and S6K (reviewed in **Manning and Cantley 2007**). As described herein, the regulation of PI3K/Akt-mediated metabolic growth and/or cellular survival responses are executed by diverse well-characterized targets of Akt catalytic activity, including the multifunctional S/T kinase GSK3 (glycogen synthase kinase 3), the pro-apoptotic factor BAD (Bcl-2 associated death promoter), the E3 ubiquitin ligase MDM2 (murine double minute 2), the FoxO (forkhead box subgroup O) family of transcription factors, and the tumor suppressor TSC2 (tuberous sclerosis 2, or tuberin), which in complex with TSC1 (also called hamartin), acts as a major upstream-acting inhibitory regulator of growth factor (GF)-dependent mTORC1 activation (**Diagram 1-9**).

1-4.1 –The role of Akt substrates in the promotion of cellular growth

Cellular growth, through which increases in cell size and/or cell number are achieved, occurs through a set of biochemical processes that promote the requisite *de novo* synthesis of cellular macromolecules including proteins required for the induced responses, storage carbohydrates that replenish or expand cellular stores, and lipids including fatty acids, which among other functions, allows for the storage of energy as fat. As described herein, these processes are coordinated with (1) the availability of the required building blocks (for example amino acids, glucose, and acetyl CoA); (2) the

cellular supply of energy needed to fuel their activity, which is usually provided in the form of ATP through the process of glycolysis (itself dependent on carbohydrate availability); and (3) the systemic input of circulated GFs like insulin, which integrate local nutrient/energy sensing mechanisms with the nutritional state of the organism as a whole. Cells also rely on a complementary set of biochemical processes that allow them to cope with nutrient starvation and low energy, during which primary biosynthetic or glycolytic programs are suppressed, while alternative mechanisms of biosynthesis based on the catabolic degradation and recycling of preexisting macromolecules are engaged. These alternative pathways are generally suppressed when organisms are well-fed, but provide the means to generate internal sources of energy and metabolites, while slowing (or ceasing) growth, and staving off death until feeding is resumed.

In response to PI3K-mediated activation, Akt can *directly* phosphorylate and modify the enzymatic activity of a number of substrates with rate-limiting “assembly line” functions in carbohydrate metabolism, thereby directly modulating the distinct enzymatic step regulated by the enzyme in question ([Diagram 1-10](#)). Examples include the Akt-mediated activating phosphorylation of 6-phosphofructo-2-kinase which was suggested to underlie the insulin-dependent stimulation of cardiac glycolysis (**Lefebvre et al 1996, Deprez et al 1997**); ATP-citrate lyase, which was identified as an Akt substrate in adipocytes, and whose Akt-mediated activation promotes the conversion of citrate to acetyl CoA (and oxaloacetate), thereby providing the essential building block for lipogenesis (**Berwick et al 2002**); and the 3B isoform of cyclic nucleotide phosphodiesterase (PDE3B), whose Akt-mediated phosphorylation in adipocytes inhibits lipolysis (**Wijkander et al 1998, Kitamura et al 1999**), thereby complementing the promotion of lipogenesis in the same cell type. In addition to its direct targeting of these enzymes with known roles as dedicated regulators of rate-limiting metabolic reactions, Akt also targets diverse signaling molecules with broader roles in cellular activity, which themselves modulate the functions of various effectors with metabolic, growth-promoting, and as discussed in **Section 1-4.7**, survival-promoting roles as well. An example directly relevant to the promotion of Akt-dependent lipogenesis described above is the requirement for mTORC1 activity, which as described in **Section 1-4.3**, can be activated downstream of Akt-mediated TSC1/2 inhibition, thereby promoting the nuclear

accumulation of the transcription factor SREBP1 (sterol-responsive element binding protein 1) and the expression of its transcriptional targets (**Porstmann et al 2008**), which are known to include various genes encoding enzymes with crucial functions in lipid and cholesterol homeostasis (**Yokoyama et al 1993**).

As shown in **Diagram 1-10**, another example is the transcriptional coactivator PGC1 α (peroxisome proliferator-activated receptor-coactivator 1 α), which not only cooperates with the transcription factor FoxO1 to transcribe genes such as *glucose-6-phosphatase* (*G6Pase*) and *phosphoenolpyruvate carboxykinase* (*PEPCK*); but also FoxO1-independently promotes the transcription of *medium-chain acetyl-CoA dehydrogenase* (*MCAD*) whose function is required for fatty acid catabolic oxidation (**Puigserver et al 2003**). In hepatic cells, the products of these genes are crucial components of gluconeogenesis, which is a glucose-generating metabolic pathway that utilizes alternative carbon sources such as pyruvate, lactate, or fatty acids; and is activated in response to the depletion of cellular stores of glucose and glycogen in order to maintain normal blood glucose levels, and to meet persisting systemic energy demands (reviewed in **Oh et al 2013**). Insulin, which is released into the bloodstream when carbohydrate abundance is restored in order to stimulate glucose uptake and the restoration of glycogen stores, accordingly decreases *G6Pase*, *PEPCK*, and *MCAD* transcription, and this transcriptional downregulation is attributed to the Akt-mediated phosphorylation and functional inhibition of FoxO1 and PGC1 α (**Li et al 2007**). The phosphorylation of PGC1 α is thought to prevent its association with transcriptional targets, while the inhibitory phosphorylation of FoxO molecules (FoxO1, FoxO3 α , and FoxO4) by Akt occurs in the nucleus at three conserved S/T residues (in FoxO1, two preferred sites at T24 and S256, and a third site at S319 preferred by SGK), two of which (T24 and S256) create 14-3-3 binding sites (reviewed in **Huang and Tindall 2007**). The interaction with 14-3-3 is known to inhibit the transcription of FoxO-targeted genes through the exclusion of phosphorylated FoxO molecules from the nucleus by virtue of their 14-3-3-mediated cytoplasmic retention, which, incidentally, is a typical function of 14-3-3 molecules (reviewed in **Dougherty and Morrison 2004, Morrison 2009**), and a common mode of Akt-mediated substrate inhibition (reviewed in **Manning and Cantley 2007**).

Additional important aspects of PI3K/Akt-mediated regulation of metabolism and growth include the synthesis of glycogen from excess glucose, which, in muscle cells for example, is the main polysaccharide form of carbohydrate storage; and protein synthesis, whose stimulation downstream of various agonists is coordinated with amino acid availability. Their regulation by GSK3, which is itself a target of Akt; and the mTORC1 complex, which can be activated downstream of Akt-mediated TSC2 and PRAS40 inhibitory phosphorylation, is summarized below.

1-4.2 – The role of Akt-mediated GSK3 inhibition in cellular growth

In the 1960s, the stimulation of nutrient uptake and the concomitant acceleration of their conversion into functional or storage macromolecules was established as the principal metabolic action of insulin in insulin-sensitive cells (reviewed in **Cohen et al 1997**). Joseph Larner and his colleagues discovered that glycogen synthase (GS), which catalyzes the rate-limiting step of glycogen synthesis, was inactivated by phosphorylation, and activated by dephosphorylation (**Friedman and Larner 1963**); and that insulin stimulated the activation of glycogen synthase (the first enzyme shown to be regulated by insulin) within minutes of insulin treatment by promoting its dephosphorylation (**Craig and Larner 1964**), further suggesting that one or more intermediate catalytic activities may be responsible for its insulin-dependent activation. Following its identification as a cAMP-dependent kinase (**Walsh et al 1968**), PKA was shown to phosphorylate GS *in vitro*, and to decrease its activity, but cAMP levels were insensitive to insulin treatment, suggesting that GS phosphorylation *in vivo* may be carried out by another enzyme. This enzyme was subsequently identified as GSK3, whose activity was first identified by Philip Cohen and colleagues from rabbit skeletal muscle extracts based on its phosphorylation and inactivation of glycogen synthase (**Embi et al 1980, Rylatt et al 1980**). The GSK3 molecule was subsequently purified by several groups throughout the 1980s from rabbit skeletal muscle (**Woodgett and Cohen 1984**) and bovine brains (**Hemmings et al 1982, Tung and Reed 1989**), in which they are highly expressed; and thereafter cloned from rat brains by James Woodgett (**Woodgett 1990, Woodgett 1991**).

Studies examining the insulin-stimulated mechanisms of fatty acid and protein synthesis, while determining that the activity of GSK3 was in fact inhibited by insulin, also identified two additional putative substrates of GSK3 activity: ATP-citrate lyase (**Hughes et al 1992**), whose activation, as mentioned above, converts citrate to acetyl-CoA, an essential building block of fatty acid chains; and eIF2B (**Welsh and Proud 1993**), a guanine nucleotide exchange factor (GEF) whose activity is essential for 43S ribosome-dependent initiation of translation and protein synthesis. Importantly, although its influence on ATP-citrate lyase activity remained unclear, these studies and others further suggested (1) that in the absence of insulin stimulation, GSK3 may play a constitutively *inhibitory* role in the control of at least two of the hallmark biosynthetic cellular responses to insulin stimulation (**Diagram 1-11**), namely GS-mediated glycogen synthesis and eIF-mediated protein synthesis; and (2) that the inhibitory activity of GSK3-mediated phosphorylation must itself be relieved in response to insulin stimulation, as previously demonstrated by Larner in the case of GS, and subsequently confirmed to occur in the case of eIF2B (**Welsh et al 1998**). The insulin-dependent relief of GSK3-mediated substrate inhibition was determined to be regulated by its N-terminal S/T phosphorylation (**Sutherland et al 1993, Welsh and Proud 1993, Cross et al 1994, Sutherland and Cohen 1994**), and following the prior demonstration in the early 1990s that PI3K is activated downstream of various RTKs including the IR, and the contemporaneous demonstration that Akt served as a central effector of PI3K activation (see **Section 1-3.1**), the search for S/T kinases that could phosphorylate GSK3 downstream of insulin stimulation converged with the quest for a *bona fide* Akt substrate, leading to the demonstration that the Akt-mediated phosphorylation of GSK3 inhibited the latter's activity in an insulin- and PI3K-dependent manner by the direct phosphorylation of the N-terminal regulatory serine residue (**Welsh et al 1994, Cross et al 1995**), thereby linking insulin stimulation with the inhibition of GSK3 and the promotion of protein and glycogen synthesis.

GSK3 is represented in mammals by two isoforms encoded by two distinct genes (*GSK3 α* and *GSK3 β*), belongs to the CMCG family of proline-directed kinases (cyclin-dependent kinase, MAPK, CDK-like kinase, and GSK), and is highly conserved throughout eukaryotes, with representatives in plants, fungi, nematodes, arthropods,

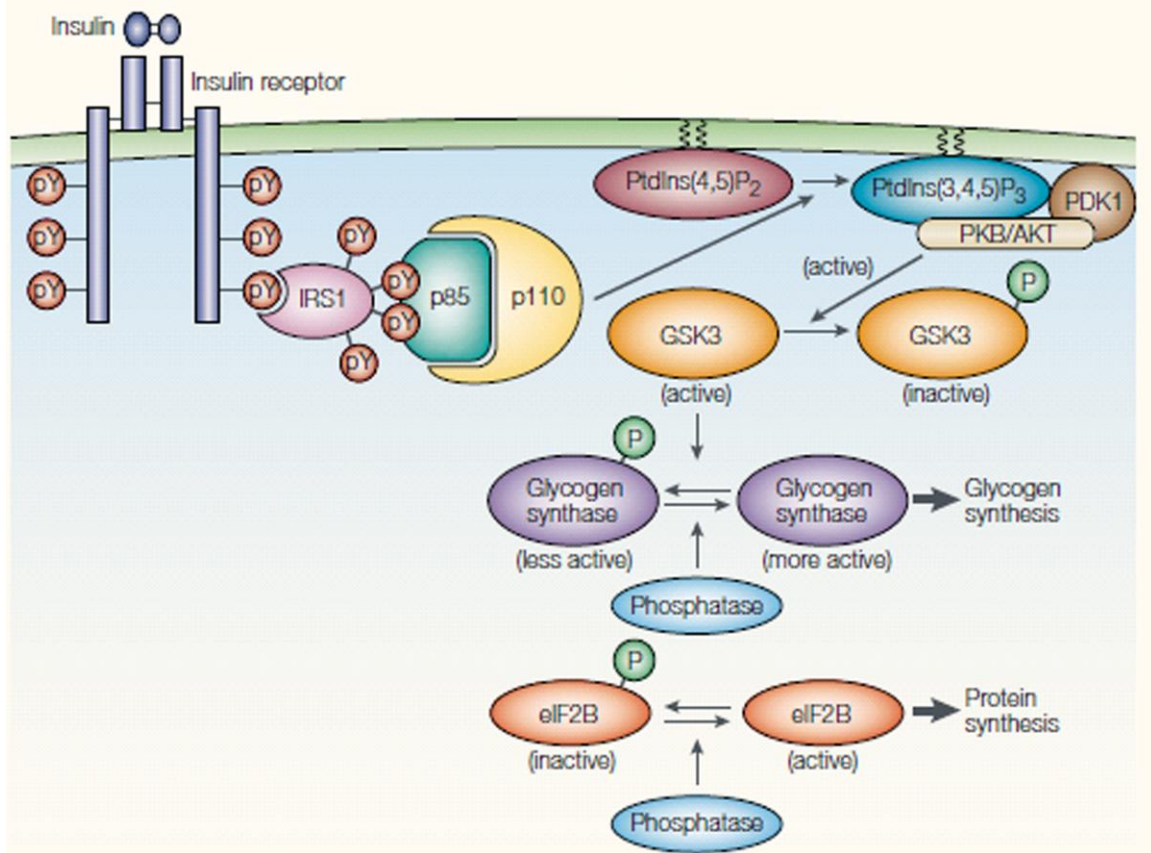


Diagram 1-11. Insulin-stimulated inhibition of GSK3 promotes glycogen and protein synthesis. Reproduced from **Cohen and Frame (2001)**. Akt phosphorylates and inhibits GSK3, relieving the latter's inhibitory effects on two of its key substrates – glycogen synthase and eIF2B. This inhibition of GSK3 and the consequent dephosphorylation and upregulation of these substrates contributes to the insulin-induced stimulation of glycogen and protein synthesis. See text for details.

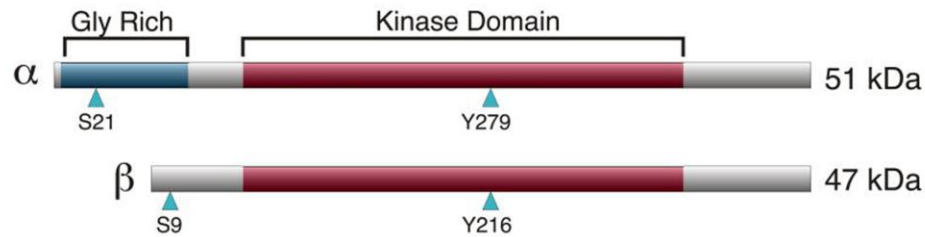
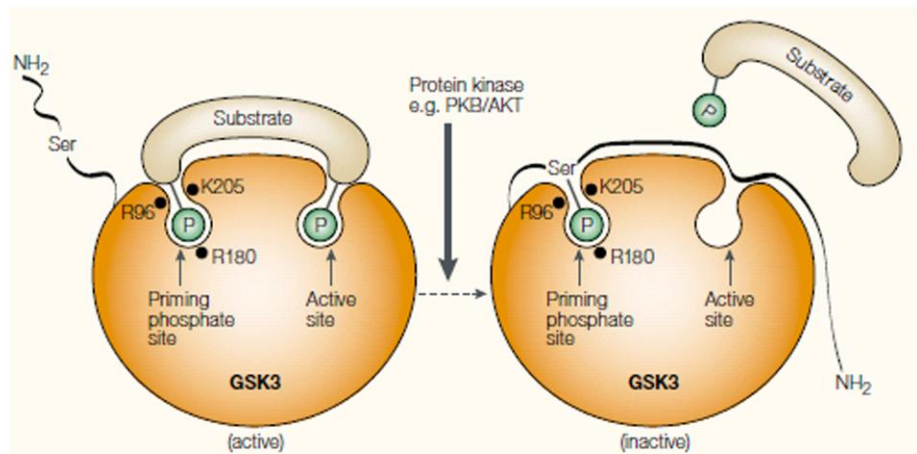
A**B**

Diagram 1-12. GSK3 structural organization and mechanism of autoinhibition. (A) Reproduced from **Doble and Woodgett (2003)**. Schematic representation of mammalian GSK3α and GSK3β. The N-termini house the S21/S9 residue whose phosphorylation inhibits GSK3 kinase activity by acting as a pseudo-substrate that competes with potential substrates for the kinase domain. The N-terminal glycine-rich domain is specific to GSK3α. The kinase domain T-loop of both isoforms is tyrosine phosphorylated at Y279/Y216, and constitutively maintained in resting cells. (B) Reproduced from **Cohen and Frame (2001)**. In the absence of inhibitory N-terminal phosphorylation, catalytically active GSK3 phosphorylates its primed substrates. Following agonist-mediated N-terminal phosphorylation by kinases such as Akt downstream of insulin stimulation, the N-terminus acts as a pseudo-substrate inhibitor by occupying the same binding site occupied by the substrate's priming phosphate, thereby excluding the substrate from the active site. R96, R180 and K205 are the key positively charged residues involved in binding the priming phosphate of the substrate or the phosphorylated amino terminus.

chordates, and vertebrates (reviewed in **Ali et al 2001, Doble and Woodgett 2003, Jope and Johnson 2004, Patel et al 2004, Kaidanovich-Beilin and Woodgett 2011**). Both GSK3 isoforms, GSK3 α (51-kDa) and GSK3 β (47-kDa), are broadly expressed, and display 85% overall primary sequence homology, including 98% identity in the kinase domain (**Woodgett 1990, Woodgett 1991**). In contrast, the relatively divergent C-termini (the last 76 residues of the isoforms) only share 36% identity, while the N-terminus, which houses the conserved regulatory serine residue mentioned above (S21 in GSK3 α , S9 in GSK3 β), encodes a glycine-rich extension in GSK3 α that accounts for the ~4-kDa difference in molecular weight between the two isoforms (**Diagram 1-12A**). Despite their high level of sequence identity, the two GSK3 isoforms are not functionally redundant, as evidenced by their distinct deficiency phenotypes (reviewed in **Doble and Woodgett 2003, Kaidanovich-Beilin and Woodgett 2011**) and their subsets of isoform specific substrates in, among other tissues, the heart (reviewed in **Force and Woodgett 2009**) and the brain (**Soutar et al 2010**). Unlike most agonist-responsive cellular kinases whose activity is *stimulated* following RTK activation, GSK3, as mentioned above, is a rare example of a kinase regulated by agonist-dependent *inhibition* rather than activation. Accordingly, the basal activity of GSK3 is relatively high in unstimulated cells, while stimulation with GFs, serum, or insulin rapidly reduces its specific activity, and this inhibition is largely accomplished through S21/S9 phosphorylation (reviewed in **Sutherland 2011**). In addition to its N-terminal phosphorylation however, which can be catalyzed by multiple members of the AGC kinase family (see below), and reflects its multifaceted role in cellular physiology, GSK3 is also regulated by subcellular localization and complex formation, which provide both a level of insulation from competing upstream elements, and also contributes to substrate preference through the influence of interacting molecules (reviewed in **Kaidanovich-Beilin and Woodgett 2011**). The Wnt-mediated inhibition of GSK3-dependent β -catenin phosphorylation, for example, which targets β -catenin for proteasomal degradation, is an exception to the general mode of GSK3 regulation, and is insulated from the inhibitory effects of N-terminal phosphorylation. In the case of Wnt signaling, the inhibition of GSK3-mediated β -catenin phosphorylation is achieved through the Wnt-mediated destabilization of the GSK3-containing β -catenin destruction complex (**Diagram 1-13**), thereby allowing the

accumulation of β -catenin protein, its interaction with TCF/LEF transcription factors, and consequent transactivation of TCF/LEF target gene transcription (reviewed in **Nusse 1999, Wu and Pan 2010**).

In addition to its distinctive modes of regulation by agonist-dependent *inhibition*, GSK3 is further distinguished as one of a few kinases with a preference for “primed” substrates, as demonstrated to be the case for GS (**Rylatt et al 1980, Parker et al 1983, Woodgett and Cohen 1984**), which is primed by casein kinase 2 (CK2, initially called GSK5). Accordingly, most (though not all) of its targeted proteins require priming at a S/T residue 4-5 residues downstream of the putative GSK3 phosphorylation site, hence its general substrate consensus sequence of (S/T)xxx(S/T)_p (reviewed in **Sutherland 2011**). Solution of the GSK3 crystal structure has provided a structural explanation for this requirement, revealing that it possesses a phosphate-binding pocket that interacts with the primed residue of the substrate, and positions the target S/T residue for phosphorylation by the GSK3 kinase domain (**Frame et al 2001**). N-terminal phosphorylation of GSK3 at S21/S9 creates a “pseudo-substrate” that competes with potential substrates for the phosphate-binding pocket (**Diagram 1-12B**), and thereby inhibits GSK3 activity towards (most) primed substrates (**Frame et al 2001**). Insulin stimulation downregulates GSK3 activity mainly through Akt-mediated S21/S9 phosphorylation (**Cross et al 1995**), but other stimuli can also lead to the S21/S9 phosphorylation of GSK3 through the activation of numerous parallel-acting kinases, including RSK downstream of Ras/MAPK activation in response to mitogens, S6K downstream of mTORC1 activation by cues such as amino acid availability, as well as by PKA and PKC downstream of their own respective activating signals, placing GSK3 downstream of most agonist-stimulated signaling pathways (reviewed in **Kaidanovich-Beilin and Woodgett 2011, Sutherland 2011**). Since the identification of GS and eIF2B, nearly 100 additional proteins implicated in diverse cellular processes including growth, development, endocrine control, cell division and circadian rhythmicity, have been classified as putative substrates of GSK3 activity. Many of the best-characterized of these candidate effectors are known to require priming phosphorylation, suggesting that their activity can be modulated by the N-terminal inhibitory phosphorylation of GSK3.

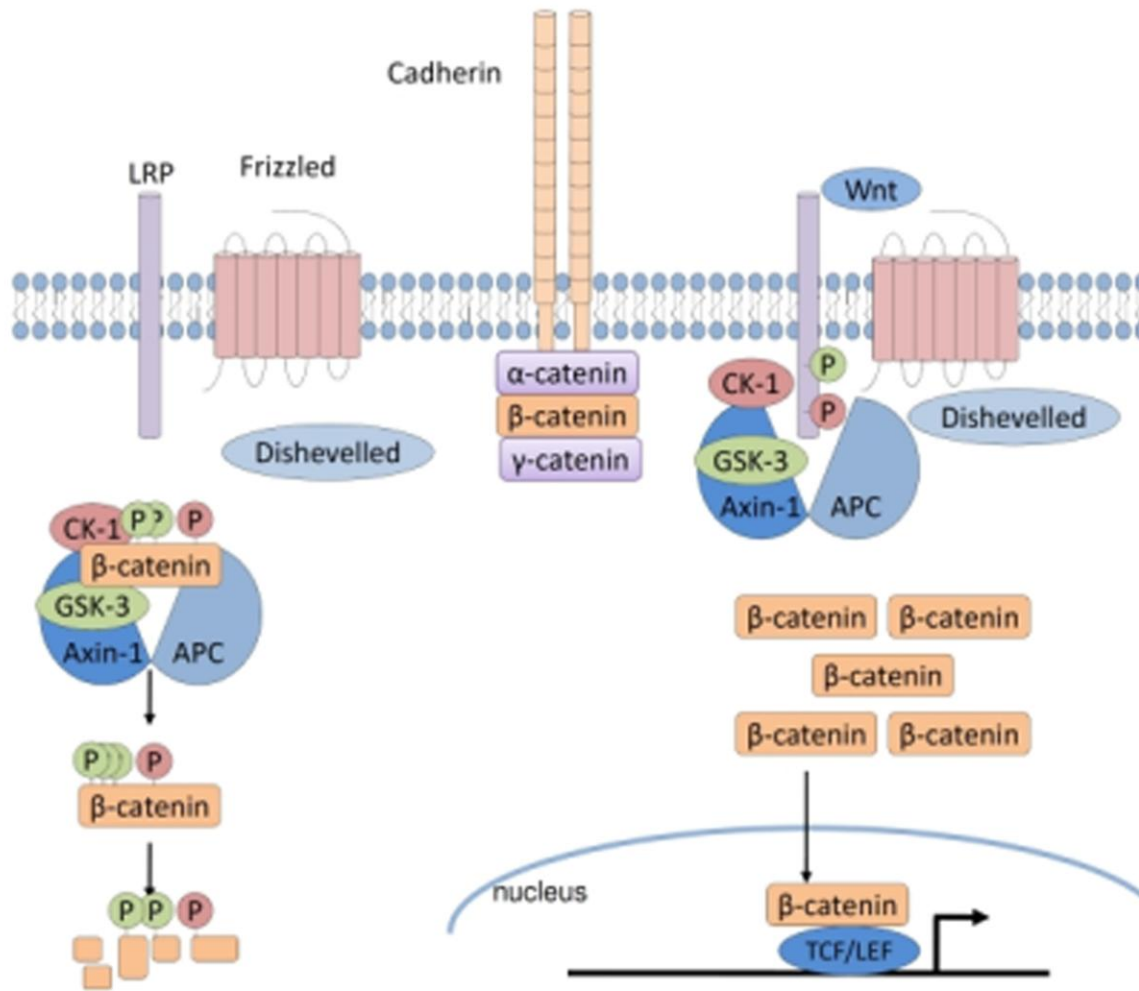


Diagram 1-13. Wnt-mediated inhibition of GSK3 kinase activity. Reproduced from Voskas et al (2010). Simplified representation of the role of GSK3 in the β -catenin destruction complex. A proportion of GSK3 in cells is present in a multiprotein complex with Axin-1, APC, and CK1. In the absence of Wnt stimulation, GSK3 (as part of the destruction complex) is active and phosphorylates axin, APC, and β -catenin. Axin, which acts as a scaffold for the complex, is stabilized by its phosphorylation; the phosphorylation of APC promotes its interaction with β -catenin, recruiting the latter to the destruction complex; and the phosphorylation of β -catenin itself targets the molecule for ubiquitination and subsequent proteasomal destruction. Upon Wnt stimulation, Dishevelled (Dvl) is engaged and the destruction complex is disrupted. CK1 and GSK-3 activities are diverted to LRP at the cell membrane. This relieves the phosphorylation and degradation of cytoplasmic β -catenin molecules, which accumulate and translocate to the nucleus, where they promotes Tcf/Lef-dependent transcriptional activation.

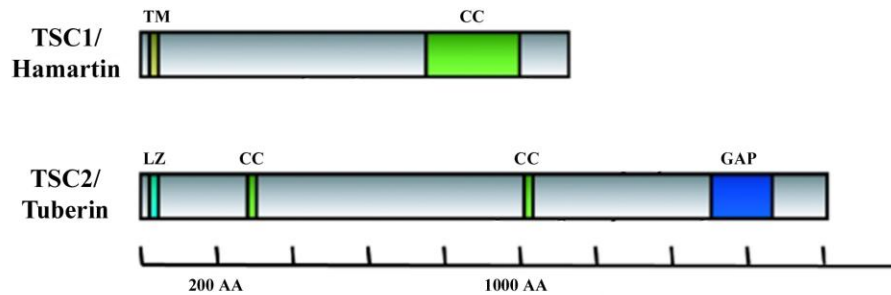
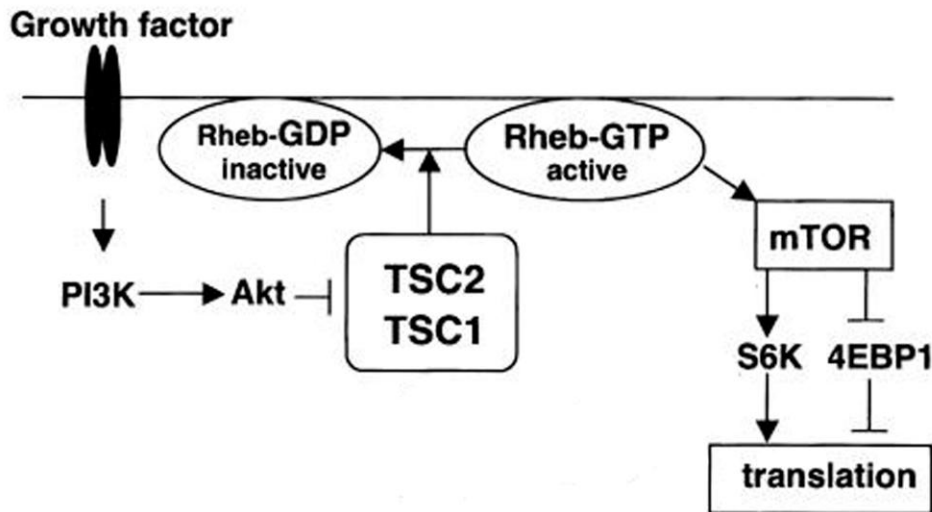
A**B**

Diagram 1-14. The TSC/Rheb junction is a direct regulator of mTORC1 catalytic function downstream of RTK stimulation. (A) Reproduced (with modification) from **Inoki et al (2005)**. The TSC1 molecule most notably encodes an N-terminal transmembrane motif through which it is anchored to endomembranes, and a C-terminal domain with several coiled-coil motifs. The TSC2 molecule encodes an N-terminal leucine zipper motif; two coiled-coil domains, one of which is centrally located in the vicinity of Akt-targeted residues; and a C-terminal GAP domain. (B) Reproduced (with modification) from **Inoki et al (2003a)**. Growth factor stimulation activates RTK/PI3K signaling, resulting in Akt activation. Akt phosphorylates TSC2, disrupting TSC1/2 GAP activity towards GTP-bound (active) Rheb. GTP-bound (active) Rheb is allowed to accumulate, promoting the growth factor-mediated activation of mTORC1 activity.

Though many of these substrates lie outside the scope of this introductory chapter, those requiring mention due to their implicit roles in growth include (1) Rictor, which has recently been suggested to be targeted by GSK3 at S1235 in response to ER stress, resulting in downregulation of mTORC2/Akt signaling (**Chen et al 2011**); (2) IRS1/2 (see **Section 1-4.6**), which are phosphorylated by GSK3 (S332 in murine IRS1, S484 in IRS2), PKC β II (the priming kinase for IRS1 at S336) and JNK (the priming kinase for IRS2 at S488) in the absence of insulin/IGF stimulation, thereby promoting their degradation, and preventing ectopic PI3K activation downstream of IR/IGFR (**Liberman and Eldar-Finkelman 2005, Sharfi and Eldar-Finkelman 2008, Liberman et al 2008**); (3) TSC2 (see **Section 1-4.4**), which is phosphorylated and activated by GSK3 (at S1341 and S1337) following AMPK (AMP-activated protein kinase)-mediated priming at S1345, thereby inhibiting mTORC1 activity in the absence of agonist stimulation or in response to energy deprivation, and forming a node of integration between GSK3-dependent cellular responses, cellular energy status, and mTORC1-dependent growth-promoting functions (**Inoki et al 2006**); and lastly, (4) a subset of substrates that serve important roles in survival, which are described in **Section 1-4.7**.

1-4.3 – Growth factor-dependent PI3K/Akt-mediated mTORC1 activation

The pathway in which mTORC1 is the central kinase monitors local nutritional status, and integrates nutrient availability with local energy supplies (reviewed in **Hietakangas and Cohen 2009, Zoncu et al 2011b**). While local nutrient abundance is sensed cell-autonomously by mTORC1-mediated processes (see **Section 1-4.5**), systemic nutritional status is predominantly communicated in metazoans by insulin and insulin-like peptides, whose secretion into the bloodstream is stimulated by feeding, and whose endocrine cellular responses are chiefly mediated through the evolutionarily conserved PI3K/Akt pathway. Whereas mTORC2 operates upstream of Akt in the latter's PI3K-mediated activation, mTORC1-dependent stimulation of mRNA translation (a crucial component of proliferative or metabolic growth) can be activated, as described herein, downstream of mTORC2-dependent Akt activation (**Diagram 1-9**). The resulting integration of systemic means of growth control with local mechanisms of nutrient and energy sensing ultimately converges on the control of protein synthesis through S6K1

and 4E-BP1 (eIF4E-binding protein 1), the first and best-characterized mTORC1 targets in metazoans, which act in parallel in the regulation of the initiation and progression of mRNA translation (reviewed in **Ma and Blenis 2009**, **Silvera et al 2010**, **Magnuson et al 2012**).

To date, two main Akt-mediated mechanisms have been described that result in mTORC1 activation, and involve two Akt substrates: TSC2, which in complex with TSC1, has been established as a major upstream negative regulator of mTORC1 activity for over a decade, and PRAS40, which was more recently identified as an inhibitor of mTORC1 activation. The TSC tumor suppressors (TSC1 and TSC2) derive their names from tuberous sclerosis, an autosomal-dominant condition characterized by hamartoma formation (a benign focal neoplasm) in organs such as the brain, skin, heart, lung, and kidney (reviewed in **Orlova and Crino 2010**). *In vivo*, the TSC1/2 molecules were shown to directly associate and form a heterodimeric complex (**Plank et al 1998**, **van Slegtenhorst et al 1997**, **Nellist et al 2001**), the disruption of which by mutations in either molecule can cause the familial or sporadic forms of TSC (**Hodges et al 2001**). Their status as *bona fide* tumor suppressors was confirmed in knockout animals, whereby the loss of either *TSC1* or *TSC2*, though embryonic lethal in homozygotes (E9.5-E12.5) due to various malformations and dysfunctions, resulted in tumor prone heterozygotes (**Onda et al 1999**, **Kobayashi et al 2001**). The deduced function of the TSC1/2 complex in the regulation of growth was established in *Drosophila*, in which the inactivation of either *dTsc1* or *dTsc2* cell-autonomously increased cell size, whereas their dual overexpression was found to decrease cell size (**Gao and Pan 2001**, **Potter et al 2001**, **Tapon et al 2001**); and moreover, heterozygosity for either *dTsc1* or *dTsc2* loss-of-function was found to rescue the lethality of insulin receptor (*DIR*) mutants (**Gao and Pan 2001**), suggesting an important inhibitory role for its function in the *Drosophila* insulin signaling pathway. The biochemical analysis of TSC1/2 functional deficiency further demonstrated that the TOR pathway was ectopically activated in *TSC1*- or *TSC2*-deficient cells (**Gao et al 2002**, **Goncharova et al 2002**, **Kwiatkowski et al 2002**, **Inoki et al 2002**), and could be suppressed by rapamycin treatment or TSC1 and TSC2 overexpression (**Kenerson et al 2002**, **Tee et al 2002**), suggesting that TSC1/2 may be an upstream-acting negative regulator of TOR/S6K1 signaling.

In 2002, various groups confirmed that the TSC1/2 complex antagonized PI3K/Akt-mediated growth by inhibiting mTOR signaling (vis-à-vis the classical rapamycin-sensitive S6K1 and 4E-BP substrates), and this inhibition was demonstrated to be relieved downstream of PI3K by the activation of Akt, which could directly phosphorylate TSC2 at a number of sites *in vitro* (S939, S981, S1130, S1132, and T1462 based on the full-length sequence), two of which (S939 and T1462) were shown to diminish the ability of TSC1/2 to inhibit S6K1 activity *in vivo* (**Inoki et al 2002**, **Manning et al 2002b**, **Potter et al 2002**, **Dan et al 2002**), thereby placing TSC1/2 function downstream of Akt, and upstream of mTORC1 activity. The TSC1 (140-kDa) and TSC2 (200-kDa) molecules are not structurally related, and bear very little resemblance to other known proteins (reviewed in **Inoki et al 2005**). With the exception of an N-terminal transmembrane domain that anchors the complex to endomembranes and a C-terminal domain with several coiled-coil motifs ([Diagram 1-14A](#)), TSC1 does not encode any other recognizable protein-protein interaction or catalytic domains (**van Slegtenhorst et al 1997**). The TSC2 molecule most notably encodes a central coiled-coil motif in the general vicinity of the cluster of Akt-targeted sites ([Diagram 1-14A](#)), an N-terminal leucine-zipper domain implicated in its association with TSC1, and a C-terminal domain with homology to RapGAP (**European Chromosome 16 Tuberous Sclerosis Consortium 1993**). The structural clues to TSC1/2 function were sparse, but mutations in TSC2 causing the severest forms of the associated neoplastic syndromes were known to map to its RapGAP domain (**Maheshwar et al 1997**), and the GTPase activity of Rap1, a Ras superfamily GTPase, was shown to be stimulated *in vitro* by TSC2, consistent with its putative function as a GAP (**Wienecke et al 1995**). However, since insulin had been found to have no effect on Rap1 activity in cells that potently activated S6K1 under the same circumstances (**Ming et al 1994**, **Zwartkruis et al 1998**), the *in vivo* target of TSC2 GAP activity was speculated to be a GTPase other than Rap1. The small GTPase Rheb (20-kDa, Ras homolog enriched in brain) emerged as a putative candidate of TSC2 GAP activity in 2003 following a screen for Ras family members with elevated levels of their GTP-bound state in TSC2-deficient MEFs (**Garami et al 2003**), and was found to (1) be a target of TSC1/2 GAP activity, (2) to be GTP-loaded in response to insulin treatment, and (3) to activate S6K1 when overexpressed; while pathological TSC2

mutants were demonstrated to be incapable of inhibiting S6K1 activation, consistent with a negative role for TSC1/2 downstream of Akt, and a positive role for Rheb downstream of TSC1/2 in the promotion of cell growth (**Garami et al 2003**, **Castro et al 2003**, **Inoki et al 2003a**, **Tee et al 2003**, **Zhang et al 2003**).

The gene encoding *Rheb* was first identified in a differential screen conducted in order to identify specifically induced mRNAs in neurons by seizure-provoking agents (**Yamagata et al 1994**), and although highly expressed in brain and muscle tissue, Rheb is broadly expressed (**Gromov et al 1995**), and highly conserved in eukaryotes, from yeast to mammals (reviewed in **Inoki et al 2005**). The involvement of Rheb in the regulation of cell growth was initially suggested by its reported overexpression in several tumor cell lines, and its capacity to induce the transformation of murine fibroblasts as a result of its ectopic expression (**Yee and Worley 1997**); while in yeast, Rheb was implicated in nutrient sensing based on the similarity of its loss-of-function phenotype with those associated with nitrogen starvation (**Mach et al 2000**). The identification of mammalian Rheb as a downstream effector of TSC1/2 *in vitro* coincided with genetic screens in *Drosophila* for genes involved in the control of growth, which identified dRheb as a positive regulator cell growth *in vivo* (**Saucedo et al 2003**, **Stocker et al 2003**), whereby the loss of dRheb function led to decreased cell size, while its overexpression achieved the opposite effect of overgrowth, both hallmarks of insulin signaling pathway mutant growth phenotypes in *Drosophila*. These studies also epistatically placed dRheb downstream of dTsc1/2, which had been shown to antagonize insulin signaling in the regulation of cellular growth and proliferation (**Potter et al 2001**, **Tapon et al 2001**); and upstream of dTOR/dS6K, consistent with its placement in mammals, and in general agreement with subsequent epistatic and biochemical analyses (reviewed in **Inoki et al 2005**), including the demonstration that RNAi-mediated knockdown of Rheb, but not any other of the 17 GTPases tested (including Rac1, Rap1, Rab5, and Cdc42), could abolish S6K activation in cultured *Drosophila* S2 cells (**Zhang et al 2003**). Rheb was thereafter demonstrated to be capable of interacting directly (though weakly) with mTOR irrespective of its guanine nucleotide-binding status, but with a requirement for the GTP-bound state for mTOR activation (**Long et al 2005a**) and

the facilitation of substrate recruitment (**Sato et al 2009**), establishing it as a direct positive regulator of mTORC1 catalytic function (**Diagram 1-14B**).

The current model of TSC1/2-dependent Rheb inhibition and PI3K/Akt-mediated Rheb/mTORC1/S6K1 activation posits that PI3K activation stimulates the Akt-mediated inhibition of TSC1/2 GAP activity towards Rheb, promoting the accumulation of the latter's GTP-loaded state, and resulting in the potentiation of mTORC1/S6K1 activation. However, important mechanistic aspects of this process remain poorly understood (reviewed in **Huang and Manning 2008**), including (1) the molecular basis of TSC1/2 inhibition resulting from Akt-mediated TSC2 phosphorylation; (2) the biochemical basis of Rheb activation with respect to the yet unidentified RhebGEF responsible for the agonist-dependent stimulation of Rheb in the process of mTORC1 activation; and (3) the mechanistic basis of Rheb-dependent mTORC1 activation, which may involve an intermediate signaling component(s). Insight into the colocalization of Rheb and its downstream effector mTORC1 has been provided by the demonstration that the signaling function of Rheb in the process of mTORC1/S6K1 activation normally requires localization to endomembranes (**Buerger et al 2006**), and is accomplished by its farnesylation (**Castro et al 2003, Basso et al 2005, Buerger et al 2006**), consistent with the most recent demonstrations (see **Section 1-3.3**) of endomembranes such as late endosomes and lysosomes as crucial subcellular locales of mTORC1 activity (**Sancak et al 2008, Sancak et al 2010, Flinn et al 2010, Zoncu et al 2011a**). With respect to the mechanism of Akt-mediated TSC1/2 inhibition, the leading theory suggests that the phosphorylation of TSC2 by Akt (and other regulators, see below) may result in a change in TSC2 subcellular localization, releasing it from TSC1 such that its inhibitory interaction with Rheb is prevented. Evidence in support of this possibility includes numerous reports that 14-3-3, which as previously mentioned, often participates in the cytoplasmic retention of its binding partners, binds TSC2 in a phosphorylation-dependent manner, and inhibits TSC1/2 function (**Liu et al 2002, Nellist et al 2002, Li et al 2003, Shumway et al 2003**). The Akt-dependent phosphorylation of TSC2 at S939/S981 was itself suggested to create a 14-3-3 binding site (**Liu et al 2002, Li et al 2002**), though the association of TSC2 with 14-3-3 was suggested not to *directly* involve Akt. Furthermore, GF stimulation was demonstrated to produce PI3K-dependent increases in cytosolic

TSC2 levels requiring the Akt-dependent phosphorylation of S939/S981 on TSC2, while TSC1 and Rheb remain predominantly membrane-localized irrespective of agonist stimulation (**Cai et al 2006**).

The Akt-mediated path of TSC2 phosphorylation and TSC1/2 inhibition, though an important negative regulator of Rheb/mTORC1 activation (**Diagram 1-15**), is neither universal, nor even the sole mechanistic means through which Akt itself can contribute to mTORC1 activation, not to mention the Akt-independent means of TSC1/2 modulation described below. For example, the Akt-dependent TSC2 phosphorylation sites are conserved in *Drosophila* (S924 and T1518), but mutations at these sites that render them unphosphorylatable do not significantly alter dTsc2 biological activity (unless overexpressed), and furthermore, rescue the lethality and growth defects of *dTsc2*-null mutants (**Dong and Pan 2004**), suggesting that in *Drosophila* at least, dTor/dS6K activation does not require Dakt-mediated dTsc2 phosphorylation (though a requirement for the Dakt-dTsc2 interaction cannot be discounted), and may rely on other Dakt-dependent or perhaps even Dakt-independent mechanisms acting in parallel. One such alternative target of Akt activity in the process of mTORC1 activation is PRAS40 (40-kDa proline-rich Akt substrate), also called AKT1S1 (**Kovacina et al 2003**), which has been identified as an inhibitory mTORC1 binding partner. The PRAS40 molecule binds Raptor in an interaction that is inhibitory towards mTORC1 catalytic activity by competing with substrates such as S6K1 and 4E-BP1 (**Wang et al 2007a**). In response to agonists such as insulin or increased nutrient abundance, PRAS40 is phosphorylated by Akt at S246, a site that is also conserved in its *Drosophila* ortholog (*lobe*), and the replacement of which with alanine (S246A) prevents the insulin-stimulated activation of mTORC1 (**Sancak et al 2007, Vander Haar et al 2007**). The PRAS40 molecule has also been demonstrated to be a target of mTOR activity itself (**Oshiro et al 2007, Fonseca et al 2007, Wang et al 2008**), whereby its mTORC1-mediated phosphorylation at S183 and S221 have been suggested to cooperate with the Akt-dependent mode of PRAS40 phosphorylation at S246 in the process of mTORC1 activation. Similar to the suggested mechanism of Akt-mediated TSC1/2 inhibition, the Akt-dependent phosphorylation of PRAS40 has been proposed to induce its release from the complex, its association with

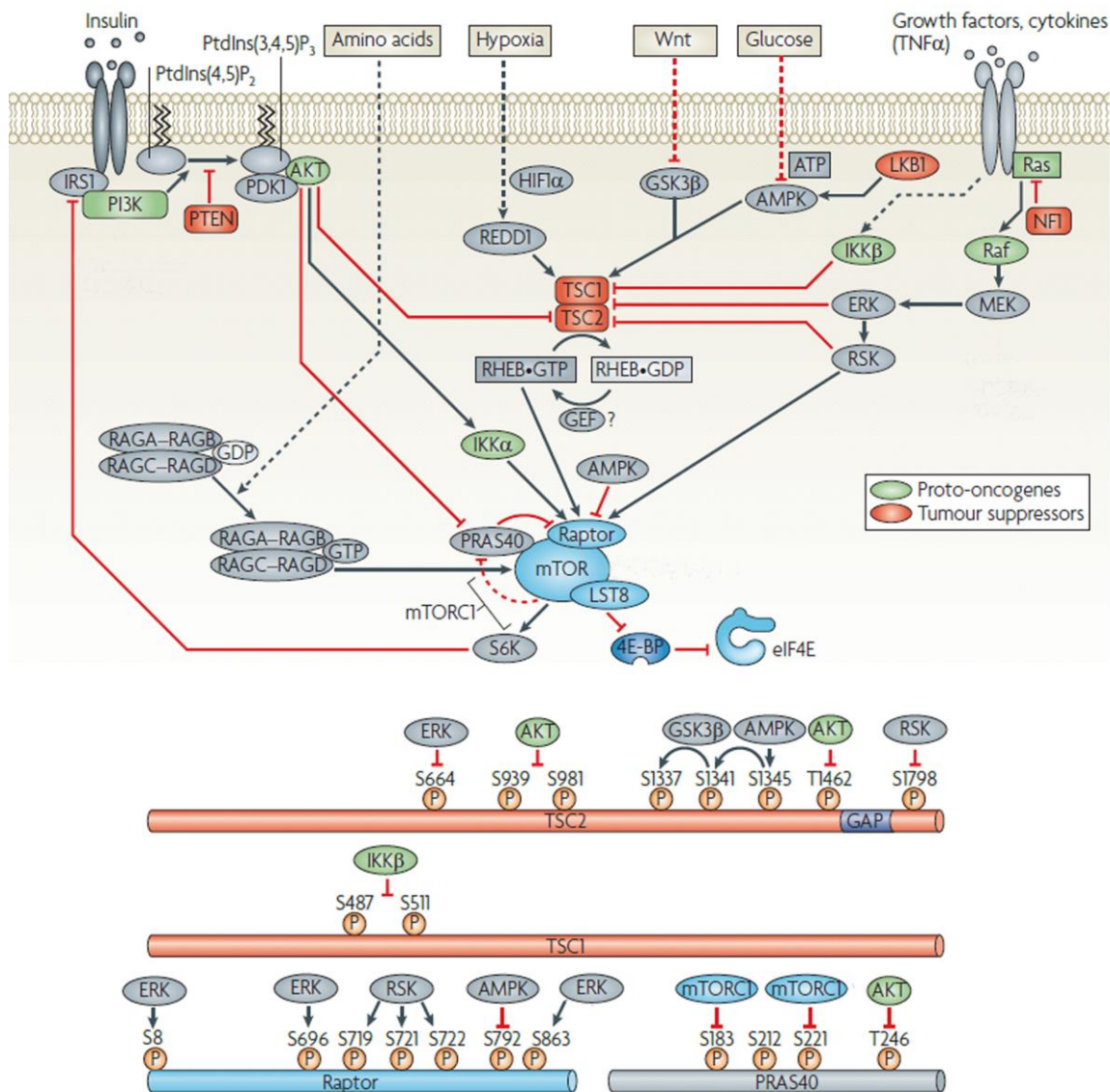


Diagram 1-15. The mTORC1 signaling regulatory network. Reproduced (with modification) from **Ma and Blenis (2009)**. Multiple upstream signaling inputs from the PI3K/Akt, Ras/ERK/RSK, TNF α /IKK β , AMPK/GSK3, LKB1/AMPK, and Wnt/GSK3 β pathways (among others) are integrated in the regulation of TSC/Rheb-mediated mTORC1 activation, and either positively or negatively regulate mTORC1 signaling. TSC1/2, Raptor, and PRAS40 are important hubs of upstream signaling integration in the process of mTORC1 activation. The hypoxia-induced REDD1-mediated promotion of TSC1/2 function; the stress-activated inhibitory phosphorylation of TSC2 (S1254) by MK2 downstream of p38 activation; and the JNK-mediated mTORC1-activating phosphorylation of Raptor at S696, S706, and S863 are not shown in this diagram, but discussed in the text. The contribution of the Rag GTPases to nutrient-dependent mTORC1 activation is discussed in **Section 1-4.5**.

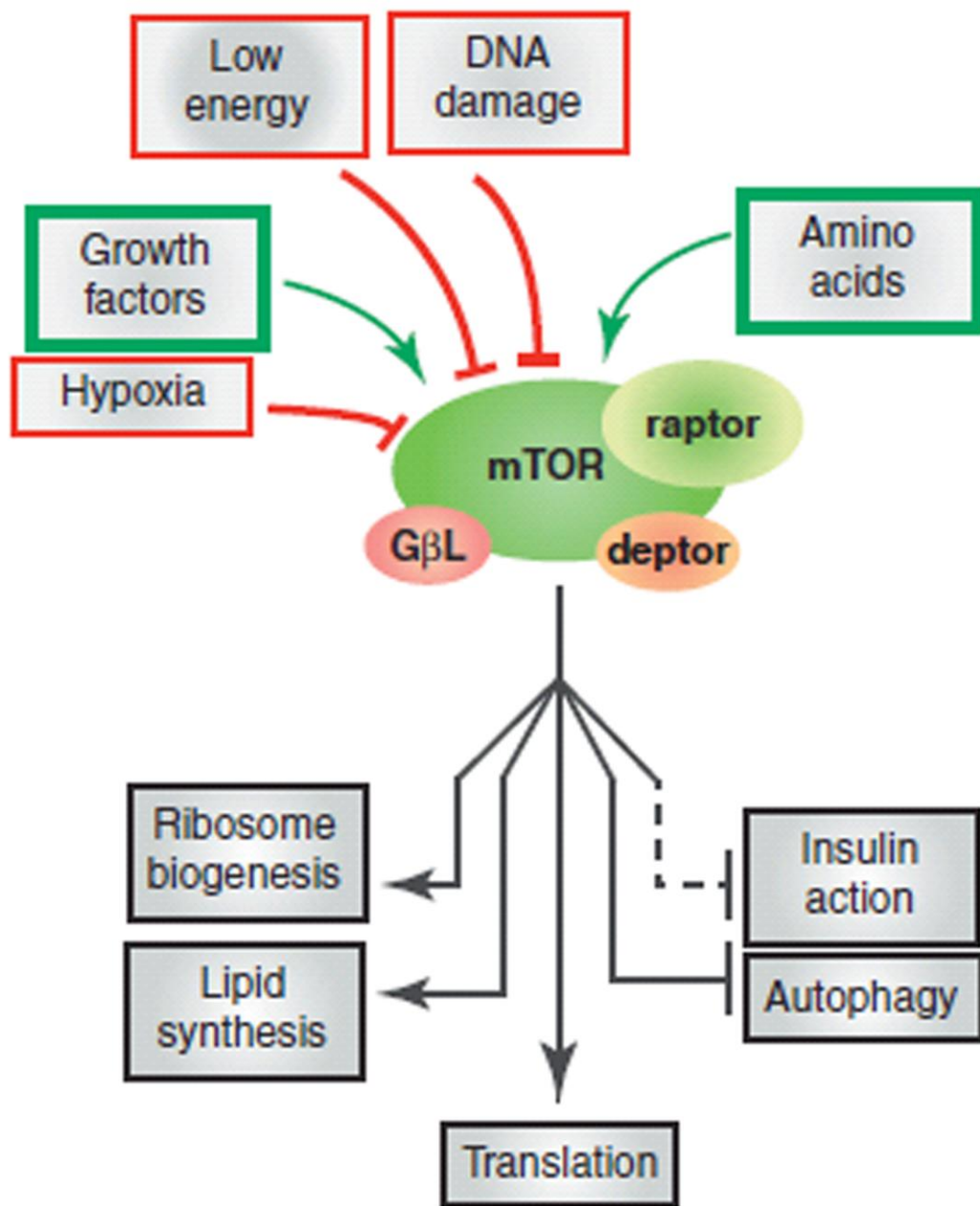


Diagram 1-16. Multiple upstream signaling inputs are integrated upstream of mTORC1. Reproduced from Efeyan et al (2012). The activation of mTORC1 promotes cellular growth by upregulating ribosome biogenesis and protein translation, while inhibiting autophagy. Growth factors and amino acids promote mTORC1 activation; whereas hypoxia, energy/nutrient deprivation, or DNA damage can result in the inhibition of mTORC1 function. The mTORC1 component mLST8 is also referred to as GβL. See text for details.

14-3-3, and its sequestration away from mTORC1, thereby relieving competition for mTORC1 substrates.

1-4.4 – TSC1/2 integrates multiple modulatory inputs in the regulation mTORC1

In contrast to the strictly GF-dependent induction of mTORC2 activity, a number of upstream regulatory inputs converge on mTORC1 function, including energy status and stress, nutrient and oxygen availability, and GF stimulation, which can context-dependently cooperate or oppose each other in the process of mTORC1 regulation (reviewed in **Ma and Blenis 2009**, **Zoncu et al 2011b**, **Tchevkina and Komelkov 2012**). In mammals, the TSC1/2 complex is heavily targeted for phosphorylation, and like IRS1, is an archetype of signal integration in the regulated cross-talk and compensation between the Ras/MAPK and PI3K/Akt signaling pathways (reviewed in **Mendoza et al 2011**). As described herein, the GAP activity of TSC2 towards Rheb can be regulated downstream of a set of kinases similar in composition to that operating on IRS1, including ERK, RSK, S6K1, GSK3, AMPK, and IKK.

Acting in parallel to the PI3K/Akt pathway, the Ras/MAPK pathway (see [Diagram 1-20](#)) has been demonstrated to activate mTORC1 by converging on TSC2 in the process of Rheb activation. Following Ras-dependent Raf/Mek/Erk activation, ERK and its substrate RSK have been shown to phosphorylate TSC2 at S664 and S1798 respectively (**Roux et al 2004**, **Ballif et al 2005**, **Ma et al 2005**), and appear to act additively to the Akt-mediated inhibitory phosphorylation at S939/S981 and T1462 in the process of TSC1/2 dissociation and the inhibition of its RhebGAP activity ([Diagram 1-15](#)), though the precise means of this synergy in the activation of Rheb remain to be defined. More recently, an additional Ras-dependent ERK/RSK-mediated avenue of mTORC1 activation has been described ([Diagram 1-15](#)), whereby the mTORC1 scaffold Raptor has been shown to be directly phosphorylated at multiple sites by both ERK (**Carrière et al 2011**), which phosphorylates Raptor at S8, S696, and S863; and RSK, which ERK-dependently phosphorylates TSC2 at S719/S721/S722 (**Carrière et al 2008**), resulting in the promotion of mTORC1 activity in a manner that may preclude PRAS40 inhibition, and synergize mTORC1-mediated phosphorylation of Raptor at S863, which

has been proposed to act as a biochemical switch for further multisite activating phosphorylation (**Wang et al 2009a, Foster et al 2010**).

In addition to MAPK-stimulated inhibitory phosphorylation, TSC2 and Raptor have also been shown to be targeted for phosphorylation by the stress-activated protein kinase (SAPK) pathways. The induction of p38 activity was demonstrated to stimulate the MK2 (MAPKAP kinase-2)-dependent phosphorylation of TSC2 at S1254 both *in vivo* and *in vitro*, promoting 14-3-3 association, TSC1/2 inhibition, and downstream S6K1 activation (**Li et al 2003**); while in response to osmotic stress, which also activates mTORC1 activity, JNK has recently been shown to phosphorylate Raptor at S696, T706, and S863 (**Kwak et al 2012**). In addition to physiological p38- and JNK-activating stress stimuli, the activity of mTORC1 can also be stimulated in response to pro-inflammatory cytokines such as TNF α through the activation of the IKK complex, a major downstream kinase of the TNF α signaling pathway (reviewed in **Pahl et al 1999**). The IKK-mediated inhibition of I κ B liberates the latter's inhibitory interaction with NF- κ B, which promotes the transcription of anti-apoptotic and survival-promoting genes in response to inflammatory cytokines, chemokines and other stress response-inducing signals. In addition to this well-documented function with respect to the NF- κ B-mediated transcriptional response however, IKK has also been demonstrated to stimulate mTORC1 activity (and by extension, the translational machinery) through a direct interaction between IKK β and TSC1 (in contrast to the Akt- and ERK/RSK-mediated phosphorylation of TSC2), resulting in the IKK-mediated phosphorylation of TSC1 at S487 and S511, and TSC1/2 dissociation (**Lee et al 2007**), which has been proposed to contribute to obesity induced insulin resistance (**Lee et al 2008**). Interestingly, the promotion of mTORC1 signaling by TNF α , which can signal through Akt (reviewed in **Perkins 2007**), has also been proposed to involve a direct association between IKK α , and mTORC1 that is Akt-dependent (**Dan et al 2007, Dan and Baldwin 2008**), though it remains unclear how this interaction contributes to mTORC1 activation.

Numerous agonists then, such as GFs, mitogens, environmental stress, and cytokines can promote mTORC1 *activation* through TSC1/2 *inhibition*, thereby favoring ribosome biogenesis and protein synthesis (**Diagram 1-16**). However, TSC1/2 can also be *positively* targeted for phosphorylation in response to energy deprivation, hypoxia, or

DNA damage, thereby *stimulating* its RhebGAP activity and *inhibiting* mTORC1 activation under conditions that do not favor growth (reviewed in **Sengupta et al 2010**). The process of protein synthesis consumes two important forms of cellular currency – ingested or recycled amino acids and energy in the form of glycolytically produced ATP – whose conservation becomes essential under nutritionally unfavorable circumstances. Whereas PI3K/Akt activation promotes glycolysis (thereby promoting ATP synthesis) and protein synthesis, the demonstration that energy (ATP) depletion severely diminished mTORC1 activation (**Dennis et al 2001**) established a reciprocal link between energy sensing and the control of translation, while the subsequent investigation of this correlation revealed the AMPK complex as the mTORC1-modulating cellular energy sensor. Chief amongst the various conditions of cellular stress that activate the heterotrimeric AMPK complex (catalytic α and regulatory β/γ subunits) are those that increase the AMP:ATP ratio, which is indicative of energy (ATP) depletion (reviewed in **Kahn et al 2005, Hardie et al 2007, McBride and Hardie 2009**). The steady state level of AMP is typically low, and modest reductions in net ATP abundance can cause relatively significant increases in the AMP/ATP ratio, leading to the binding of AMP to the AMPK γ subunit, which allows LKB1-mediated α subunit phosphorylation (see below), AMPK activation, and AMPK-mediated inhibition of energy-consuming cellular functions, as well as the activation of ATP-generating metabolic processes. AMPK activity can also be regulated by glycogen, which in “times of plenty” binds the AMPK β subunit, and inhibits AMPK activity (**McBride et al 2009**). This bimodal energy-sensing mechanism allows the AMPK complex to monitor both ATP levels (immediately available energy) and glycogen stores (energy in reserve), thereby ensuring a high level of sensitivity and responsiveness to energy status. Following the observation that AMPK activation in skeletal muscle or hepatocytes inhibited the activation of mTORC1 effectors (**Krause et al 2002, Horman et al 2002, Bolster et al 2002**), the TSC2 molecule was demonstrated to be a major effector of AMPK-dependent mTORC1 inhibition in the energy response pathway, and has been shown to be AMPK-dependently phosphorylated at S1345 (**Diagram 1-15**), *enhancing* its GAP activity towards GTP-bound (active) Rheb, thereby acting as a molecular brake on the mTORC1-mediated promotion of protein synthesis (**Inoki et al 2003b**).

As suggested by TSC2-deficient cells, in which mTORC1 can still be inhibited (though only modestly) in response to cellular energy depletion (**Hahn-Windgassen et al 2005, Gwinn et al 2008**), a TSC2-independent mechanism of mTORC1 inhibition has also been described to occur through the direct AMPK-mediated phosphorylation of Raptor at S792, resulting in the latter's inhibitory association with 14-3-3, thereby depriving the mTORC1 complex of its obligate component (**Gwinn et al 2008**), and establishing Raptor itself as a hub of signal integration downstream of AMPK, as well as ERK, RSK, JNK, and mTORC1 itself as described above. The AMPK-mediated inhibition of mTORC1 has also been shown to require the activity of the tumor suppressor LKB1 (liver kinase B1, also known as S/T kinase 11, or STK11), which phosphorylates and activates AMPK in response to energy depletion, and the loss of which in LKB1-deficient mice results in elevated levels of mTORC1 activation (**Shaw et al 2004, Corradetti et al 2004**). The phosphorylation of TSC2 at S1341 and S1337 by GSK3 also potentiates its GAP activity towards Rheb (**Diagram 1-15**), and as mentioned previously (**Section 1-4.2**), requires priming at the S1345 site targeted by AMPK (**Inoki et al 2006**). The expression of a TSC2 variant that cannot be phosphorylated by GSK3 renders mTORC1 activation resistant to energy deprivation, suggesting that AMPK and GSK3 are cooperative in their activation of TSC1/2 GAP activity downstream of their respective stimulatory cues (**Inoki et al 2006**). Interestingly, considering (1) the fact that both RSK and Akt, which phosphorylate TSC2 and *inhibit* TSC1/2 GAP activity, are capable of phosphorylating and inhibiting GSK3; and (2) the fact that glycogen, the catalytic product of GS, whose activation is promoted by GSK3 inhibition, further investigation of TSC1/2 regulatory dynamics should reveal whether Akt/RSK-mediated relief of the GSK3-mediated activation of TSC1/2 is part of the molecular clockwork responsible for the GF-mediated *stimulation* of mTORC1 activity and/or the suppression of AMPK activity upon restoration of optimal AMP:ATP ratios. Most recently, an AMPK-independent mechanism of mTORC1 inhibition in response to energy deprivation has also been suggested to cooperate with AMPK-mediated phosphorylation of TSC2 and Raptor, whereby the PRAK (p38-regulated/activated protein kinase)-mediated phosphorylation of Rheb at S130 in response to p38 SAPK activation was shown to

suppress its GTP-loading, resulting in diminished mTORC1 activation (**Zheng et al 2011**).

The cellular supply of ATP, which is generated through glycolysis, is also dependent on an adequate supply of oxygen, and whereas chronic hypoxic stress can contribute to LKB1/AMPK-mediated mTORC1 inhibition, transiently hypoxic conditions can also acutely limit energy expenditure by inhibiting metabolically costly processes like protein synthesis (**Shaw et al 2004, Liu et al 2006**). The transcription factor HIF1 α (hypoxia inducible factor 1 α) can drive the insulin-stimulated transcription of genes such as *GLUT1* (**Zelzer et al 1998**), which is the main glucose transporter in most tissues, but short-term hypoxic stress also results in the stabilization of HIF1 α (reviewed in **Liu et al 2012**), driving the transcription of hypoxia-stimulated genes such as *REDD1* (regulated in development and DNA damage responses 1), whose protein product has been suggested to compete with 14-3-3 for TSC2 binding (**Diagram 1-15**), thereby preventing TSC1/2 inhibition (and consequent mTORC1 activation) under hypoxic conditions in both mammals and *Drosophila* (**Brugarolas et al 2004, Reiling and Hafen 2004, Sofer et al 2005, DeYoung et al 2008**). Interestingly, environmental stresses unrelated to energy deprivation can also signal through AMPK, as DNA damage has also been demonstrated to result in mTORC1 inhibition through a mechanism that may involve two evolutionarily conserved transcriptional targets of p53 – sestrin 1 and 2 – which have been demonstrated to potently activate AMPK, thereby additionally linking genotoxic stress to the inhibition of the translational machinery (**Feng et al 2007, Budanov et al 2008**). Lastly, like hypoxia, ER stress can also upregulate *REDD1* expression through transactivation of the transcription factor ATF4 (activating transcription factor 4), resulting in mTORC1 inhibition (**Whitney et al 2009**), and further diversifying the panoply mTORC1-modulating stimuli.

1-4.5 – Activation of mTORC1 by amino acid/nutrient availability

Amino acids (AA) are essential building blocks not only for protein synthesis, a process in which mTORC1 is intimately involved, but also as intermediates in the synthesis of DNA, glucose, and ATP (reviewed in **Bender 2012**). In 1970, the importance of AA availability in the growth and homeostasis of organisms was

recognized when the deprivation of a single amino acid – leucine – resulted in severe weight loss and muscular atrophy in rats, followed by death (**Said and Hegsted 1970**). This discovery was preceded in the mid-1960s by Nobel Laureate Christian de Duve's characterization of autophagy as a self-degradative mechanism associated with the lysosomal system, following his discovery of lysosomes as a distinct intracellular membranous entity in 1955 (reviewed in **Klionsky 2007, Kroemer et al 2010**). The “autophagosome” forms in a largely *de novo* process in response to stimuli ranging from developmental cues to nutrient/AA deprivation, and sprouts as a bilayered lipid membrane that engulfs the surrounding cytoplasm and organelles, expands through vesicular addition, and fuses with a lysosome, which supplies acid hydrolases that digest the ingested biomass, allowing the context-dependent reallocation of building blocks (**Diagram 1-17A**). In the late 1970s, the induction of autophagy was shown to be inhibited by insulin treatment (**Pfeifer 1977**) or AA addition (**Mortimore and Schworer 1977**), and conversely, to be promoted by the withdrawal of AAs; while morphological studies conducted in the 1980s provided significant insight into the morphological features of autophagic progression (reviewed in **Klionsky 2007**). The identification of TOR in the early 1990s led to two important observations: (1) rapamycin-mediated TOR inhibition could induce autophagy (**Blommaert et al 1995**); and (2) rapamycin treated or TOR-deficient yeast cells were indistinguishable from starved cells (**Barbet et al 1996**). Prior to the distinction made between the mTORC1 and mTORC2 complexes (see **Section 1-3.3**), pilot studies in cultured cells initially indicated an absolute requirement for AAs in mTOR signaling (**Hara et al 1998, Wang et al 1998**), and the investigation of this requirement indicated that AA deficiency could not be compensated for by other known mTOR-activating stimuli such as GF stimulation. As such, the withdrawal of AAs, particularly branched chain amino acids such as leucine and isoleucine, were shown to rapidly inhibit mTOR activation, while the replenishment of AA abundance to starved cells was demonstrated to strongly stimulate mTOR activation (reviewed in **Jacinto and Hall 2003, Hay and Sonenberg 2004, Avruch et al 2009**). Combined, these studies established a molecular link between nutrient availability, mTOR, and autophagy in the control of growth under nutritionally favorable conditions, and growth arrest in response to starvation (**Diagram 1-17B**).

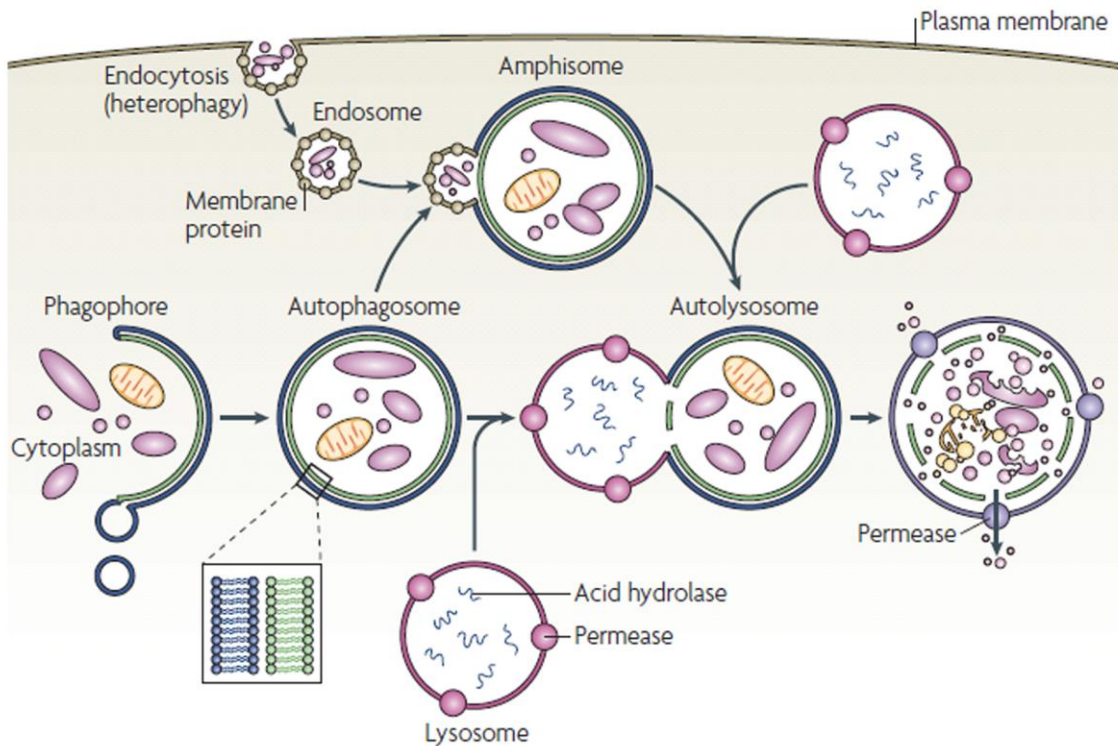
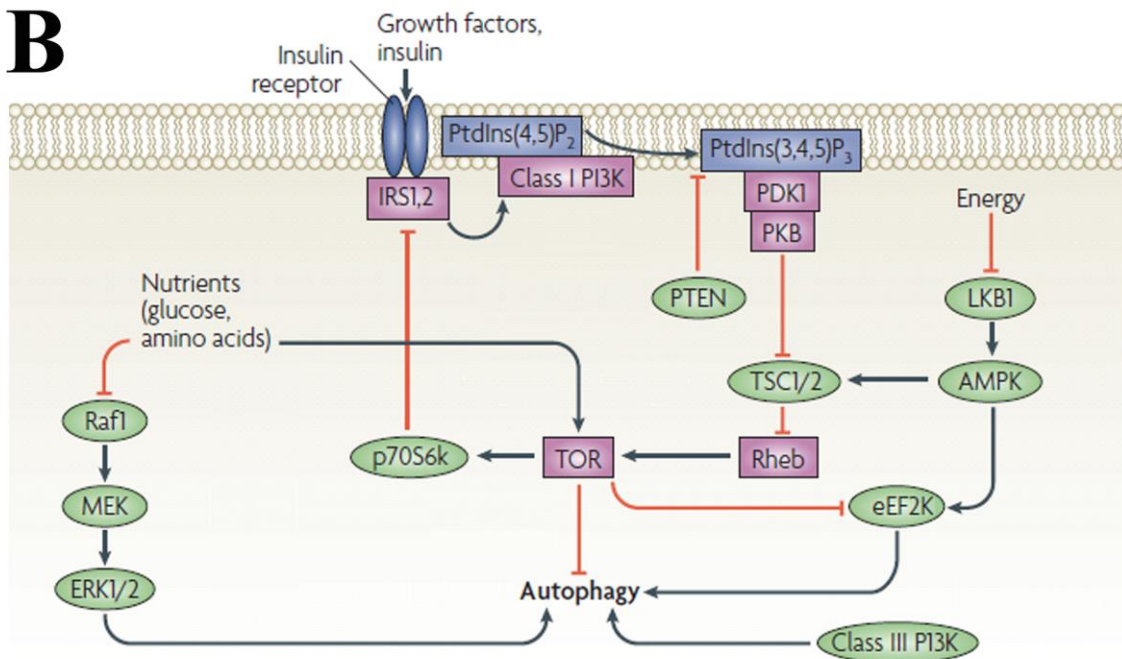
A**B**

Diagram 1-17. The regulation of autophagy in mammalian cells. Reproduced (with modification) from Klionsky (2007). (A) Sequestration begins with the formation of a

phagophore that expands into a bilayered autophagosome that engulfs a portion of the cytoplasm. Prior to fusion with a lysosome, the autophagosome may additionally fuse with an endosome in a process referred to as heterophagy, whereby the cell internalizes and degrades biomass that originates outside of the cell, in contrast to autophagy in which the cell consumes its own biomass. The product of endosome-autophagosome fusion is known as an amphisome, and the completed autophagosome or amphisome ultimately fuses with a lysosome, which supplies acid hydrolases. These enzymes in the resulting autolysosome break down the inner membrane from the autophagosome and degrade the cargo. The resulting macromolecules are released through permeases and recycled in the cytosol. **(B)** Green circles represent components whose functions directly or indirectly stimulate autophagy, whereas the purple boxes correspond to factors that inhibit autophagy. Inhibitory interactions are indicated with red lines, while activating interactions are shown as black arrows. The role of TOR in the regulation of autophagy is discussed in the text. See **Backer (2008)**, **Wang et al (2009b)**, and **Wu et al (2006)** for a description of the respective roles of Class III PI3K (Vps34), Raf/Mek/Erk non-canonical signaling, and eEF2K (Elongation factor-2 kinase) function in the regulation of autophagy.

With the establishment of mTORC1 as the “classical” target of rapamycin, the observation that the loss of TSC1/2 function conferred a degree of resistance to AA deprivation suggested that the TSC1/2 complex may be involved in the relay of AA abundance to mTORC1, and that branched AAs such as leucine may lead to mTORC1 activation through the inhibition of TSC1/2 and the stimulation of Rheb (reviewed in **Yang and Guan 2007, Wang and Proud 2009**), whose association with mTORC1 was shown to be inhibited in response to AA deprivation (**Long et al 2005b**). However, this supposition was challenged by studies demonstrating that cells lacking TSC1/2 function (through the loss of either TSC1 or TSC2), though leading to Rheb/mTORC1 hyperactivation, retained sensitivity to AA deprivation and exhibited diminished mTORC1 activity under conditions of starvation (**Smith et al 2005**). Furthermore, though Rheb is required for the AA-dependent stimulation of mTORC1 activity, AA deprivation had no apparent effect on Rheb GTP-loading (**Long et al 2005b, Roccio et al 2006**), suggesting that the AA-dependent regulation of mTORC1 relies on a distinct mechanism with respect to the sensing of AA abundance, which converges with and modulates Rheb-dependent mTORC1 function independently of AA availability. Although our understanding of the precise mechanism through which amino acid availability is conveyed to mTORC1 is incomplete, a number of recent studies have uncovered some of the components of AA-dependent mTORC1 activation. Recent reports featuring a series of elegant studies conducted by David Sabatini and his colleagues have been instrumental in establishing (1) the lysosomal membrane as a hub of GF-dependent Rheb activation and AA-induced mTORC1 activation, and (2) the V-ATPase/Ragulator/RagGTPase signaling module as a mechanistic link between the nutrient-sensing apparatus and mTORC1 activation (reviewed in **Hietakangas and Cohen 2009, Zoncu et al 2011b, Efeyan et al 2012**).

The initial link between Rag GTPases and TOR signaling was established in yeast, where the Rag ortholog Gtr2 was demonstrated to act in a vacuolar membrane-associated complex, and to be required for the cell’s exit from rapamycin- or AA deprivation-induced growth arrest; while genetic analyses hinted at the existence of a growth control mechanism originating (in yeast) at the vacuolar membrane (**Dubouloz et al 2005**). The major leap of understanding resulted from the search for novel modifiers of

mTORC1 activity through both biochemical methods in mammalian cells and genetic screens in *Drosophila*, which revealed Rag GTPases as crucial mediators of AA signaling to mTORC1 (**Kim et al 2008, Sancak et al 2008**). In metazoans, the Rag family consists of four related members (A through D) that form a heterodimeric complex consisting of RagA or RagB, which are highly similar and functionally redundant; and RagC or RagD, which are also highly similar, leading to four possible combinations (**Schürmann et al 1995, Hirose et al 1998, Sekiguchi et al 2001**). As described below, AAs were shown to regulate the guanine nucleotide-binding status of Rag heterodimers, which themselves serve as an mTORC1-binding molecular switch in the GTP-bound state, rendering mTORC1 susceptible to activation by another GTPase-dependent molecular switch, the Rheb molecule, which converges on mTORC1 downstream of agonist-dependent TSC1/2 inactivation. Mutagenesis studies determined that the association of the Rag heterodimer with mTORC1 is strongest when the components have opposite GTP-loading states (**Sancak et al 2008**), whereby in the absence of amino acids, RagA/B is GDP-loaded, while RagC/D is GTP-bound, forming an inactive conformation with respect to mTORC1 binding, while increased AA abundance induces a transition to the reciprocal GTP-loading state of the active conformation, with RagA/B being GTP-bound, and RagC/D being GDP-bound (**Diagram 1-18**). Interestingly, whereas Rheb is thought to directly activate mTORC1 activity, the Rag-mTORC1 association, which is attributed to a direct interaction between the active heterodimeric Rag conformer and Raptor (**Sancak et al 2008, Sancak et al 2010**), is suggested to modulate mTORC1 activity by controlling its translocation to the lysosomal surface in response to AA-dependent cues that promote its recruitment (see below), thereby colocalizing it with both the AA-sensing apparatus, and Rheb, its membrane-anchored GF-mediated activator. Accordingly, mTOR was shown to be diffusely distributed under starvation conditions, but upon AA-stimulation, to rapidly cluster into a punctate pattern of intracellular structures including lysosomes and late endosomes; and furthermore, in cells expressing mutant forms of RagA or RagB that are constitutively GTP-bound, Rags and mTORC1 were found to be colocalized at the lysosomal surface irrespective of AA availability, rendering the mTORC1 pathway immune to downregulation by AA starvation (**Kim et al 2008, Sancak et al 2008**).

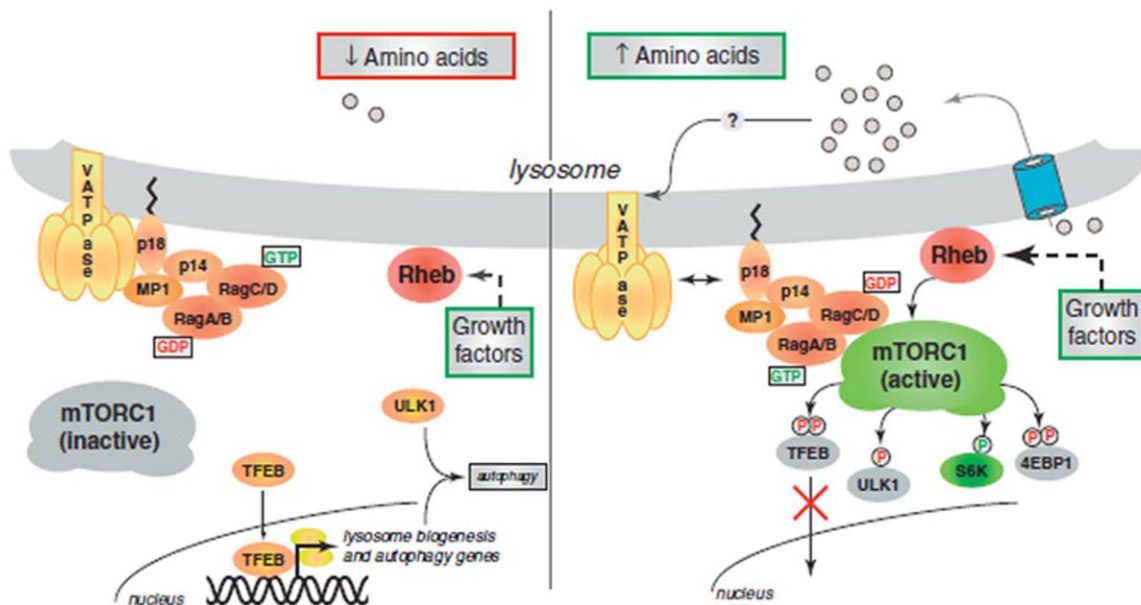


Diagram 1-18. Nutrient-dependent regulation of mTORC1 at the lysosomal surface. Reproduced from **Efeyan et al (2012)**. Amino acids regulate the recruitment of mTORC1 to the lysosomal surface, where mTORC1 is activated by Rheb. Under conditions of amino acid starvation (left panel), the V-ATPase/Ragulator/Rag GTPase complex is in the inactive conformation and unable to bind to mTORC1, resulting in the latter's cytoplasmic retention. When amino acid abundance is replenished (right panel), its increased abundance is detected (at least in part) via a lysosomal 'inside-out' mechanism, and transduced to the V-ATPase/Ragulator complex. The Ragulator complex acts as a GEF for the RagA/B GTPases, which switch their nucleotide loading to a GTP-bound state, and become activated. In turn, active Rag GTPases recruit mTORC1 to the lysosomal surface, where Rheb, which is activated downstream of TSC1/2 inhibition, stimulates mTORC1 kinase activity. Active mTORC1 phosphorylates several targets, including S6K, 4E-BP1, the autophagy regulator ULK1 and the transcription factor TFEB. Phosphorylated S6K and 4E-BP1 favor protein synthesis; phosphorylation of ULK1 blocks autophagosome formation, whereas phosphorylation of TFEB prevents it from entering the nucleus and activating a catabolic transcriptional program. Inhibitory phosphorylation is indicated by a red "P", while activating phosphorylation is indicated by a green "P".

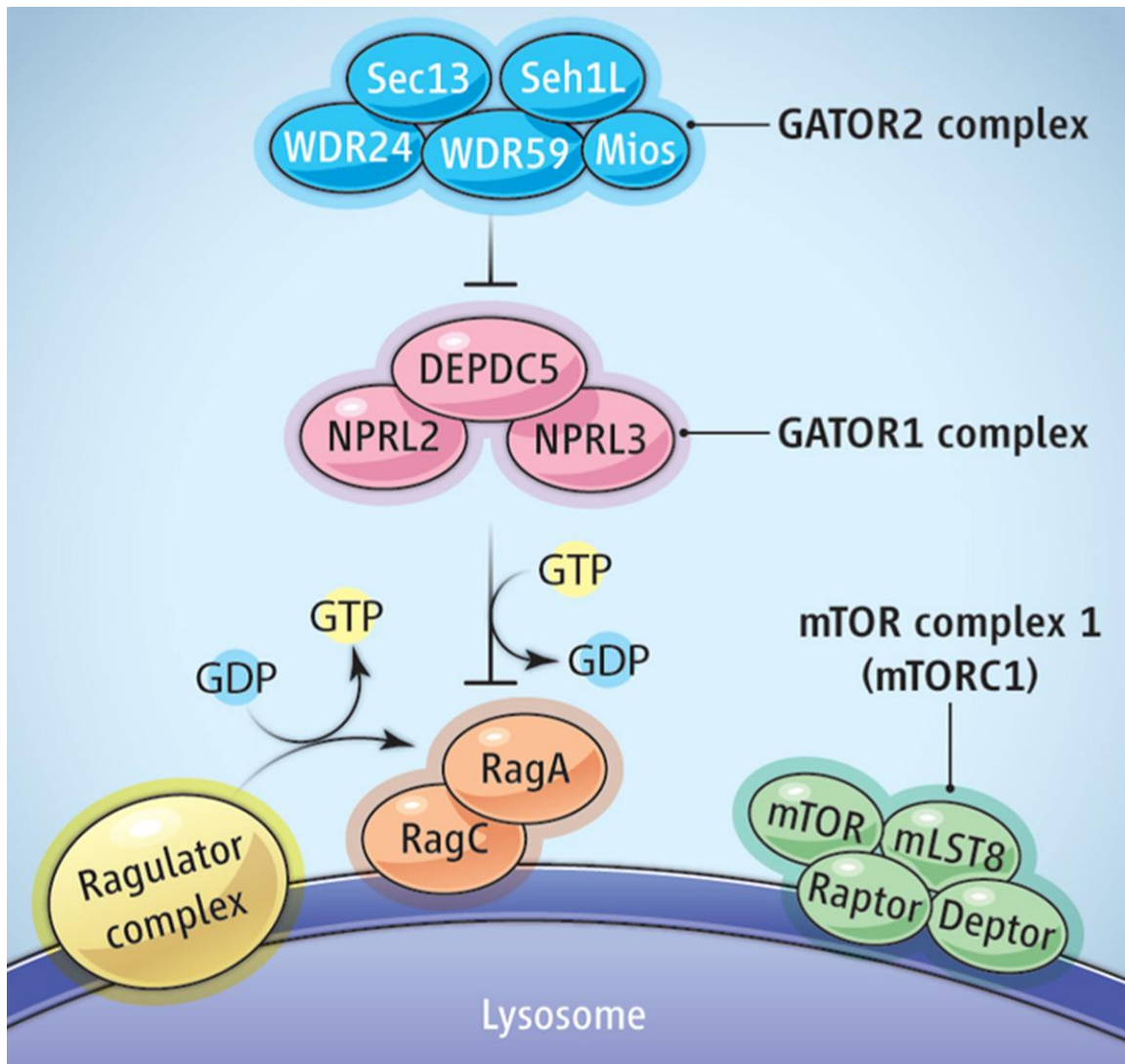


Diagram 1-19. Regulatory role of the GATOR complexes in the Rag-dependent lysosomal recruitment of mTORC1. Reproduced from Shaw (2013). Schematic representation of the function of GATOR complexes in the amino acid-sensing branch of mTORC1 activation. The heteropentameric GATOR2 complex is a negative regulator of the GATOR1 heterotrimeric complex, which itself inhibits mTORC1 activation by functioning as a GAP for RagA/B, thereby opposing the Ragulator's amino acid-dependent RagA/B GEF activity, and preventing the lysosomal localization of mTORC1.

The Rag GTPases, like Rheb GTPases, constitutively localize to the cytoplasmic surface of lysosomes and other endomembranes, but unlike Rheb (or Ras family GTPases) do not encode a lipid modification (farnesylation) motif, implying that a hypothetical Rag-binding molecule may mediate its lysosomal association. Mass spectrometry analysis of Rag immunoprecipitates identified a complex of three small proteins referred to as the “Ragulator”, consisting of MP1 (MEK-binding partner 1, encoded by *LAMTOR3*), the endosomal adaptor proteins p14 (encoded by *LAMTOR2*), and the lipid raft adaptor p18, which is encoded by *LAMTOR1* (**Sancak et al 2010**). The Ragulator complex was shown to predominantly reside on the lysosome, where it tethers Rags to the lysosomal surface; and to be required for the AA-dependent recruitment of mTORC1 as determined by the genetic disruption of Ragulator integrity, which results in the mislocalization of Rags to the cytoplasm, and the impairment of AA-induced mTORC1 recruitment to the lysosome (**Sancak et al 2010**). Furthermore, though the forced targeting of mTORC1 to the lysosomal surface was demonstrated to effectively bypass the requirement for AAs, Rags, and the Ragulator complex, rendering them dispensable for mTORC1 activation, the synthetic targeting of mTORC1 to the lysosomal surface did not bypass the requirement of Rheb function for its activation, since the RNAi-mediated knockdown of Rheb abolished the otherwise constitutive activation of lysosomally-anchored mTORC1 (**Sancak et al 2010**). The conceptual paradigm that emerged from these studies posited that the lysosome served as the major site for the integration of the independently regulated processes of GF-mediated Rheb activation and Rag/Ragulator-mediated AA-dependent mTORC1 lysosomal localization. Thus, the primary function of the Rag/Ragulator appears to be that of a scaffold that AA-dependently enables the association of mTORC1 with its activator Rheb at the lysosomal surface, while Rheb, the GF-dependent ignition switch, provides a mechanistic basis for the requirement of both “on” switches in the process of mTORC1 activation (**Diagram 1-18**).

What remained unclear in the understanding of the Ragulator/Rag mTORC1 recruitment module, however, was (1) the biochemical and mechanistic basis of AA-sensing upstream of the Rag/Ragulator activation response, and (2) the regulatory inputs, with respect to the molecules responsible for the RagGEF and RagGAP activities that

reciprocally impinge on Rag-dependent mTORC1 recruitment. The specific localization of mTORC1 to the lysosomal surface in mammals, and similar observations made in yeast, in which TORC1 is localized (constitutively, unlike in mammals) to the surface of vacuoles (**Binda et al 2009**); and the long-established role of vacuoles (the yeast equivalent of lysosomes) and lysosomes as the end-points of autophagosomes and a major storage sites for AAs in both mammals and yeast (**Harms et al 1981, Kitamoto et al 1988**, reviewed in **Klionsky et al 1990**); suggested that in addition to its role as a scaffold for the AA-mediated recruitment of mTORC1 to the lysosome (in mammals), the Rag/Ragulator may also serve as an interface for the transduction of lumenally-sensed AA availability at the lysosomal/vacuolar surface. Using a cell-free system, the lysosome itself was shown to contain all the molecular components required for AA-sensing and Rag activation, whereby the influx of AAs induced the recruitment of mTORC1 to the lysosomal surface, while conversely, the induction of lysosomal AA “leakage” resulted in the suppression of mTORC1 membrane recruitment (**Zoncu et al 2011a**). In order to identify lysosome-associated proteins that participate in the process of AA-dependent mTORC1 activation, Roberto Zoncu and his colleagues in the Sabatini laboratory screened cultured *Drosophila* S2 cells by transiently knocking down the expression of various genes with known roles in lysosomal biogenesis and function, and using S6K1 activation as a read out, identified the vacuolar H⁺-ATPase (V-ATPase), which is an ATP-driven endomembrane proton pump whose function and diverse roles in cellular physiology are described in the concluding section of the introductory chapter, as a positive regulator of AA-dependent mTORC1 activation. The existence of a direct interaction between V-ATPase and the Ragulator/Rag complex was investigated *in vitro*, and confirmed by mass spectrometry analysis of immunoprecipitates of cultured HEK293T cells expressing epitope-tagged constructs of Ragulator components or RagB; and immunoblot assays against endogenous V-ATPase subunits which further delineated that the Ragulator and Rags directly interact with multiple V-ATPase subunits (**Zoncu et al 2011a**). This study suggested an important role for the V-ATPase complex as a transducer of the “inside-out” AA-sensing signal by virtue of its engagement in direct and extensive AA-sensitive interactions with the Ragulator on the cytoplasmic surface of the lysosome, thereby additionally implicating the V-ATPase complex in the AA-dependent

branch of mTORC1 activation (*Diagram 1-18*). Importantly, in addition to its novel role in nutrient-dependent growth-promoting signals, our own experiments, which characterize both genetic and biochemical interactions between the V-ATPase complex and Akt activity (as well as their clinical implications) are the focus of the remaining chapters of this dissertation, which discuss the findings of Zoncu et al (**2011a**) in further detail, and delineate our proposed role for V-ATPase function in the GF-mediated potentiation of Akt activation, as well as downstream mTORC1/S6K1 activation.

Though the precise molecular mechanism through which V-ATPase conveys its signal to the regulator complex remains to be determined, following the establishment of the V-ATPase/Ragulator/Rag/mTORC1 signaling axis as an important lysosomal AA-sensing module, the most recently published reports from Sabatini's group have identified putative RagGEF and RagGAP complexes that fill (at least partially) two conceptual gaps in the mechanistic paradigm of AA-dependent mTORC1 recruitment. First, two important additional components of the Ragulator complex were identified (HBXIP and C7orf59), and found to interact with the heterotrimeric MP1/p14/p18 Ragulator complex, thereby forming a *pentameric* Ragulator (**Bar-Peled et al 2012**). Both HBXIP and C7orf59 were shown to be required for Rag and mTORC1 lysosomal localization, and the pentameric (rather than trimeric) conformation of the Ragulator complex alone was determined to possess GEF activity towards RagA and RagB, and required V-ATPase for its AA-dependent interaction with Rags (**Bar-Peled et al 2012**). Soon thereafter, the so-called GATOR complex, consisting of two subcomplexes called GATOR1 and GATOR2, was reported to function as a regulator of putative GATOR1-mediated RagGAP activity (*Diagram 1-19*), providing the negatively acting regulatory counterbalance (itself negatively regulated by GATOR2) to the Ragulator's positively acting RagGEF activity (**Bar-Peled et al 2013**). This study demonstrated that the inhibition of GATOR1, a heterotrimer consisting of DEPDC5, Nprl2, and Nprl3, renders mTORC1 signaling resistant to AA deprivation, consistent with its demonstrated GAP activity towards RagA and RagB *in vitro*; whereas conversely, the inhibition of GATOR2, a heteropentamer composed of Mios, WDR24, WDR59, Seh1L, and Sec13, suppressed mTORC1 signaling, with epistatic analysis suggesting that GATOR2 negatively regulates the DEPDC5 subunit of GATOR1 (**Bar-Peled et al 2013**). The

regulatory inputs operating on GATOR2 itself currently remain to be elucidated, and will surely garner intense study considering the detection of GATOR2 mutations, Rag deregulation, and V-ATPase overexpression in cancer, with implied roles in tumorigenesis and metastasis (**Bar-Peled et al 2013, Fais 2007**).

The AA-dependent presence of mTORC1 at the lysosomal surface also has significant implications with respect to its aforementioned evolutionarily conserved control over autophagy. Under optimal nutritional conditions, active mTORC1 suppresses phagophore formation through its targeted phosphorylation and inhibition of the ATG1 complex (autophagy-related gene 1, ULK1/2 in mammals), which is an essential regulator of autophagy induction (reviewed in **Chang et al 2009, Mizushima 2010, Mizushima et al 2011, Wong et al 2013**). In yeast, TORC1 promotes the dissociation of ATG1 from its regulatory binding partner ATG13, thereby inhibiting autophagy (**Kamada et al 2000**), while in *Drosophila* and mammals, both members of the ATG1/ATG13 complex, which is stable regardless of nutritional status (**Chang and Neufeld 2009, Hosokawa et al 2009, Jung et al 2009**), are TORC1-dependently phosphorylated and inactivated (**Hosokawa et al 2009, Kim et al 2011**), thereby inhibiting ectopic autophagy under favorable nutritional conditions, while mTORC1 downregulation in response to AA starvation relieves mTORC1-dependent ATG1 inhibition, which contributes (along with AMPK-mediated activating phosphorylation of ILK1 in response to energy stress) to the induction of the autophagic response. Following nutrient withdrawal and TORC1 inhibition, phagophore formation is triggered, and culminates in the fusion of mature autophagosomes with lysosomes generating hybrid organelles that enable cargo digestion (**Diagram 1-17A**). Importantly, the control over autophagy exerted by mTORC1 is Rag-dependent, as the expression of constitutively active forms of Rag suppresses the autophagic response to nutrient deprivation, whereas the expression of inhibitory mutants results in constitutive autophagosome formation irrespective of AA abundance (**Kim et al 2008**). Interestingly, the restoration of AA levels by autophagy during starvation leads to the “re-recruitment” of mTORC1 to the autophagolysosomal surface, promoting the reformation of primary lysosomes, and thereby assuring the maintenance of sensitivity to dynamically changing nutritional conditions (**Dubouloz et al 2005, Kim et al 2008**).

Whereas developmentally-programmed and/or nutritionally-imposed autophagy in *Drosophila* larval development and the implication of TORC1 in its regulation is discussed in **Chapter 2**, the *in vivo* investigation of Rag-mediated mTORC1 regulation in mice has revealed that, unlike the mid-gestational (~E12.5) embryonic lethality observed in cases of GF-sensing deficiency in, for example, TSC1/2 (**Onda et al 1999, Kobayashi et al 2001**), Rheb (**Goorden et al 2011**), or mTOR mutants (**Murakami et al 2004, Gangloff et al 2004**), the inability of mTORC1 to sense amino-acid deprivation in knockin mice expressing constitutively GTP-bound (and therefore active) RagA mutants did not compromise viability during embryonic development (during which nutrient sensing and supply is maternally determined), but dramatically compromised the neonatal survival of homozygous mutants (**Efeyan et al 2013**). The perinatal death in RagA^{GTP/GTP} mice was shown to occur within 24 hours *post partum*, in the fasting period following the post-natal interruption of placental nutrient provision and the initiation of suckling-based feeding. During this transitional period, mTORC1 inhibition caused by nutrient and glucose deprivation normally triggers autophagy as a means of supplying the building blocks of gluconeogenic molecules required for the sustenance of plasma glucose concentrations, which allows survival through this period of otherwise lethal starvation (**Efeyan et al 2013**). As such, the inability of RagA^{GTP/GTP} newborn pups to downregulate mTORC1 activation and induce autophagy in response to nutrient and glucose starvation was demonstrated to result in the reduction of hepatic gluconeogenesis, thereby accelerating compensatory glycogen breakdown, leading to hypoglycaemia, the exhaustion of energy stores, and neonatal death. Importantly, serum withdrawal, which Rag-*independently* inhibits GF-mediated mTORC1 activation through Rheb inhibition, efficiently triggered autophagy in cultured MEFs of all examined genotypes, including RagA^{GTP/GTP}, suggesting that *in vitro* at least, constitutive RagA activity does not inhibit autophagy induction by *all* signals. However, considering the evident lethality of RagA^{GTP/GTP} mutants and the demonstrated efficacy of rapamycin treatment in delaying the lethality phenotype strongly suggests that the inhibition of mTORC1 by the stimulation of Rag GTPase activity (resulting in Rag inactivation) in response to starvation is a crucial regulator of autophagy *in vivo* during the perinatal fasting period (**Efeyan et al 2013**).

1-4.6 – mTORC1/S6K1-mediated negative feedback regulation of PI3K/Akt activity

In unicellular protozoans such as yeast, in which a direct correlation exists between nutrient availability and metabolic growth (**Barbet et al 1996**), the TORC1 complex is constitutively maintained at the vacuolar membrane by the yeast equivalent of the Rag/Ragulator complex, the Gtr/EGO complex, where its activity is coordinated with (and largely determined by) endogenously sensed nutrient availability (reviewed in **Loewith and Hall 2011**). In metazoans on the other hand, in which TORC1 activity is dependent on the combined inputs of Rag/Ragulator-mediated AA-sensing and TSC/Rheb-mediated GF-dependent signaling, the requisite point of mechanistic integration is provided by the Rag/Ragulator-dependent recruitment of mTORC1 to its GF-mediated activator Rheb at the lysosomal surface. Accordingly, whereas the TSC1/2-Rheb module is progressively linked to the GF-mediated control of mTORC1 activation in metazoan lineages of increasing complexity (**van Dam et al 2011**), the protozoan paralogs of these molecules are primarily involved in AA acid uptake (**Urano et al 2000**, **van Slegtenhorst et al 2004**), and do not directly impinge on TORC1 function as evidenced by: (1) the recent demonstration that the TSC2 regulatory domains targeted for phosphorylation by the plethora of agonist-activated upstream kinases in metazoans are not conserved in the earliest protozoan paralogs, which consist of little more than a rudimentary precursor GAP protein (**Serfontein et al 2010**); and (2) the demonstration that Rheb neither functions upstream of TORC1, nor is required for viability in yeast (**Urano et al 2000**).

As such, though the evolutionarily ancestral means of modulating TORC1 activity can accommodate the nutrient availability-based growth programs of single-celled protozoans, the onset of multicellularity marked the beginning of a shift in the control of TORC1-mediated cellular processes away from cell-autonomous determinants of AA abundance, and towards receptor-based signaling pathways whose activation by systemically circulated GFs and mitogens coordinate *organismal* growth and development by engaging the appropriate TORC1-mediated cellular responses, while AA abundance itself determines the Rag-mediated colocalization of mTORC1 with its GF-dependent activator Rheb, and ensures that local nutritional state can accommodate the

context-specific responses elicited by systemic cues. Moreover, in higher metazoans, TORC1 integrates numerous upstream-acting signaling cues, including not only growth factor-dependent Akt-mediated TSC1/2 phosphorylation and Rheb activation, but as described above, the modulation of TSC1/2 activity by mitogens, cytokines, as well as by nutrient or energy availability (**Diagram 1-16**), cumulatively implicating the TSC2/Rheb/mTOR signaling module and its all-important control over protein synthesis in numerous developmental and physiological processes. Accordingly, equally sophisticated reciprocal mechanisms of context-dependent feedback (**Diagram 1-20**), cross-talk, and compensation have been described that fine tune the magnitude *and* duration of receptor-mediated signaling processes, and ensure the maintenance of sensitivity to stimulatory agonists; while also preventing the accrue ment of pathological levels of upstream activating cues (reviewed in **Mendoza et al 2011**). One of the best-characterized feedback-targeted molecules is the IRS1 molecule, which is negatively feedback-regulated by various pathways, including the mTORC1/S6K1 signaling module downstream of IRS1-mediated PI3K/Akt activation, whereby the activation of mTORC1 can stimulate both its own, as well as S6K1-mediated inhibitory phosphorylation of IRS1. The mTORC1 molecule itself targets IRS1 for inhibitory phosphorylation at two residues in the vicinity of the p85-binding motifs (S636/S639), thereby interfering with PI3K recruitment and activation (**Ozes et al 2001, Gual et al 2003, Hiratani et al 2005, Tzatsos and Kandr or 2007**), while downstream of mTORC1 activation, S6K1 can phosphorylate IRS1 at (1) in the PTB domain at S312 (in human IRS1), which occurs latently and interferes with receptor binding while also promoting IRS1 degradation (**Pederson et al 2001, Greene et al 2003, Briaud et al 2005, Shah et al 2004, Harrington et al 2004, Carlson et al 2004**); (2) S270, which it phosphorylates in response to TNF α , causing its 14-3-3-mediated sequestration from the receptor (**Zhang et al 2008a**); and (3) S1101, which is located in the C-terminal portion of the IRS1 molecule, and shown to be stimulated in response to nutrient-dependent mTORC1 activation (**Tremblay et al 2007**).

In addition to its role in targeting the IRS1-dependent activation of PI3K signaling, the mTORC1/S6K signaling module has also more recently been shown to conduct its feedback inhibitory function by phosphorylating and regulating IRS1-

independent means of PI3K/Akt signal transduction. Following mTORC1 activation, S6K1 has been demonstrated to phosphorylate the mTORC2 component Rictor at T1135 (in human Rictor), promoting the latter's interaction with 14-3-3 (**Dibble et al 2009, Treins et al 2010**). However, although the substitution of this residue to alanine (rendering it unphosphorylatable) was found to cause an increase in Akt HM phosphorylation at S473 (**Dibble et al 2009, Julien et al 2010**), the S6K1-mediated phosphorylation of Rictor at T1135 itself was not found to significantly alter mTORC2 activity in cultured cells or *in vitro* kinase assays in any of the studies investigating its function (**Dibble et al 2009, Treins et al 2010, Julien et al 2010, Boulbés et al 2010**), suggesting the involvement of additional unidentified modifications and/or interactions *in vivo*. Further upstream of Rictor, mTORC1 has been found to phosphorylate the RTK-associated adaptor Grb10, which is a negative regulator of insulin signaling frequently downregulated in cancer (**Yu et al 2011**). Grb10-deficiency in knockout mice results in PI3K/Akt hyperactivation in insulin-sensitive tissues (**Charalambous et al 2003, Wang et al 2007b**), and was identified as a direct mTORC1 substrate whose rapamycin-sensitive phosphorylation at S501 and S503 promoted its stabilization, leading to feedback inhibition of both PI3K/Akt and MAPK signaling (**Yu et al 2011, Hsu et al 2011**), further establishing the mTORC1 axis as a prominent feedback-regulatory mechanism of receptor-dependent PI3K/Akt activation.

The gradual integration of TORC1-mediated control over protein synthesis into RTK-responsive upstream-acting signal transduction pathways therefore also led to an increase in the sophistication of mTORC1-mediated feedback mechanisms on said RTK-based signaling pathways. In humans, the loss of negative feedback phosphorylation of IRS1 at S312, which can be executed by a number of kinases including S6K1 downstream of mTORC1, has been implicated in the insulin resistance commonly observed in cases of chronic nutrient overload and mTORC1 activation such as obesity (reviewed in **Zick 2005, Gual et al 2005**), and has been evoked to at least partially account for: (1) the benign nature of TSC1 or TSC2-deficient tumors (**Shah et al 2004, Harrington et al 2004, Manning et al 2005**), and (2) the failure of a number of rapamycin analogues to efficiently inhibit growth in some tumors (reviewed in **Guertin and Sabatini 2009, Liu et al 2009a**). In contrast to the implied importance of S6K1-

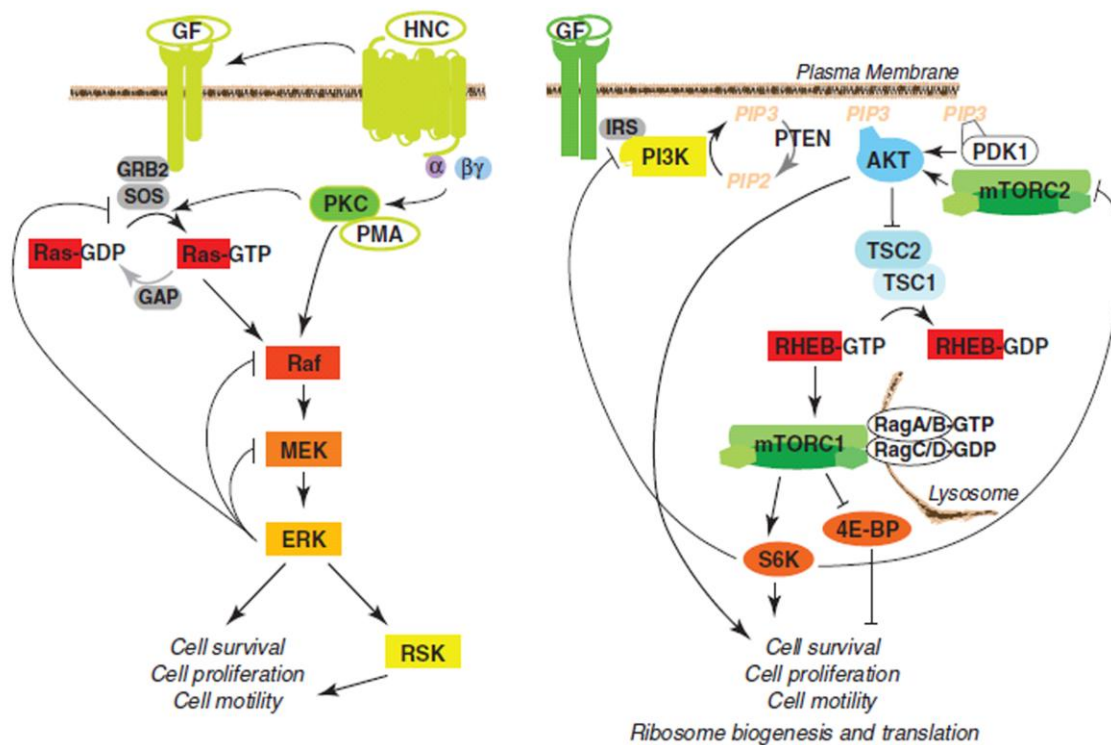


Diagram 1-20. Negative feedback loops operating downstream of growth factor receptors. Reproduced from **Mendoza et al (2011)**. The left panel depicts major mechanisms of negative feedback within the Ras/MAPK pathway. In quiescent cells, Raf, Mek, and Erk are largely cytoplasmic and inactive. Growth factors promote RTK auto-phosphorylation, generating binding sites for the Grb2 adaptor molecule, which is constitutively associated with the RasGEF SOS (son of sevenless). As a RasGEF, the SOS molecule catalyzes guanine nucleotide exchange, and active Ras-GTP then recruits Raf to the membrane, where it is activated. Polypeptide hormone, neurotransmitter, and chemokine (HNC) activation of GPCRs can also feed into the MAPK cascade by trans-activating upstream RTKs and/or by activating PKC. Cell-permeable phorbol esters such as PMA (phorbol-12-myristate-13-acetate) bind and activate PKC by mimicking the natural PKC ligand diacylglycerol. Activation of the Raf/Mek/Erk/Rsk kinase cascade, in addition to promoting the appropriate downstream mitogenic cellular responses, also provides an Erk-mediated negative feedback loop that limits the magnitude and duration of the receptor-borne Ras-dependent signal. The right panel depicts main mechanisms of negative feedback within the PI3K/Akt pathway. Receptor activation recruits adaptors such as IRS and Gab, which in turn recruit PI3K to the receptor complex. The agonist-dependent activation of PI3K at the receptor complex stimulates PIP₃ production, promoting Akt and PDK1 PH domain-mediated membrane recruitment and activation. The mTORC1/S6K1 pathway negatively feeds back on PI3K/Akt signaling by targeting IRS and Rictor for inhibitory phosphorylation, thereby diminishing both PI3K/PDK1-dependent and mTORC2-mediated Akt activation. See text for details.

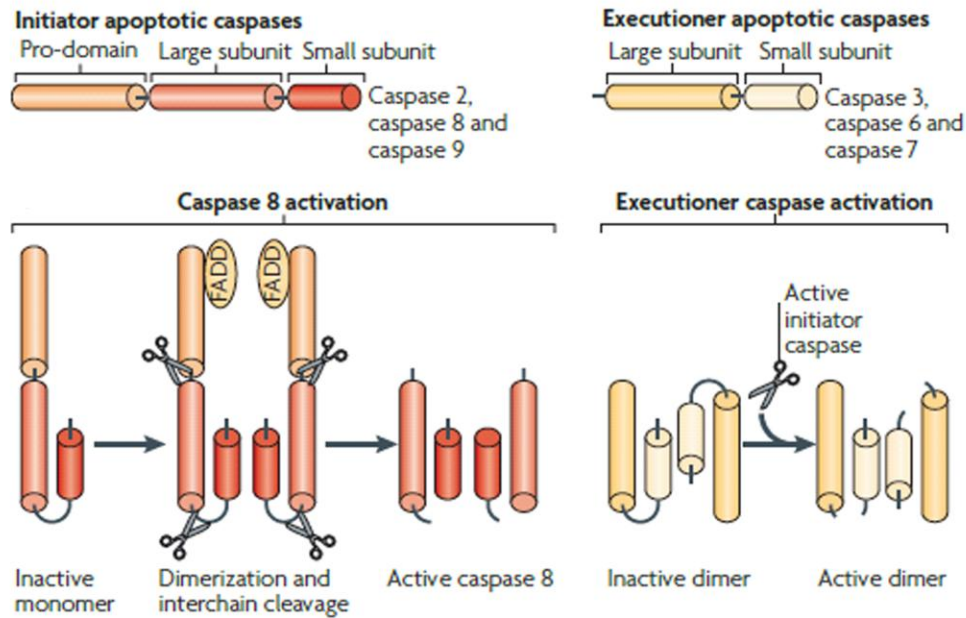
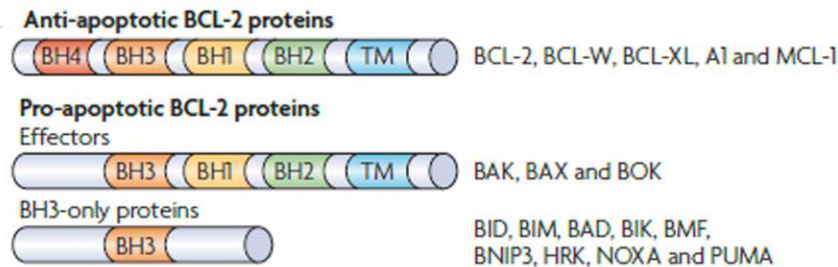
A**B**

Diagram 1-21. Caspases and the Bcl-2 family of proteins. Reproduced from **Tait and Green (2010)**. (A) Caspases (Cys Asp acid proteases) cleave substrates in a highly specific manner after an aspartate residue in short tetrapeptide (X-X-X-Asp) motifs. Whereas executioner caspases have more than a hundred substrates, the repertoire of initiator caspase substrates is limited, and includes self-cleavage, BID (BH3 interacting-domain death agonist), and executioner caspases. Initiator Caspase-8 activation first requires dimerization of inactive caspase monomers at the death receptor complex, which is followed by interdomain cleavage of the initiator caspase. In contrast, executioner caspases are constitutively present in cells as dimers, and are activated by initiator caspase-dependent cleavage, leading to intramolecular rearrangements and the formation of an enzymatically active dimer. (B) Pro-apoptotic Bcl-2 proteins can be subdivided into effectors (molecules that cause MOMP) or BH3-only molecules (responsible for relaying the apoptotic signal to the effectors). Anti-apoptotic Bcl-2 proteins prevent or delay MOMP by sequestering BH3-only proteins or by inhibiting activated Bax or Bak.

mediated feedback inhibition in *normal* mammalian physiology, TORC1-mediated feedback inhibition of PI3K/Dakt signaling, which occurs during normal *Drosophila* development *in vivo*, has recently been suggested to be defined by a feedback loop that depends on TORC1 itself, and to be dS6K-*independent* (**Kockel et al 2010**). The switch to dS6K-dependent means was suggested to occur strictly in the context of ectopically enhanced TORC1 activity, such as the loss of dTSC2 function, which itself, as mentioned above, does not require direct phosphorylation by Dakt for normal growth despite the conserved presence of the putatively targeted residues known to be phosphorylated by Akt in mammals (**Dong and Pan 2004**). This is consistent with the prior genetic, biochemical, and pharmacological demonstration that dS6K, though activated, like its mammalian counterpart, by the combined activities of TORC1 and dPDK1, resides in a branch of the insulin signaling pathway that is distinct from, and *independent* of, the PI3K/Dakt signaling node (**Radimerski et al 2002**), establishing its now decade-long perception as an intermediately integrated signaling network (by metazoan standards) with respect to the *parallel*-acting rather than upstream-acting PI3K/Dakt signaling pathway.

1-4.7 – The role of Akt substrates in the promotion of cellular survival

Apoptosis is a genetically programmed cellular response that leads to cell death. Its homeostatic induction occurs in normal physiology throughout metazoans, and its deregulation is implicated in diverse pathologies including cancer and neurodegenerative diseases (reviewed in **Elmore 2007**). The induction of apoptosis culminates in the proteolytic activation of “executioner” caspases (**Diagram 1-21A**), which are themselves proteolytic enzymes responsible for the defining morphological characteristics of apoptosis including diminished cell volume, membrane blebbing, and nuclear fragmentation (reviewed in **Tait and Green 2010**). In the mid-1990s, around the same time that numerous studies were establishing Akt as an important PI3K effector downstream of various GFs (see **Section 1-3.1**), the promotion of survival associated with the growth factor stimulation of cultured PC-12 cells, which like developing neurons *in vivo* rely on specific neurotrophic factors like NGF (nerve growth factor) for survival, was found to *require* the activation of PI3K, whereas Ras, though required for

differentiation, was dispensable for NGF-mediated survival (**Yao and Cooper 1995**). Shortly thereafter, Akt itself was implicated in the promotion of cellular survival in response to apoptotic insults such as UV radiation, and downstream of growth factors such as PDGF and IGF1 in neuronal cells, cytokines like IL-3 in leucocytes, and the attachment of adherent cells to the extracellular matrix (**Yao and Cooper 1995, Kulik et al 1997, Dudek et al 1997, Khwaja et al 1997, Songyang et al 1997, Coffey et al 1998b**).

In mammalian cells, the cellular apoptotic machinery is initially induced through one of two signaling cascades termed the intrinsic and extrinsic pathways (**Diagram 1-22**), which converge on the activation of the executioner Caspase-3 and Caspase-7 molecules. The intrinsic pathway, which can be induced by cytotoxic insults such as growth factor withdrawal, DNA damage, or ER stress (reviewed in **Letai 2006, Tait and Green 2010**), is primarily controlled through interactions between pro- and anti-apoptotic members of the Bcl-2 (B cell lymphoma 2) family, whereby the activation of pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins leads to Bax (**BCL-2-associated X** protein) and Bak (**Bcl-2 antagonist or killer**) activation, resulting in mitochondrial outer membrane permeabilization (MOMP), and marking the point of no return for cell survival. Importantly, the activation of either Bax or Bak is essential for MOMP, as evidenced by the profound resistance of mice lacking both molecules to the induction of apoptosis by most stimuli that act through the intrinsic pathway (**Wei et al 2001**). Conversely, acting in opposition to the pro-apoptotic BH3-only and effector members of the Bcl-2 family are anti-apoptotic Bcl-2 proteins, which delay or prevent MOMP by binding BH3-only proteins and the activated Bax or Bak effectors (**Diagram 1-21B, Diagram 1-22**).

Following the implication of PI3K signaling in the promotion of survival, one of the first substrates of Akt to be identified with direct implications in the regulation of survival was the BH3-only pro-apoptotic BAD molecule (Bcl-2 antagonist of death), which was shown to be phosphorylated by Akt at S136, forming a high affinity 14-3-3 binding site (**Zha et al 1996, Datta et al 1997**). In its unphosphorylated state, BAD is free to bind and inhibit anti-apoptotic members of the Bcl-2 family such as Bcl-x_L (reviewed in **Chipuk et al 2010**), but upon its phosphorylation and 14-3-3-mediated

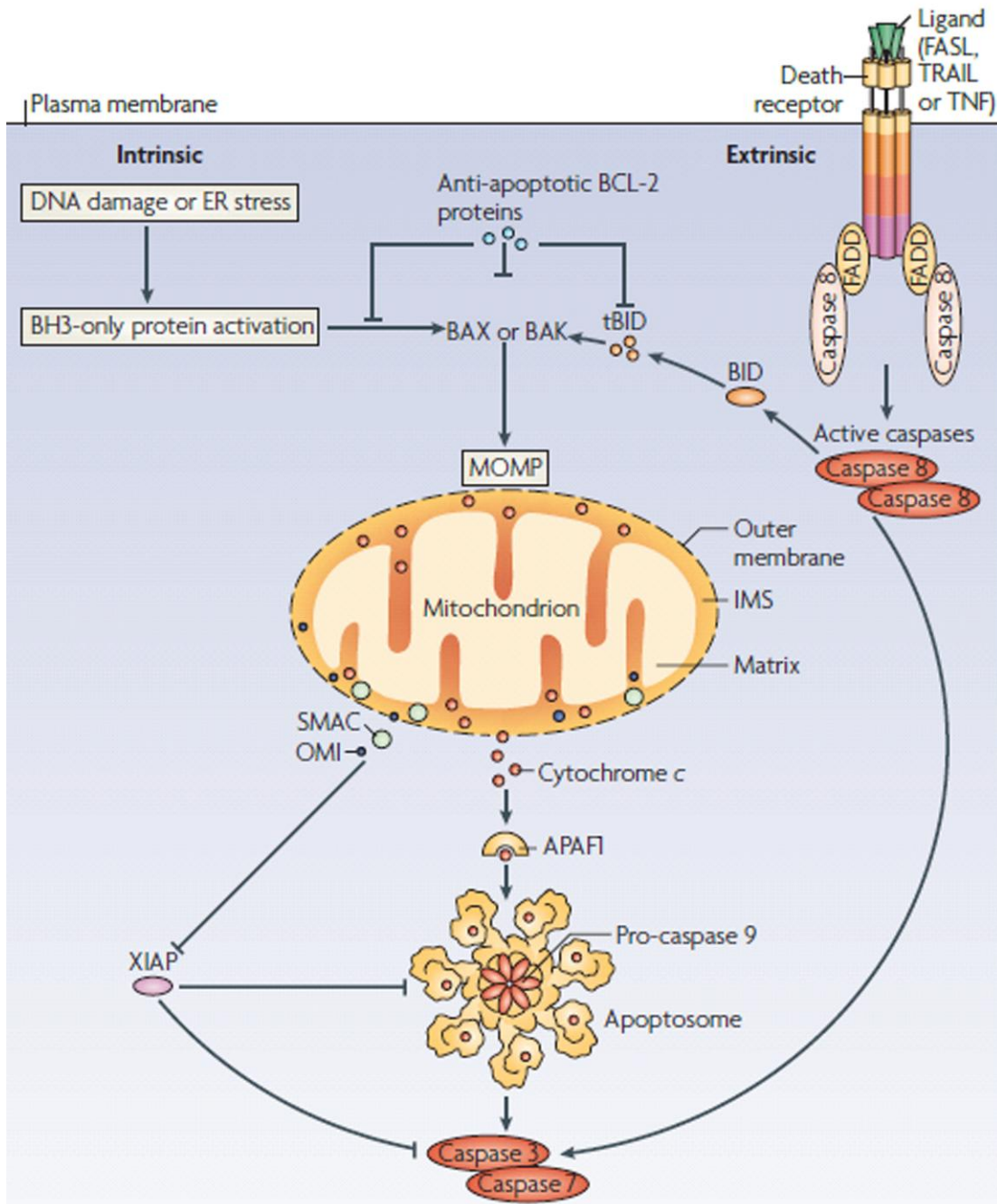


Diagram 1-22. The intrinsic and extrinsic pathways of apoptosis. Reproduced from **Tait and Green (2010)**. The intrinsic pathway is activated by factors including DNA damage and ER stress, and induces executioner Caspase (3 and 7) activation through Bax- or Bak-mediated mitochondrial outer membrane permeabilization (MOMP). The extrinsic pathway is activated by death receptor stimulation, leading to the activation of initiator Caspase-8, and subsequent executioner Caspase activation. Extrinsic apoptotic stimuli can also transactivate the intrinsic pathway through the Caspase 8-mediated cleavage of the BH3-only molecule BID, the truncated product of which (tBID) promotes Bax or Bak activation.

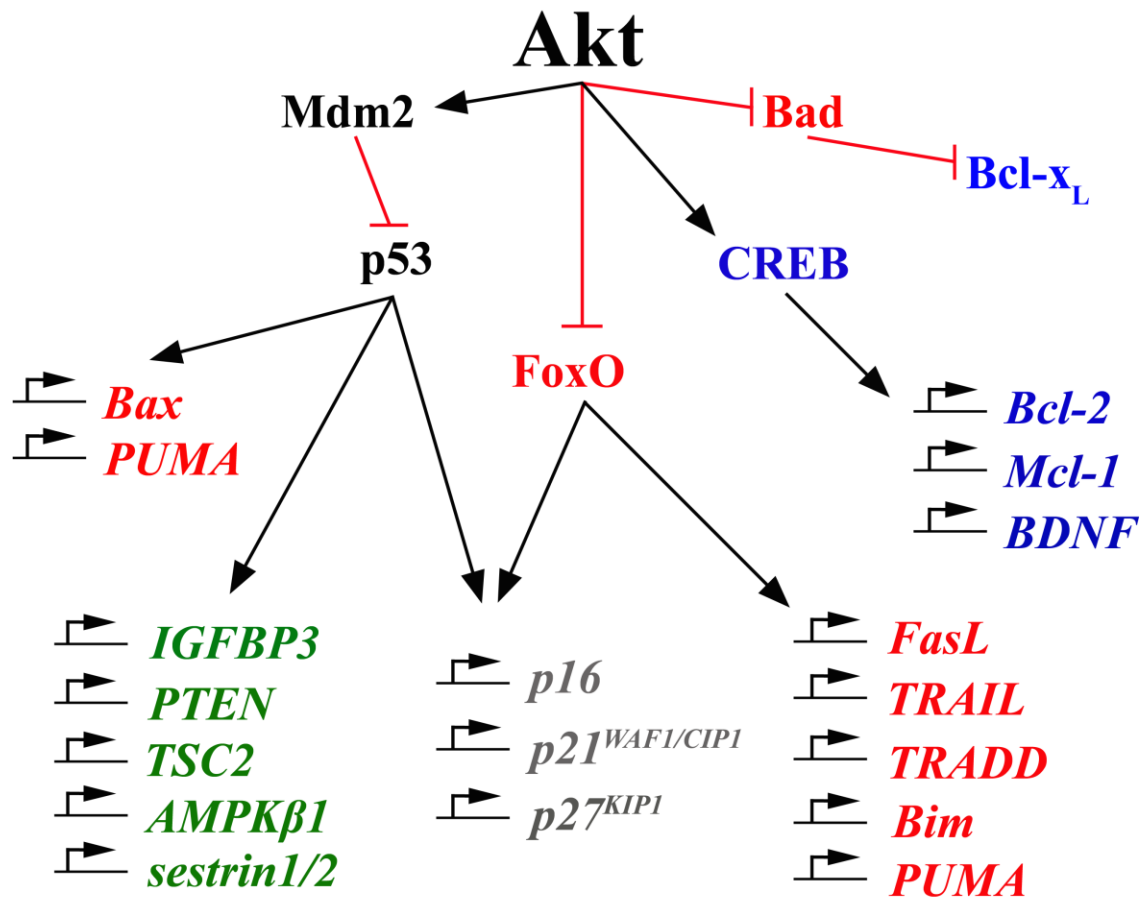


Diagram 1-23. The survival-promoting signaling network downstream of Akt. The activation of Akt can promote survival through a number of its substrates, notably including: the BH3-only pro-apoptotic molecule Bad, thereby suppressing the latter's inhibitory function towards anti-apoptotic members of the Bcl-2 family; the transcription factor CREB, whose activation promotes the transcription of anti-apoptotic target genes; the transcription factor FoxO, whose function as a transcriptional activator of pro-apoptotic and cyclin-dependent kinase (CDK)-inhibiting genes is suppressed by Akt-dependent inactivation; and E3 ubiquitin ligase Mdm2, whose activation by Akt promotes p53 degradation, thereby suppressing the latter's transcriptional activation of pro-apoptotic, CDK-inhibiting, and PI3K/Akt/mTORC1-inhibiting genes. See text for details. Activating interactions are shown as black arrows and inhibitory interactions are shown as red lines. Survival-promoting genes (in italics) and proteins are shown in blue; pro-apoptotic genes and proteins are shown in red; genes encoding inhibitors of cell cycle progression are depicted in grey, and genes encoding negative regulators of PI3K/Akt and mTORC1 signaling are shown in green.

sequestration, its inhibitory role towards anti-apoptotic Bcl-2 family members is relieved, allowing the latter to resume their protective functions (*Diagram 1-23*). Importantly, much as TSC1/2 serves as a crucial integration point for mTORC1 activation, BAD is in its own right an important node of cross-talk with respect to survival signaling as it is targeted at multiple residues (S122 and S155, in addition to S136) by kinases such as RSK, PKA, PAK (p21-activated kinase), and CaMKK (Calmodulin-dependent protein kinase kinase) downstream of diverse signaling pathways that can impinge on cellular survival (**Datta et al 2000**). Although the expression of an unphosphorylatable BAD molecule in a BAD-null background was found to resensitize cells to apoptotic stimuli (**Datta et al 2002**), endogenous BAD expression is not ubiquitous, and varies depending on cell type *in vivo* (**Kitada et al 1998**), and furthermore, cells lacking BAD expression can still be protected from apoptosis by Akt activation (**Kennedy et al 1999**), suggesting that additional targets also contribute to the Akt-mediated promotion of survival.

A number of these additional Akt targets protect cells from apoptotic stimuli by impinging on the transcriptional element of the apoptotic response, whereby the transcription of pro-apoptotic molecules is downregulated, whereas the transcription of survival-promoting factors is activated (*Diagram 1-23*). Activated Akt can phosphorylate the transcription factor CREB (cAMP response element-binding protein) at S133, thereby promoting the transcription of its targets (**Du and Montminy 1998**), which include genes encoding anti-apoptotic factors such as Bcl-2 (**Wilson et al 1996**) and Mcl-1 (**Wang et al 1999**), as well as the brain-derived neurotrophic factor BDNF (**Tao et al 1998**). The phosphorylation of the tumor-suppressing FoxO transcription factors by Akt (which as previously described in **Section 1-4.1**, occurs at multiple residues, and creates 14-3-3-binding motifs) inhibits their function as transcriptional activators for a number of genes encoding pro-apoptotic molecules. The pro-apoptotic targets of FoxO transcription factors most notably include extracellular ligands such as FasL (the ligand for the death receptor Fas), TRAIL (TNF-related apoptosis-inducing ligand), and TRADD (TNF receptor type 1 associated death domain), which can activate the extrinsic apoptotic pathway in target cells; as well as intrinsically acting pro-apoptotic factors such as the BH3-only proteins Bim (Bcl2-interacting mediator of cell death) and PUMA (reviewed in **Zhang et al 2011**). Many of these transcriptional targets have been shown to be

upregulated in the apoptotic response to GF withdrawal, including the induction of FasL in neurons (**Brunet et al 1999**), as well as the induction of Bim and PUMA in hematopoietic cells (**Dijkers et al 2000, You et al 2006**).

There are many similarities and overlap in the mode of regulation and function of FoxO molecules, which in addition to cell death, also transcriptionally regulate cell cycle arrest in the absence of Akt signaling (reviewed in **Burgering and Medema 2003**) and oxidative stress (reviewed in **Storz 2011**), and the mode of regulation and function of the tumor suppressor p53 (reviewed in **Dansen and Burgering 2008, Zhang et al 2011**). Various intrinsic and extrinsic stress signals including DNA damage, hypoxia, nutrient starvation and oncogene activation serve as inputs into the regulation of p53 function; in response to which p53 specifically regulates the transcriptional expression of a set of its target genes, many of which overlap with FoxO targets. The transcriptional activation of p53 targets, depending on cell type, physiological context, and degree of stress, initiate cell cycle arrest, DNA repair, and if necessary, apoptosis (reviewed in **Vogelstein et al 2000, Levine et al 2006a, Vousden and Prives 2009, Beckerman and Prives 2010, Feng 2010**). The capacity of both FoxO and p53 to arrest cell cycle progression stems from their transcriptional activation of target genes encoding tumor suppressors such as the cyclin-dependent kinase inhibitors p16, p21^{WAF1/CIP1}, and p27^{KIP1} (**Diagram 1-23**); while their orchestration of apoptosis relies on multiple mechanisms, but favors the upregulation of pro-apoptotic BH3-only and Bcl-2 molecules such as Bim by FoxO, Bax by p53, and PUMA by both FoxO and p53 (reviewed in **Beckerman and Prives 2010, Zhang et al 2011**). However, in addition to its role in the *induction* of cell cycle arrest and apoptosis, p53 also plays an important role in the *suppression* of Akt-dependent survival pathways by inducing the expression of another set of target genes, all of which negatively regulate PI3K/Akt- and mTORC1-dependent signaling pathways in response to stress (reviewed in **Levine et al 2006b, Feng et al 2010**), and include *IGF-BP3* (**Buckbinder et al 1995**), which binds to circulating IGF molecules and prevents IGF1R stimulation, *PTEN* (**Stambolic et al 2001**), *TSC2* and *AMPK β 1* (**Feng et al 2005, Feng et al 2007**), as well as *sestrin1* and *sestrin2* (**Budanov and Karin 2008**), whose functions have been described in previous sections.

Accordingly, several regulatory loops have been described between p53 and the PI3K/Akt- and mTORC1-mediated signaling pathways, the balance of which either promotes stress response, apoptotic death, or the renewal of the commitment towards growth. For example, while the stimulation of mTORC1 activity can lead to the downstream activation of the protein phosphatase PP2A, resulting in the inactivation of p53 through its PP2A-mediated dephosphorylation at S15 (**Kong et al 2004**); the S15 residue, phosphorylation of which promotes p53 activity, can itself be targeted downstream of starvation-induced AMPK activation, thereby reinstating p53-mediated transcription of Akt/mTORC1-inhibitory signaling components (**Imamura et al 2001, Feng et al 2005, Jones et al 2005**). Similarly, the PI3K/Akt pathway can also impinge on p53 transcriptional activity through its capacity to phosphorylate the E3 ubiquitin ligase MDM2 at S166 and S186, thereby activating its ligase function, which in turn leads to p53 ubiquitination and degradation (**Zhou et al 2001, Ashcroft et al 2002, Gottlieb et al 2002**); whereas MDM2 can be bound and inhibited by p14^{ARF} in response to stress signals, thereby allowing p53-dependent transcriptional responses (reviewed in **Harris and Levine 2005**). Moreover, in addition to its regulation of p53 integrity through the activation of MDM2, Akt can also indirectly modulate the transcriptional activity of p73, a member of the p53 family that also participates in the induction of cell death by inducing the transcription of many of the same pro-apoptotic factors as p53, including PUMA and Bax (reviewed in **Roos and Kaina 2006**). The p73 molecule can be bound by the transcriptional co-activator YAP1 (Yes-associated protein1), which promotes the transcription of p73 target genes (**Strano et al 2001**). The activation of Akt can interfere with the YAP1-mediated activation of p73 by directly phosphorylating YAP1 at S127, thereby promoting its association with 14-3-3, and resulting in YAP1 cytoplasmic retention, which sequesters it from p73 in the nucleus (**Basu et al 2003**). Furthermore, the activation of Akt downstream of GF or cytokine stimulation has also been implicated in the transcriptional activation of pro-survival genes through its context-specific cross-talk with the NF- κ B pathway. As previously described in **Section 1-4.4**, the activation of the IKK complex downstream of pro-inflammatory cytokines can activate mTORC1 signaling by phosphorylating TSC1 and inactivating TSC1/2 RhebGAP function (**Lee et al 2007**). The IKK complex itself, however, can be activated by the Akt-mediated

phosphorylation of IKK α at T23, thereby inducing the degradation of I κ B (the NF- κ B inhibitor), and allowing NF- κ B-mediated transcription of anti-apoptotic factors (**Kane et al 1999, Ozes et al 1999, Romashkova and Makarov 1999**).

Lastly, depending on signaling context and the identity of the apoptotic stimulus (intrinsic or extrinsic), the *inhibition* of GSK3 by Akt (among numerous other upstream regulatory elements) in response to GF stimulation can, on the one hand, *suppress* apoptosis through the intrinsic pathway; whereas conversely, its *activity* is required for survival in response to extrinsic apoptotic stimuli such as TNF α - or death-receptor activation (reviewed in **Beurel and Jope 2006**). The requirement of GSK3 β activity for survival was established by the demonstration that *GSK3 β* -deficient mice develop normally through early/mid embryonic development, but perish around E14 following massive TNF α -induced hepatocyte apoptosis, which was determined to be caused by the loss of the GSK3 β -dependent NF- κ B-mediated survival response (**Hoeflich et al 2000**). These findings were foreshadowed by the observed capacity of lithium, thereafter shown to inhibit GSK3 (**Klein and Melton 1996**), to potentiate the cytotoxic response of tumor cells to TNF both *in vitro* and in animal studies (**Beyaert et al 1989**); and subsequently recapitulated by the identification of NF- κ B-responsive survival-promoting signaling elements directly induced by GSK3 β kinase activity (**Schwabe and Brenner 2002, Demarchi et al 2003**). Prior to the distinction between the opposite roles of GSK3 in intrinsic versus extrinsic apoptotic stimuli, the pro-survival function attributed to GSK3 β activity was perplexing in light of the previously demonstrated pro-apoptotic role of GSK3 in a wide variety of signaling contexts such as growth factor deprivation and PI3K inhibition (reviewed in **Grimes and Jope 2001**), including the demonstration in 1998 by Geoffrey Cooper that overexpression of catalytically active GSK3 β induced apoptosis in both Rat-1 fibroblasts and neuronal PC-12 cells in a p53-dependent manner (**Pap and Cooper 1998**), which was reported shortly after his aforementioned demonstration of the survival-promoting effects of PI3K/Akt signaling in 1995 (**Yao and Cooper 1995**).

With respect to its pro-apoptotic role in the intrinsic pathway, which converges on Bax/Bak-mediated mitochondrial disruption, and is subject to suppression by GF-mediated PI3K/Akt-dependent GSK3 inhibition, GSK3 exerts its control over survival both directly and indirectly. For instance, in the absence of survival promoting signals

that inhibit its activity, GSK3 can activate Bax by directly phosphorylating it at S163 (**Linseman et al 2004**), while its phosphorylation of the anti-apoptotic Bcl-2 family member Mcl-1 enhances the latter's degradation (**Maurer et al 2006**). In addition to its direct modulation of the function of Bcl-2 family members, however, the relief of GSK3 inhibition can also promote the apoptotic signaling cascade by directly impinging on the activity of numerous transcription factors through which the apoptotic machinery is upregulated. Although the majority of GSK3 resides in the cytoplasm, a small contingent of GSK3 β is dynamically localized to the nuclear and mitochondrial compartments, where it is known to be in a relatively greater state of activation in comparison to the predominantly cytoplasmic majority of GSK3 β molecules (**Bijur and Jope 2003**). Whereas the cytosolic pool of GSK3 β has been specifically implicated in the extrinsic TNF-induced anti-apoptotic response (**Meares and Jope 2007**), the size and/or activity of the nuclear pool of GSK3 can be increased under apoptotic conditions (**Bijur and Jope 2001**), and the increased activity of GSK3 β towards nuclear targets, many of which are crucial pro-apoptotic transcription factors such as p53 (reviewed in **Beurel and Jope 2006**), mediates its promotion of the intrinsic pathway of apoptosis. Accordingly, the stimulation of PI3K/Akt signaling diminishes the levels of nuclear GSK3 β (**Bijur and Jope 2001**), and more recently, the pharmacological inhibition of GSK3 was found to attenuate DNA damage-induced activation of Bax, and to inhibit the translocation of p53 to mitochondria (**Ngok-Ngam et al 2013**), which is known to induce apoptosis independently of the p53-mediated effects on gene transcription (**Marchenko et al 2000**, **Mihara et al 2003**, reviewed in **Moll et al 2005**).

1-4.8 – Identification of novel Akt interactions and signaling components

In addition to the proposed role of mTORC2-mediated HM phosphorylation as a determinant of differential Akt substrate recognition (see **Section 1-3.3**), the investigation of physiological mechanisms governing the context-dependence of Akt function has highlighted two interrelated factors – subcellular localization and the identity of interacting proteins, whose deduced significance with respect to isoform-specific function and substrate specificity is currently best understood in the context of adipocyte function (reviewed in **Gonzalez and McGraw 2009**, **Fayard et al 2010**). These two crucial

variables have been the main focal points in the effort to reconcile first, the redundancy shown by often unrelated extracellular inputs (GFs, cytokines, cellular adhesion, etc) in their ability to PI3K-dependently stimulate Akt activity to comparable degrees *in vitro*, and second, the diversity of critical cellular processes that Akt activity impinges on through its numerous putative or recognized substrates. In the effort to formulate a mechanistic paradigm that accounts for modes of differential context-dependent regulation *in vivo*, a number of Akt-interacting molecules with both inhibitory and activating properties have been identified and characterized over the past decade (**Diagram 1-24**), including the APE molecule (Akt phosphorylation enhancer), which binds the C-terminal domain of Akt and *enhances* its basal phosphorylation at both the HM and T-loop sites *in vitro* (**Anai et al 2005**); the Ft1 protein, which also associates with the C-terminal portion of Akt, and *enhances* its activity by promoting the PDK1-Akt interaction (**Remy and Michnick 2004**); TRB3 (tribbles homolog 3), which *attenuates* GF-mediated Akt activation in hepatocytes by binding the Akt kinase domain and precluding T-loop phosphorylation (**Du et al 2003**); and three E3 ligases, of which TRAF6 (TNF receptor associated factor 6) catalyzes K63-linked ubiquitination, thereby promoting Akt membrane localization and activation (**Yang et al 2009**), whereas K48-linked ubiquitination, which targets activated Akt molecules for degradation, can be carried out by the TTC3 (tetratricopeptide repeat domain 3) and MULAN (MUL1, mitochondrial E3 ubiquitin protein ligase 1) E3 ligases (**Suizu et al 2009**, **Wu et al 2011**, **Bae et al 2012**).

Further underscoring the importance of a more complete understanding of Akt activation and function is its frequent hyperactivation in pathological conditions like cancer (see **Chapter 4**). Accordingly, though the elucidation of the regulatory mechanisms governing agonist-stimulated mTORC2 activation have lagged behind that of mTORC1 (reviewed in **Zoncu et al 2011b**), many of the identified modifiers of Akt activity have also been found to be deregulated in pathological conditions (**Diagram 1-24**). Examples include: (1) the oncogene *Tcl-1*, whose protein product directly interacts with Akt by binding its PH domain, thereby *promoting* its activation and nuclear translocation and *enhancing* proliferation and survival (**Laine et al 2000**, **Pekarsky et al 2000**, **Hiromura et al 2004**, **Pekarsky et al 2008**), and whose overexpression in human

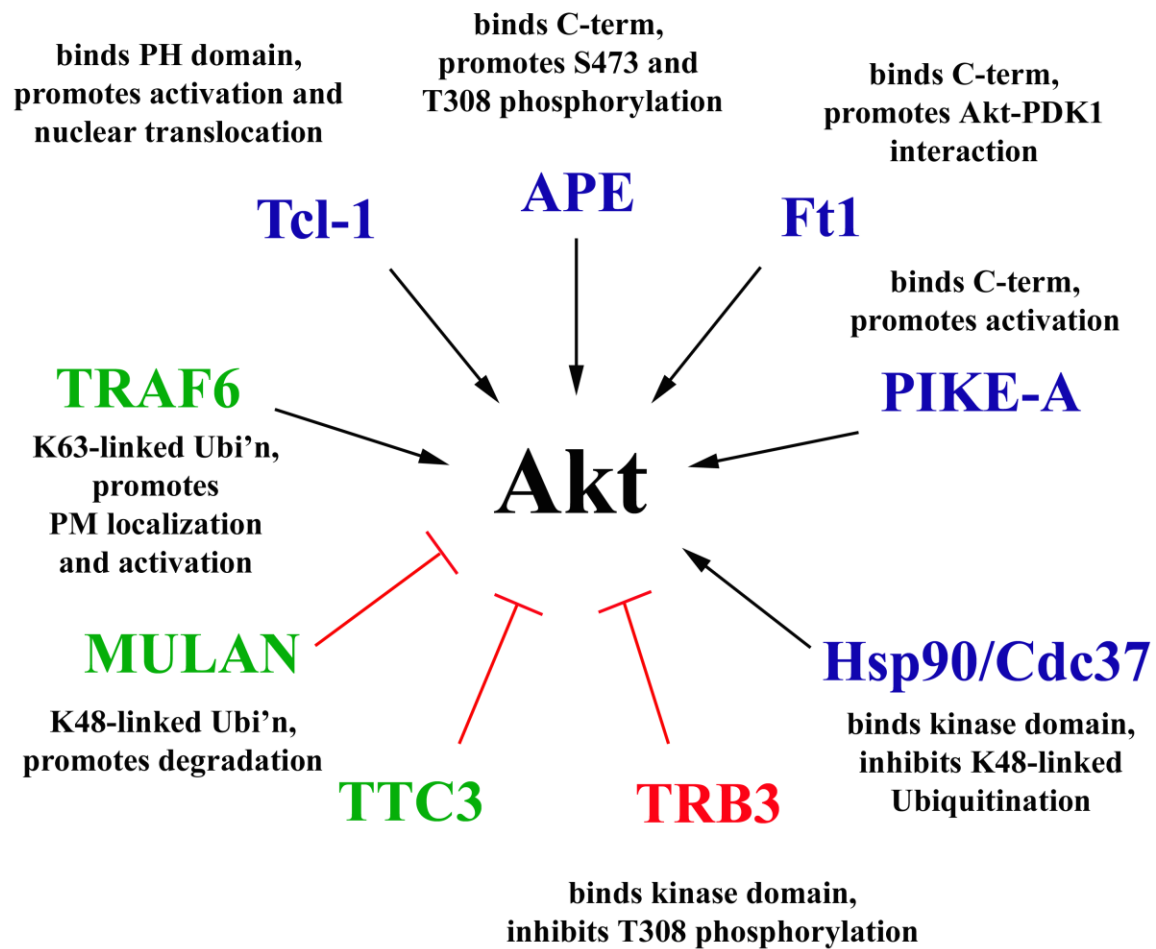


Diagram 1-24. Akt-interacting proteins with modulatory functions. Molecules represented in blue are positive regulators of Akt function, whereas TRB3 is a negative regulator. Molecules in green are E3 ubiquitin ligases. Black arrows represent a positive interaction, and red lines designate inhibitory ones. See text for details

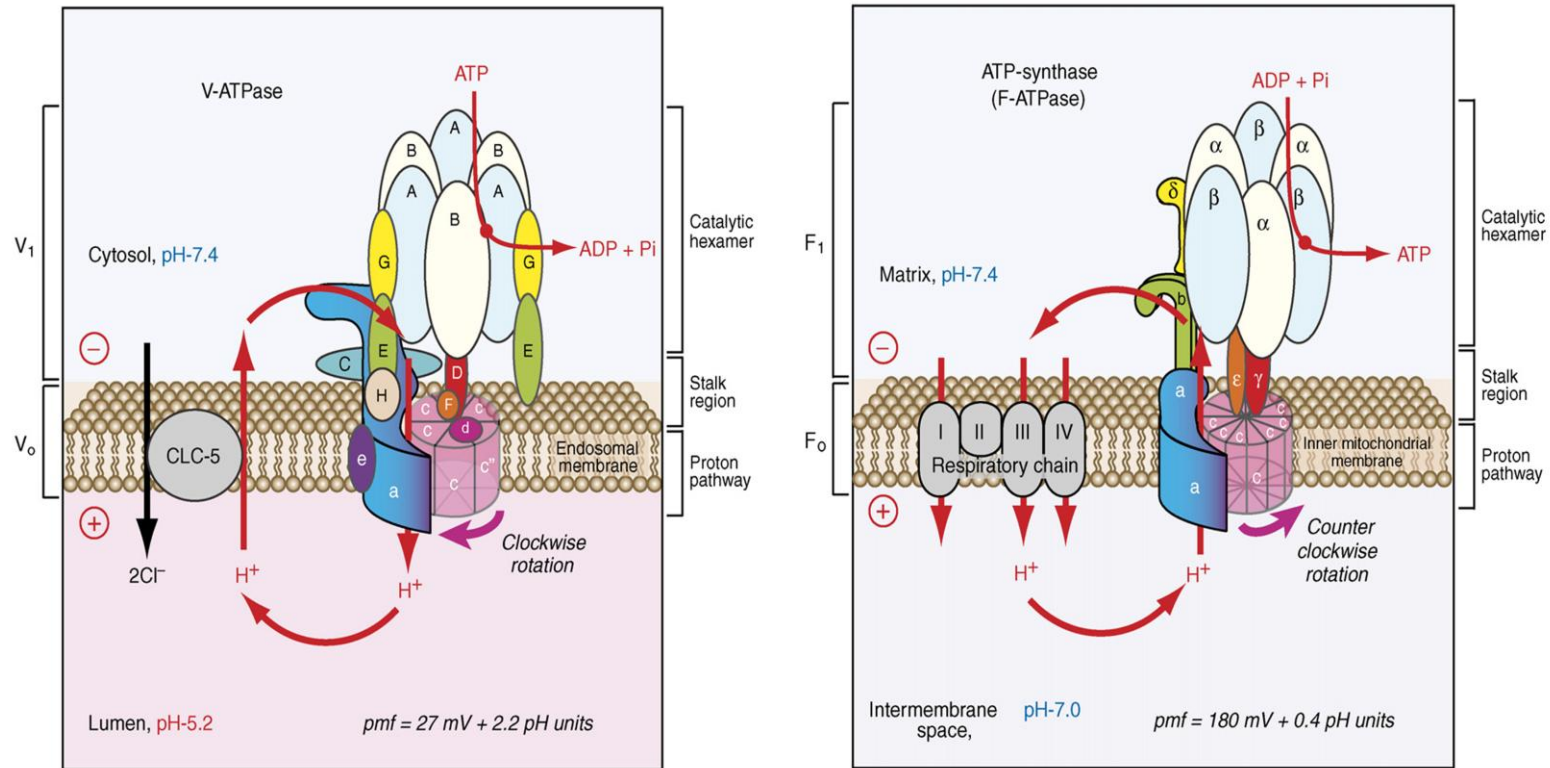


Diagram 1-25. Comparative structural models and functional roles of V-ATPase and F-ATPase. Reproduced from **Marshansky and Futai (2008)**. Homologous subunits (such as the A and B subunits of V-ATPase and the α and β subunits of F-ATPase) are shown in the same colors. In the V-ATPase complex, ATP hydrolysis drives clockwise rotation of the central stalk and integral V_0 domain, leading to the translocation of protons from the cytosol to the progressively acidified endomembrane lumen, and generating an electrochemical proton gradient (or proton-motive force) across the membrane. In endosomes, V-ATPase activity also drives the neutralizing current mediated by electrogenic CLC5 exchanger. F-ATPase pumps protons in the opposite direction, and the counter-clockwise rotation of its integral domain (F_0) is coupled to ATP synthesis, and driven by the proton-motive force generated by the function of respiratory chain enzymes. See text for details.

T cell prolymphocytic leukemia contributes to pathogenesis (**Noguchi et al 2007**); (2) the plasma membrane-associated CTMP molecule (C-terminal modulatory protein), whose influence on Akt activity remains controversial, with opposing accounts of its function as a negative (**Maira et al 2001**) and positive (**Ono et al 2007**) regulator of Akt activation, but whose characterization as a pro-apoptotic factor (**Miyawaki et al 2009**, **Piao et al 2009**, **Hwang et al 2012**) is consistent with a negative role in the Akt-dependent promotion of survival, and whose expression has been reported to be downregulated in primary glioblastomas (**Knobbe et al 2004**, **Knobbe et al 2005**); (3) the GTPase PIKE-A (PI3K enhancer A), whose direct interaction with the Akt C-terminal domain is thought to stimulate Akt activation, and promote the survival and invasiveness of glioblastoma cells (**Ahn et al 2004a**, **Ahn et al 2004b**, **Liu et al 2008**); and (4) the Hsp90/cdc37 chaperone complex, which has been shown to bind the Akt kinase domain, thereby protecting the Akt molecule from K48-linked ubiquitination and proteasomal degradation (**Basso et al 2002**), and consistent with the reduction of Akt activity observed in *cdc37*-deficient human colon cancer cells (**Gray et al 2007**, **Smith et al 2009**). The development and refinement of proteomics-based approaches to the investigation of protein-protein interactions, including high-throughput yeast two-hybrid cDNA library screens, protein-fragment complementation, fluorescence or bioluminescence resonance energy transfer (FRET/BRET), and mass spectrometry (reviewed in **Yates et al 2009**, **Choudhary and Mann 2010**, **Petschnigg et al 2011**, **Mohr and Koegl 2012**) has greatly contributed to the multidisciplinary “decoding” of signaling networks.

These *in vitro* screening methods have led to the identification of many of the Akt-interacting molecules described above, including Ft1, APE, Tcl-1, and PIKE-A; as well as modulatory partners of other prominent members of the mTORC1/S6K1 and PI3K/Akt signaling axes, notably including the various regulatory elements operating upstream of mTORC1 (such as the Ragulator, GATOR1 and GATOR2 complexes, see **Section 1-4.5**); and the TUSC4 molecule (tumor suppressor candidate 4), which was recently identified as a PDK1-interacting and inhibiting molecule (**Kurata et al 2008**). However, genetic screens in protozoan model organisms such as yeast (reviewed in **Widmann et al 1999**, **Forsburg 2001**); as well as simple metazoans, including invertebrates such as *C. elegans* (**Jorgensen 2002**, **Kaletta and Hengartner 2006**) and

Drosophila (reviewed in **St Johnston 2002, Neumüller and Perrimon 2010**), whose numerous advantages as screening subjects include the *a priori* establishment of the detected interaction's relevance *in vivo* within the signaling context investigated, have long been (and remain) prolific tools for the identification of functionally interacting molecules within specific cellular processes or signaling pathways, and highly useful physiological models for diverse human diseases. Dr. Manoukian's screen in *Drosophila* for enhancers of embryonic Dakt activity (see **Chapter 2**) identified a strong genetic interaction with the vacuolar H⁺-ATPase, whose major physiological functions (in addition to its aforementioned novel role in the regulation of mTORC1 activity) are summarized below.

1-5 – The vacuolar H⁺-ATPase

The evolutionarily related membrane-spanning F-type and V-type ATPases (**Diagram 1-25**), whose reversible catalytic activities are specifically coupled to proton transport, are the cornerstones of cell-autonomous bioenergetic processes, and are present in all three domains of life – bacteria, which predominantly expresses the F-type; archaea, which are generally obligate anaerobes, and predominantly express the V-type; and eukaryotes, which possess both (reviewed in **Mulkidjanian et al 2007**). The F-type ATPase (also called ATP-synthase) is thought to represent the ancestral *bacterial* cation-translocating ATPase responsible for ATP *synthesis* in most bacteria, as well as the mitochondria and/or chloroplasts of all eukaryotic cells, in which it is coupled to the proton motive force generated by aerobic glycolysis (reviewed in **Marshansky and Futai 2008**). The vacuolar-type H⁺-ATPase (V-ATPase) is thought to be the ancestral *archaeal* form, and in eukaryotic cells (which unlike prokaryotes, are highly compartmentalized), are located in a variety of endomembranes such as clathrin-coated, synaptic, recycling, storage, Golgi-derived, and secretory vesicles; as well as endosomes, lysosomes (or vacuoles in plants and fungi), phagosomes, and in certain cell types, the inner surface of the plasma membrane, where they predominantly function as ATP *hydrolysis*-driven proton pumps that acidify their respective lumenal (or in the case of

plasma membrane V-ATPases, extracellular) compartments (reviewed in **Beyenbach and Wiczorek 2006, Toei et al 2010, Ma et al 2011**).

Whereas plasma membrane V-ATPases predominantly play cell-type specific roles such as renal pH homeostasis and uric acid secretion (**Wagner et al 2004**), bone resorption in osteoclasts (**Toyomura et al 2003, Sørensen et al 2007**), and sperm maturation through the maintenance of seminal fluid acidity by the clear cells of the epididymis (**Pietrement et al 2006**); the acidification of endomembranes by intracellular V-ATPases plays a crucial role in various inducible and constitutive cellular processes, including cell surface receptor endocytosis and recycling (reviewed in **Katzmann et al 2002, Maxfield and McGraw 2004**), intracellular membrane traffic and exocytic hormone secretion (reviewed in **Marshansky and Futai 2008**), as well as lysosomal protein degradation, the coupled uptake of small molecules such as nutrients and neurotransmitters, and neural or hormonal/GF-mediated signal transduction (reviewed in **Forgac 2007, Marshansky and Futai 2008**). Accordingly, the breadth of physiological processes depending on V-ATPase function is reflected by: (1) its requirement for normal development in both protozoans (reviewed in **Beyenbach and Wiczorek 2006**) and metazoans including *Drosophila* (**Davies 1996, Allen et al 2005**), mice (**Inoue et al 1999, Sun-Wada et al 2000**), *C. elegans* (**Oka et al 2001**), as well as *Xenopus* and other non-mammalian vertebrates (**Adams et al 2006**); and (2) the diversity and number of human diseases associated with its deregulation, including neurological disorders such as Alzheimer's and Parkinson's disease (reviewed in **Wolfe et al 2013**), bone diseases like osteoporosis (reviewed in **Yuan et al 2010, Qin et al 2012**), viral infection (reviewed in **Gong et al 2007**), diabetes (**Rojas et al 2004, Sun-Wada et al 2006**), and cancer (reviewed in **Fais et al 2007, Spugnini et al 2010**).

1-5.1 – V-ATPase structure and function

Eukaryotic V-ATPases are highly conserved, large, membrane-spanning multi-subunit complexes containing at least fourteen different “core” subunits (some of which are present in multiple copies per complex) that are organized into 2 domains. The peripheral 500-kDa ATP-hydrolyzing V₁ domain is a cytosolic cylinder composed of eight different subunits (A-H), and responsible for ATP binding and hydrolysis, which

powers the rotation of the V_1 domain, and couples ATP hydrolysis with proton translocation through the integral 250-kDa V_0 domain. Its recruitment to the membrane and reversible association with the V_0 domain is a crucial (though poorly understood) regulatory checkpoint along the endocytic/exocytic tract (reviewed in **Toei et al 2010**), and the signaling pathways involved in this important regulatory step notably include PKA (**Alzamora et al 2010**), AMPK (**Gong et al 2010**), and PI3K/Akt (**Sautin et al 2005**), which has been implicated in the glucose-mediated assembly of V-ATPase in renal epithelial cells. The V_0 domain spans the membrane and harbors the rotary mechanism, and consists of five different subunits (a , c , c' , c'' , and d) that, along with an additional accessory subunit (e), form the proton translocation pore.

As shown in **Diagram 1-26**, the A and B subunits make up the ATP hydrolyzing motor of the peripheral V_1 domain, are each present in three copies per complex, and arranged in alternating fashion in a hexameric ring (A_3B_3). In both prokaryotic and eukaryotic V-ATPases, most of the catalytic site residues for ATP hydrolysis are contributed by the A-subunit (**MacLeod et al 1998**, **Zhang et al 2008b**, **Maher et al 2009**), which is encoded in *Drosophila* by the *Vha68(1-3)* genes, with *Vha68-2* being the most widely expressed isoform (**Allan et al 2005**), and mutant alleles of which were used in our genetic studies described in **Chapter 2**. The B subunit on the other hand, seems to play a regulatory role. Though sequence analysis of the B subunit has revealed significant homology with other nucleotide-binding proteins (including the related A subunit), it lacks the central glycine-rich loop required for catalytic activity, consistent with the aforementioned assignment of catalytic function to the A subunit, in which the glycine-rich loop is conserved (reviewed in **Ma et al 2011**). The N-terminal β -barrel domain of the B subunit does however bind F-actin (**Lee et al 1999b**, **Holliday et al 2000**, **Chen et al 2004**, **Zuo et al 2008**), microfilaments of which are implicated in intracellular traffic, cell motility, and cell division. These actin filaments are essential for the survival of most cells due to their provision of internal mechanical support, and as tracks for the intracellular transport of various cargos (reviewed in **Pollard and Cooper 2009**).

The D and F subunits occupy the central cavity created by the A_3B_3 cylinder, forming a stalk that serves as a rotor for the A_3B_3DF sub-complex (**Yokoyama et al 2000**, **Imamura et al 2003**). The E and G subunits heterodimerize to form at least two

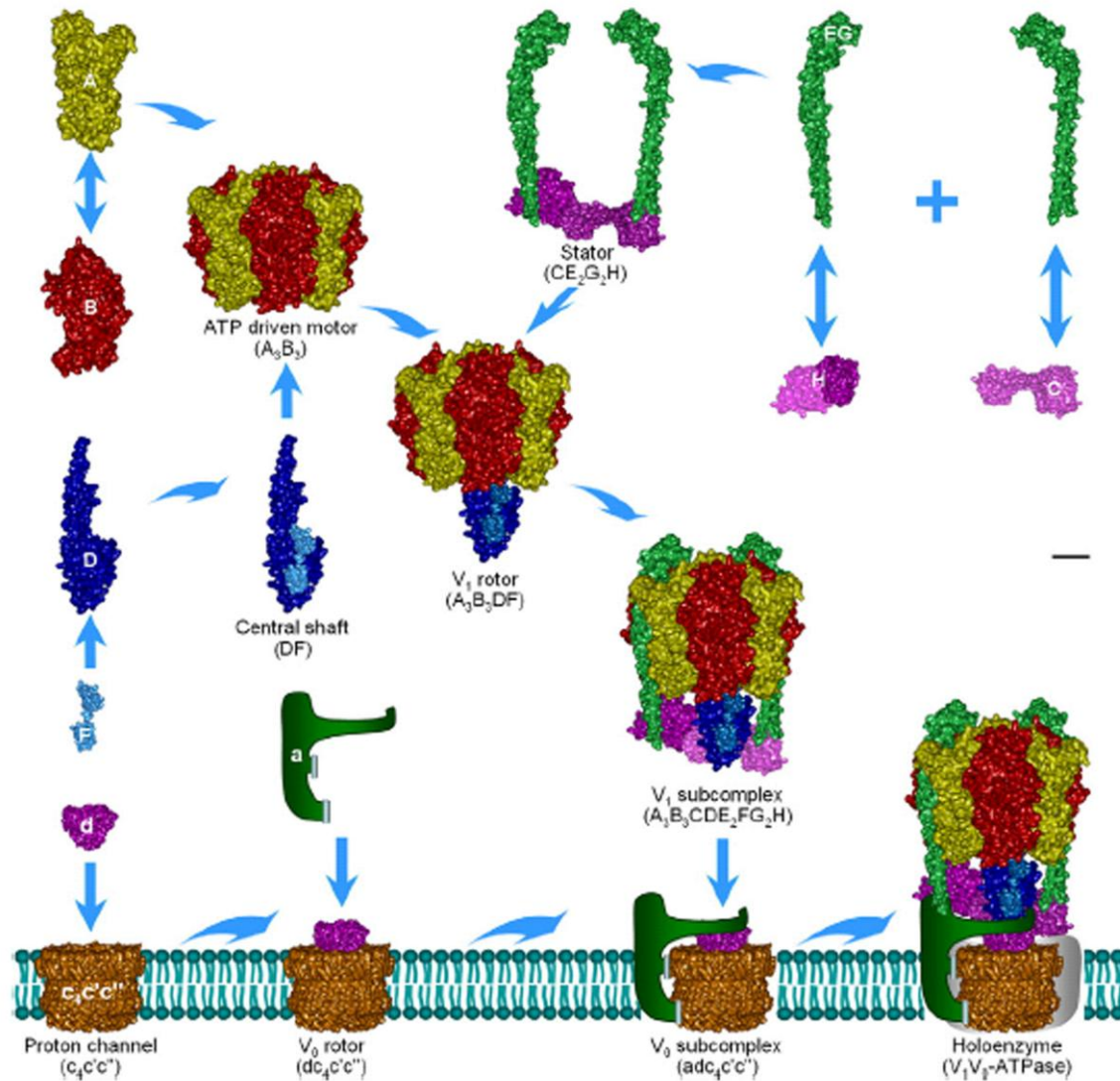
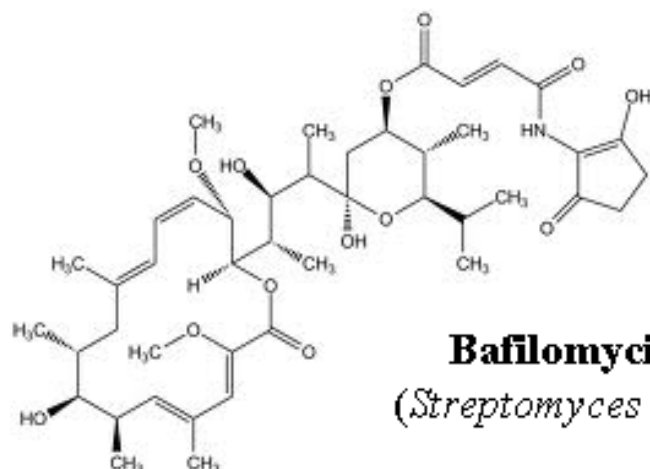
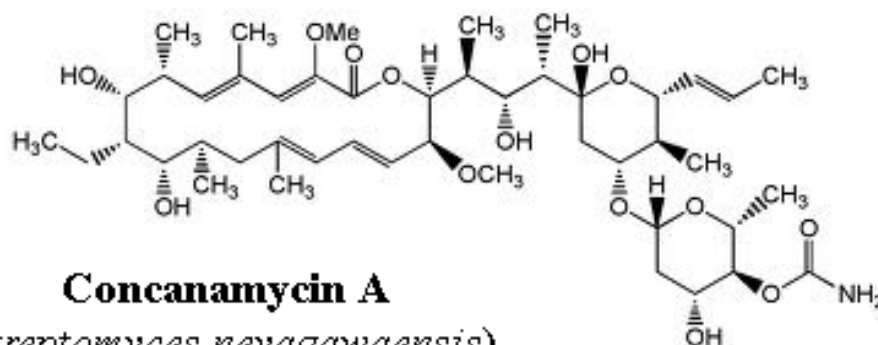


Diagram 1-26. Molecular assembly of the V-ATPase holoenzyme. Reproduced from **Ma et al (2011)**. Three copies of subunits A and B (A₃B₃) form the ATP driven motor, which is the catalytic core. The D and F subunits make up a central shaft (DF) that fills the central cavity of the A₃B₃ cylindrical motor. The catalytic core and central shaft form the V₁ rotor (A₃B₃DF). The E, G, H, and C subunits form a stator (CE₂G₂H) whose addition to the V₁ rotor completes the assembly of the V₁ subcomplex (A₃B₃CDE₂FG₂H). The subunits *a*, *d*, and the proton channel c₄c'c'' combine to form the integral V₀ subcomplex (adc₄c'c''), which recruits the V₁ subcomplex, thereby producing the V-ATPase holoenzyme. The scale bar represents 50 Å.



Bafilomycin A1
(*Streptomyces griseus*)



Concanamycin A
(*Streptomyces neyagawaensis*)

Diagram 1-27. Chemical structure of the V-ATPase inhibitors **Bafilomycin A1** and **Concanamycin A**. Bafilomycin A1, molecular formula: $C_{35}H_{58}O_9$, molecular weight: 622.83. Concanamycin A, molecular formula: $C_{46}H_{75}NO_{14}$, molecular weight: 866.1.

(but possibly three) peripheral stalks (**Xu et al 1999, Radermacher et al 2001, Rizzo et al 2003, Wilkens et al 2004**), while a high affinity interaction between the C and G subunits provides a base for the EG stalks, thereby forming the CE₂G₂H (or CE₃G₃H) sub-complex (**Hildenbrand et al 2010**), which serves as a stator (the stationary part of the rotary holoenzyme) for the A₃B₃DF catalytic sub-complex (**Diagram 1-26**).

Importantly, the association of the V₁ domain (A₃B₃CDE_{2/3}FG_{2/3}H) with the V₀ integral domain requires the presence of all eight minimal subunits of V₁, as the loss of any single subunit can prevent its efficient recruitment to the endomembrane surface, and results in the cytoplasmic retention of the misassembled V₁ domain (**Doherty et al 1993, Tomashek et al 1997**). The core components of the membrane-embedded V₀ domain (*a*, *c*, *c'*, *c''*, and *d*) are integral membrane proteins with the exception of the *d* subunit, which is a peripheral membrane protein tightly associated with the proton translocation pore formed by the other four subunits (**Bauerle et al 1993**). The *d* subunit, for which the *a* subunit acts as a swivel, associates with the DF central rotor (**Diagram 1-26**), effectively linking ATP hydrolysis-driven rotation of the V₁ domain with that of the hexameric translocation pore formed by the *c*, *c'*, and *c''* subunits (*c*₄, *c'*, *c''*), and thereby initiates proton translocation (**Imamura et al 2005, Nakano et al 2008**). As in the case of the V₁ domain, all the core subunits must be present for V₀ assembly, the lack of any of which results in highly diminished stability (**Graham et al 1998**), and the loss of V-ATPase function (**Doherty et al 1993, Tomashek et al 1997**). Importantly, the interface between the *a* subunit and the translocation pore is the mechanistic target of bafilomycin A1 (a macrolide antibiotic synthesized by the bacterium *Streptomyces griseus*) and concanamycin A (a related macrolide produced by *Streptomyces neyagawaensis*), which are potent and specific V-ATPase inhibitors that prevent ATP catalysis, V₁/V₀ rotation, and proton flux (**Gagliardi et al 1999, Bowman et al 2004, Wang et al 2005, Bowman et al 2006**), and whose use *in vitro* has greatly contributed to the study of V-ATPase-dependent cellular functions (**Diagram 1-27**, see **Chapter 3**).

1-5.2 – V-ATPase-mediated regulation of endomembrane acidification and traffic

In eukaryotic evolution, the compartmentalization of intracellular space has allowed the additional regulatory dimension of spatial restriction in the regulation of

certain biochemical reactions and pathways. The luminal compartments of these diverse endomembranes have different properties in terms of ion concentrations, redox states, and degrees of acidification; and generally become more acidic as the exocytic or endocytic routes approach their destination (**Diagram 1-28**). In the endocytic pathway, early endosomes are mildly acidic (~pH 6.5) in comparison to the cytosol (~pH 7.2), sorting and recycling endosomes are intermediately acidic (~pH 6.5-6.0), whereas so-called “destination” endomembranes such as late endosomes/multivesicular bodies (pH 6.0-5.0) and lysosomes (pH 5.0-4.5), which require acidification for the activity of degradative enzymes and the export of degradation products, are progressively more acidic (reviewed in **Weisz 2003, Maxfield and McGraw 2004, Woodman and Futter 2008, Jefferies et al 2008**). Similarly, in the exocytic pathway, pH ranges from neutral in the endoplasmic reticulum, to nearly neutral in the trans-Golgi (pH ~6.8), to mildly acidic in (for example) insulin storage granules (~pH 6.2), and with highest levels of acidity (pH 5-5.5) in secretory granules, which require a low pH for the processing of pro-hormones to their mature forms, and the generation of a proton motive force for the exocytosis of small molecules such as neurotransmitters (**Futai et al 1998, Nishi and Forgac 2002, Tompkins et al 2002**, reviewed in **Paroutis et al 2004, Sun-Wada et al 2004, Forgac 2007**). Although pH can be modulated by various factors including proton leakage, chloride channels (ClC), or Na⁺/K⁺-ATPases (**Faundez and Hatzell 2004, Grabe and Oster 2001, Jentsch 2007, Scheel et al 2005**), luminal acidification, which is tightly regulated, is primarily carried out by V-ATPases, which harness ATP-hydrolysis to transport protons across their respective membranes.

The required level of acidification in each compartment therefore results from the combined contributions of V-ATPase and other operative transporters, channels, and/or buffers that harness the proton motive force generated by V-ATPase-mediated proton influx; as well as their respective context-dependent regulatory elements (reviewed in **Paroutis et al 2004**). Accordingly, the distribution, assembly, abundance, and regulation of V-ATPase are the major factors governing the steady-state pH of individual endomembranes, and are themselves determined by cellular function, membrane composition, cytosolic environment, and composition of the V₁/V₀ complex with respect to the subunits encoded by multiple isoforms. The intrinsic determinant of differential V-

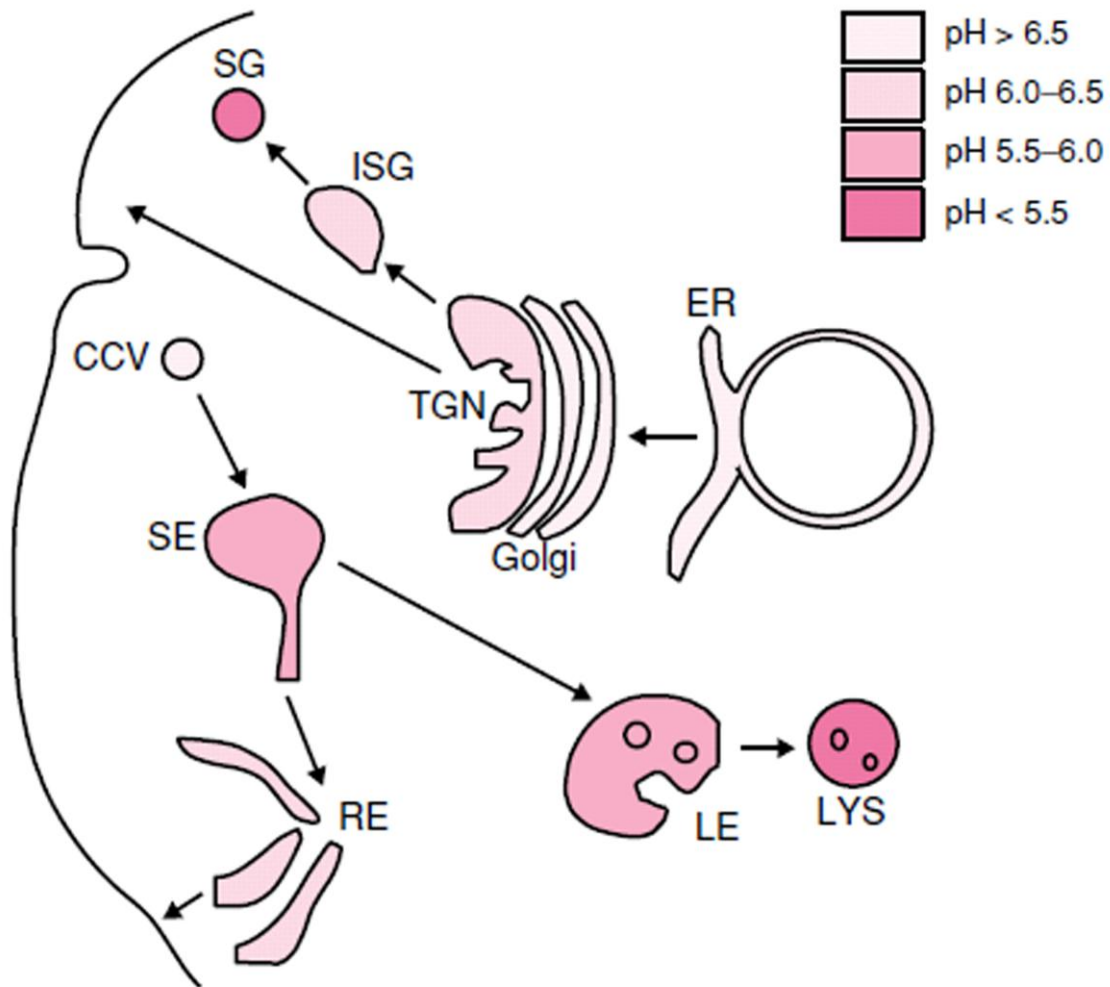


Diagram 1-28. Acidified compartments along the biosynthetic and endocytic pathways. Reproduced from Weisz (2003). The levels of compartmental acidification and the flow of membrane traffic along the biosynthetic and endocytic pathways are depicted as a function of their typical range of pH. ER (endoplasmic reticulum); TGN (trans-Golgi network); ISG (immature secretory granule, or storage granule); SG (secretory granule); CCV (clathrin-coated vesicle, or early endosome); SE (sorting endosome); RE (recycling endosome); LE (late endosome); LYS (lysosome). See text for details.

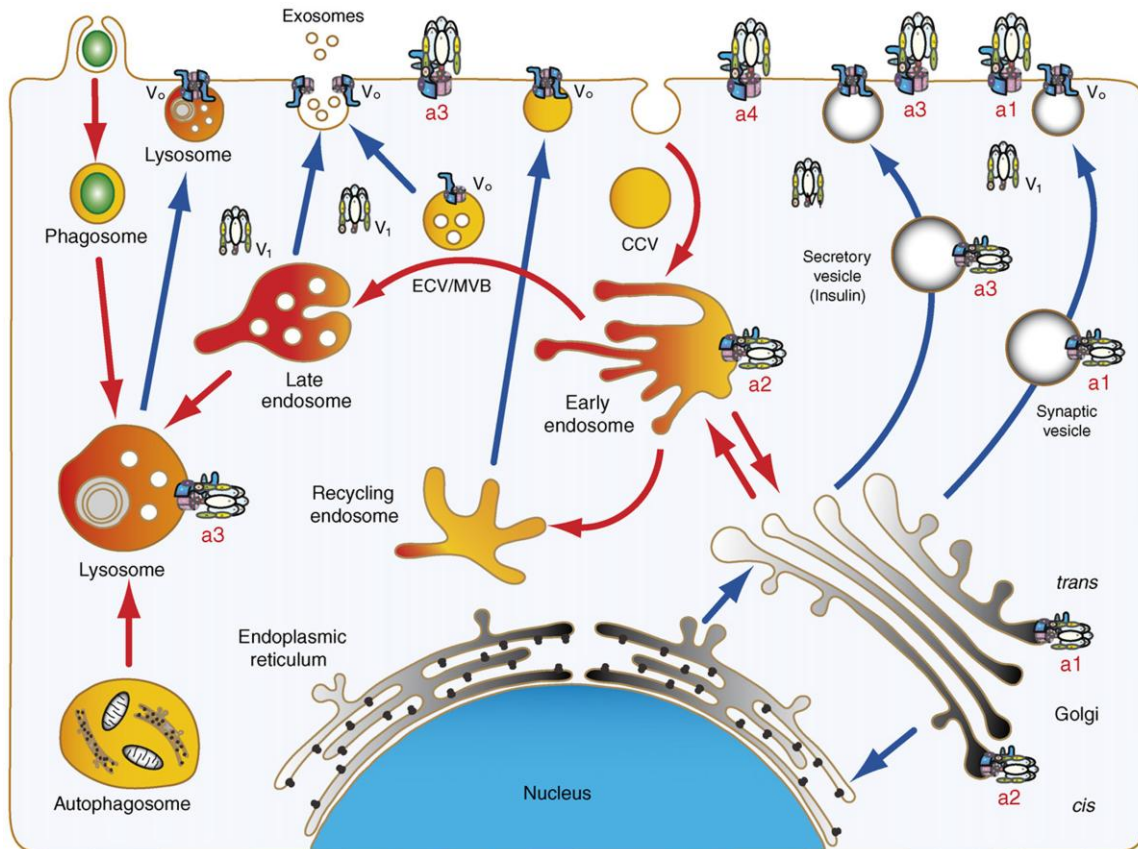


Diagram 1-29. Differential targeting of α isoforms and vesicular trafficking of V-ATPase. Reproduced from Marshansky and Futai (2008). Graphic depiction of the endocytic compartments (yellow/red) and exocytic (gray) pathways of eukaryotic cells. Vesicular trafficking steps are indicated for endocytosis in red arrows and for exocytosis in blue arrows. Differential targeting of V-ATPase is cell-specific and compartment-specific. V-ATPase complexes with the $\alpha 1$ -isoform are targeted to the Golgi, and involved in synaptic vesicle fusion and secretion, and therefore also found on the synaptic plasma membrane. V-ATPase complexes with the $\alpha 2$ -isoform are targeted either to early endosomes or to the Golgi. In early endosomes, the $\alpha 2$ -isoform functions as a pH sensor by recruiting small GTPases in an acidification-dependent manner, and is involved in the formation of endosomal carrier vesicles, which are also known as multivesicular bodies (ECV/MVB). These vesicular intermediates are involved in the trafficking between early and late endosomes, or in exosome formation and secretion. V-ATPase complexes with the $\alpha 3$ -isoform are targeted to lysosomes, and in some cells are involved in lysosomal secretion, and therefore also localized at the plasma membrane. V-ATPase complexes with the $\alpha 4$ -isoform are specifically targeted to the plasma membrane in some cells types, such as the proximal tubule cells of the kidney.

ATPase endomembrane localization resides in the α subunit (**Kawasaki-Nishi et al 2001a**). Yeast, which otherwise encode single isoforms of the other V-ATPase subunits, encode two isoforms of subunit α , one of which (Vph1) targets the V-ATPase to the vacuole, while the other (Stv1) results in localization to the Golgi (**Kawasaki-Nishi et al 2001b**). Many V-ATPase subunits are encoded by multiple isoforms in mammalian cells, including the α subunit, which is encoded by four isoforms ($\alpha 1-4$), of which $\alpha 1$, $\alpha 2$ and $\alpha 3$ are broadly expressed, whereas $\alpha 4$ is kidney specific and predominantly localized to the apical membrane of specialized renal cells (reviewed in **Wagner et al 2004**). As in yeast, the α subunit isoforms are each *preferentially* localized to distinct endomembrane compartments (**Diagram 1-29**), though it is important to note that V-ATPase localization is not static, and often subject to relocalization along cell-type specific paths of endomembrane traffic (reviewed **Toei et al 2010**).

Insulin signaling requires V-ATPase function at multiple steps, including: (1) the aforementioned PI3K-dependent effects of glucose on V-ATPase assembly and endomembrane acidification in renal epithelial cells (**Sautin et al 2005**); (2) the involvement of V-ATPase activity in both insulin secretion from pancreatic β cells (**Sun-Wada et al 2006**) and insulin-stimulated glucose uptake in cultured adipocytes (**Choi et al 2007**); as well as (3) IR recycling to the plasma membrane in primary hepatocyte cultures (**Balbis et al 2004**). Furthermore, in keeping with its involvement in exocytic events, whereas the $\alpha 3$ isoform of the V_0 domain was implicated in β cell insulin secretion (**Sun-Wada et al 2006**), the $\alpha 1$ isoform has been shown to be required for a late step in synaptic vesicle exocytosis in *Drosophila* neurons (**Hiesinger et al 2005**). In addition to exocytic events however, V-ATPase activity is also implicated in diverse endocytic events, including: (1) infection by viruses such as influenza A-virus, SARS coronavirus, and HIV; bacteria such as mycobacterium tuberculosis; as well as the internalization of exogenous molecules such as the diphtheria and anthrax toxins (reviewed in **Forgac 2007, Marshansky and Futai 2008**); (2) the regulation of phagosome maturation in *Dictyostelium* (**Rupper et al 2001a, Rupper et al 2001b**, reviewed in **Duhon and Cardelli 2002**) and zebrafish (**Peri and Nüsslein-Volhard 2008**); (3) the induction of endomembrane fusion between autophagosomes and

lysosomes (Yamamoto et al 1998); and (4) as discussed in **Chapter 3**, the biogenesis and maintenance of the endosomal network.

1-6 – Rationale behind our genetic and biochemical characterization of V-ATPase as a positive regulator of Akt activity

In mammalian systems, signaling by insulin and IGFs occurs at both the plasma membrane and in endosomes (Di Guglielmo et al 1998, Bevan 2001), and the endosomal apparatus plays an important role in signal duration and termination (Authier et al 2002). Following ligand-dependent activation and subsequent endocytosis, V-ATPases begin the process of acidifying the early endosome, allowing for the attainment of a slightly acidic luminal environment in sorting/recycling endosomes, and the dissolution of the receptor-ligand complex, after which the unoccupied receptor is either recycled to the membrane, or degraded in the late endosome/lysosome (reviewed in Forgac 2007). As discussed in **Chapter 3** however, in addition to its role as a conduit for the degradation or recycling of receptors, the endocytic network has received increasing attention over the past decade as a *source* of receptor-borne signaling, establishing this transitory endosomal locale as a hub of signaling in its own right. The crucial function of V-ATPase in the maintenance and acidification of endosomal compartments is consistent with a positive and potentially crucial role for this molecule in the modulation of growth-promoting and developmental signals through numerous cell surface receptors, including established roles downstream of GPCRs, Notch, EGFR, Wnt, and insulin/IGF.

Having detected a genetic interaction between Akt and the A subunit of V-ATPase in *Drosophila*, our characterization of V-ATPase as a positive regulator of Dakt signaling and intracellular acidification in *Drosophila* is described in **Chapter 2**, including the *Dakt* and *Vha68-2* mutant organismal growth phenotypes during the larval and pupal stages of *Drosophila* development, the cell-autonomous role of *Vha68-2* in the regulation of growth and intracellular acidification in larval tissues, and its epistatic placement downstream of the PTEN/PI3K junction. These overlapping *in vivo* growth phenotypes observed in both *Dakt* and *Vha68-2* loss-function mutant cells/organisms were complemented by our biochemical analyses of Akt phosphorylation both in *Vha68-2* mutant larval tissues, as well as in larval tissues treated with the V-ATPase inhibitor

bafilomycin A1, with each case resulting in comparable levels of downregulation in the activating phosphorylation of Akt at its crucial T-loop and HM S/T phosphorylation sites, and furthermore, in the exclusion of activated Akt molecules from the nuclei of bafilomycin-treated larval cells. These findings suggested to us that V-ATPase, through its function as a crucial regulator of intracellular acidification (which is itself upregulated in response to growth factor stimulation), may positively regulate Akt activity by promoting its localization and activation at both endomembranes (whose integrity significantly relies on V-ATPase function), and the nucleus (where many of Akt's regulatory targets are located). In **Chapter 3**, the possibility that Akt activation at the endomembrane surface and its subsequent nuclear localization is dependent on V-ATPase function is biochemically examined in NIH-3T3 fibroblasts treated with the V-ATPase inhibitor bafilomycin. The consequences of V-ATPase inhibition with respect to Akt activity and subcellular distribution are examined through the subcellular fractionation, compartmental enrichment, and immunoblot analysis of bafilomycin treated NIH-3T3 cultures, while the further subfractionation of the enriched endosomal compartments through an Optiprep density gradient, and its subsequent biochemical analysis by immunoblotting suggested that the pharmacological inhibition of V-ATPase activity with bafilomycin diminished the recruitment of Akt to early endosomal populations that is normally observed in growth factor-stimulated samples. Lastly, the relevance of this regulatory relationship to the targeted inhibition of V-ATPase activity in the clinical context of cancer pharmacotherapy is discussed in **Chapter 4**, in which our preliminary experiments examining the cytotoxicity of bafilomycin treatment in glioblastoma-derived and multiple myeloma-derived human cell lines suggest that in these two forms of cancer known to harbor mutations that result in PI3K/Akt overactivation, the inhibition of V-ATPase through small molecule inhibitors may present a viable clinical avenue for the target-directed pharmacotherapy of cancer.

CHAPTER 2

GENETIC CHARACTERIZATION OF VHA68-2 AS A CELL- AUTONOMOUSLY ACTING POSITIVE REGULATOR OF PKB/AKT SIGNALING IN *DROSOPHILA*

*** As further reiterated in their respective figure legends, the contents of Diagrams 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, and 2-14 have been reproduced from previously published articles, and are included herein for the reader's convenience.*

2-1 – Introduction

As described in the introductory chapter, the evolutionarily conserved PI3K/Akt signaling pathway is a crucial element in the promotion of cellular survival and growth in *Drosophila* (**Diagram 2-1**). In contrast to mammals, in which many components of the PI3K/Akt signaling pathway (including PI3K and Akt themselves) are represented by multiple isoforms, the *Drosophila* genome predominantly encodes single representatives of the corresponding components of the pathway, simplifying the assessment of genetic relationships and molecular functions that are often complicated by the presence of multiple isoforms of given molecules in mammals. Akt is represented in *Drosophila* by a single orthologous molecule, Dakt (**Franke et al 1994, Andjelković et al 1995**), a kinase-dead mutant allele of which (*Dakt¹*) was identified and characterized by my supervisor, Dr. Armen Manoukian, and his colleagues (**Staveley et al 1998**). Consistent with the contemporaneous emergence of PI3K/Akt signaling as an important determinant of cellular survival in mammals (reviewed in **Coffer et al 1998a**), their study provided genetic evidence implicating Dakt as an important anti-apoptotic factor in *Drosophila* embryonic development.

In insects, embryonic development consists initially of repeated cycles of nuclear division without cytokinesis or embryonic growth (**Zalokar and Erk 1976, Foe and Alberts 1983**). After several rounds of genomic replication and synchronous mitotic divisions, the resulting several thousand nuclei migrate to the margins of the egg, where the plasma membrane invaginates from the surface around each nucleus, thereby forming true cells (**Diagram 2-2**); and it is at this point that the transition from maternal to zygotic control of the cell cycle program begins, initiating gastrulation, and culminating with the formation of the 1st instar larval hatchling (**Edgar and Schubiger 1986, Edgar and O'Farrell 1989**). Whereas in normal embryos, a small number of cells naturally succumb to apoptosis in highly reproducible patterns (**Abrams et al 1993, reviewed in McCall and Steller 1997**), germ-line clones (GLCs) of *Dakt¹* (which lack both maternal and

zygotic Dakt function) are embryonic lethal, and the examination of fertilized but unhatched GLC *Dakt*¹ embryos revealed widespread ectopic apoptosis based on Acridine Orange staining (which detects cells with membrane blebbing) and terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labeling (TUNEL, which detect fragmented DNA) *in situ* assays (**Staveley et al 1998**). Furthermore, the induction of ectopic apoptosis in *Dakt*¹ GLC mutants was detectable as early as stage 8 of embryonic development (shortly after blastoderm cellularization, see [Diagram 2-3](#)), whereas normally occurring programmed apoptosis only begins to occur at stage 11 following the completion of neuroblast formation (**Staveley et al 1998**). Importantly, the inhibition of caspase activation, but not the loss of *reaper* (*rpr*), *grim*, and *hid* (which in normal embryos, are responsible for the induction of apoptosis in cells that do not successfully complete their developmental program) suppressed ectopic apoptosis in *Dakt*¹ embryos, suggesting that the observed effect is not a consequence of developmental failure, and is distinct from the *rpr/grim/hid*-mediated developmentally dictated apoptotic program (**Staveley et al 1998**). The conserved role of Dakt as a survival-promoting factor was subsequently further extended to include the upstream-acting PI3K/PTEN signaling junction, as either the overexpression of a dominant-negative allele of *Drosophila* PI3K (*Dp110*^{D954A}), or the ectopic expression of *dPTEN*, induced extensive apoptosis in developing embryos (**Scanga et al 2000**), consistent with their respectively positive and negative roles in the rheostatic regulation of Dakt activation.

The fertilized egg contains all the biomass needed for the cellularization of the syncytial blastoderm, as well as subsequent zygotic transcriptional activation, cell division, and gastrulation (reviewed in **Tram et al 2002**). This maternal deposition of energy stores allows for embryonic development without the need for external nutritional supplementation through feeding, consistent with the highlighted role of PI3K/Akt signaling in the promotion of *survival* during embryonic development. After the successful completion of zygotic development, which depletes these maternal energy stores, most cells stop proliferating and enter into an extended quiescent phase (G0 or G1), from which they are released only after hatching and onset of larval feeding (**Edgar and O'Farrell 1990, Knoblich et al 1994, Lane et al 1996**). Over the span of

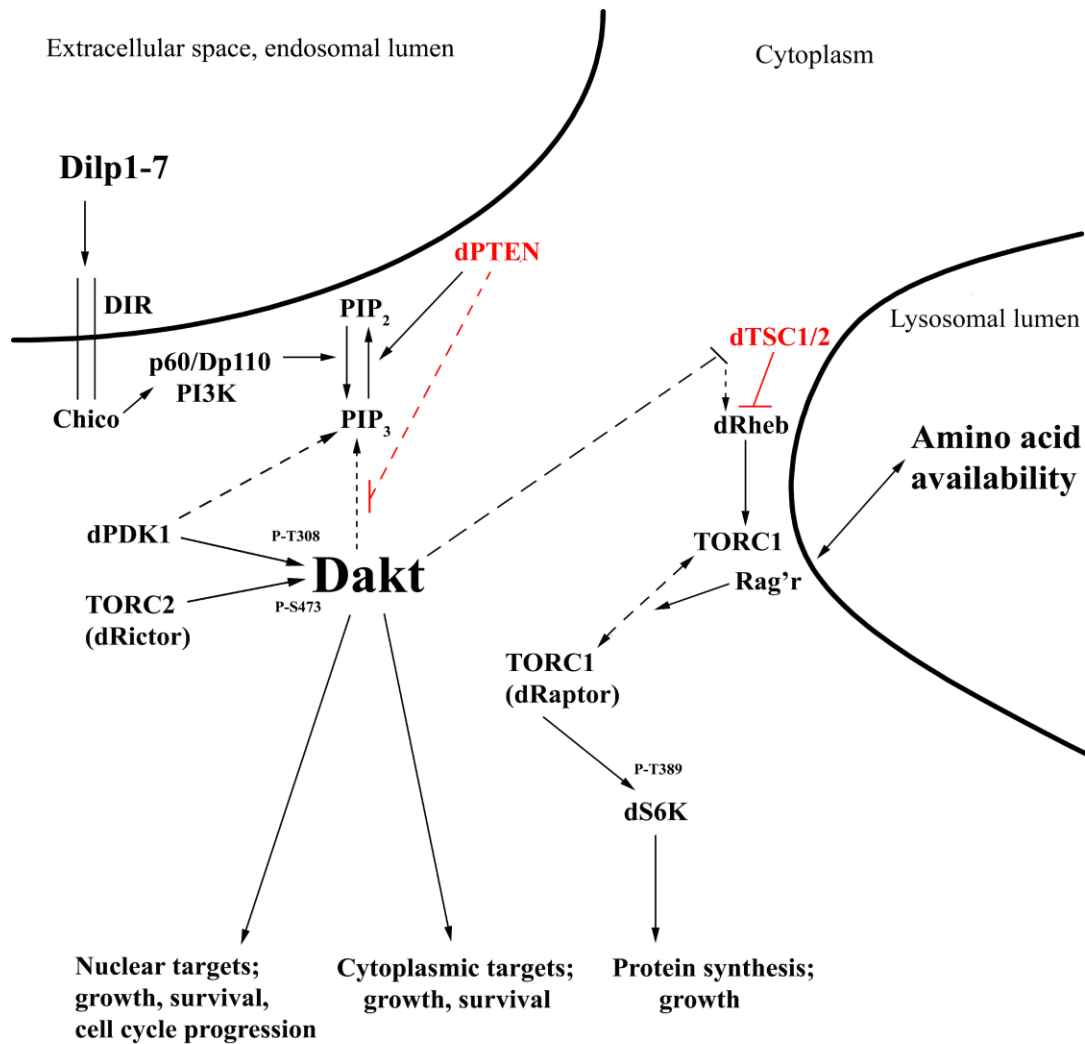


Diagram 2-1. Coordination of ISP-mediated and nutrition-dependent signal transduction in *Drosophila*. Stimulation of the *Drosophila* insulin receptor (DIR) by its systemically circulated ligands (Dilp1-7) occurs at the cell surface or subsequently in internalized signaling membranes such as endosomes and MVBs. Receptor activation recruits chico (IRS1-4 homolog), which acts as a docking protein for p60 (the regulatory subunit of PI3K), activating Dp110 (PI3K) catalytic activity. Dp110 stimulates PIP₃ accumulation at the cytoplasmic surface of the signaling membrane, thereby promoting the PH domain-dependent translocation of Dakt (and dPDK1) to the membrane surface, where it can be phosphorylated by dPDK1 (at T308) and TORC2 (at S473), resulting in its activation. This process is antagonized by dPTEN, which promotes the conversion of PIP₃ back to PIP₂, thereby terminating Dakt membrane recruitment. Dakt activation promotes growth and survival through its numerous substrates, and inhibits the dTSC1/2 complex, relieving the latter's inhibition of dRheb, which is then free to promote TORC1 activity, resulting in dS6K HM activation (at T389). The Dilp- and nutrient-dependent modes of TORC1 activation converge at the dRheb/TORC1 junction, which requires nutrient-dependent Ragulator-mediated recruitment of the cytoplasmic TORC1 complex to the lysosomal surface.

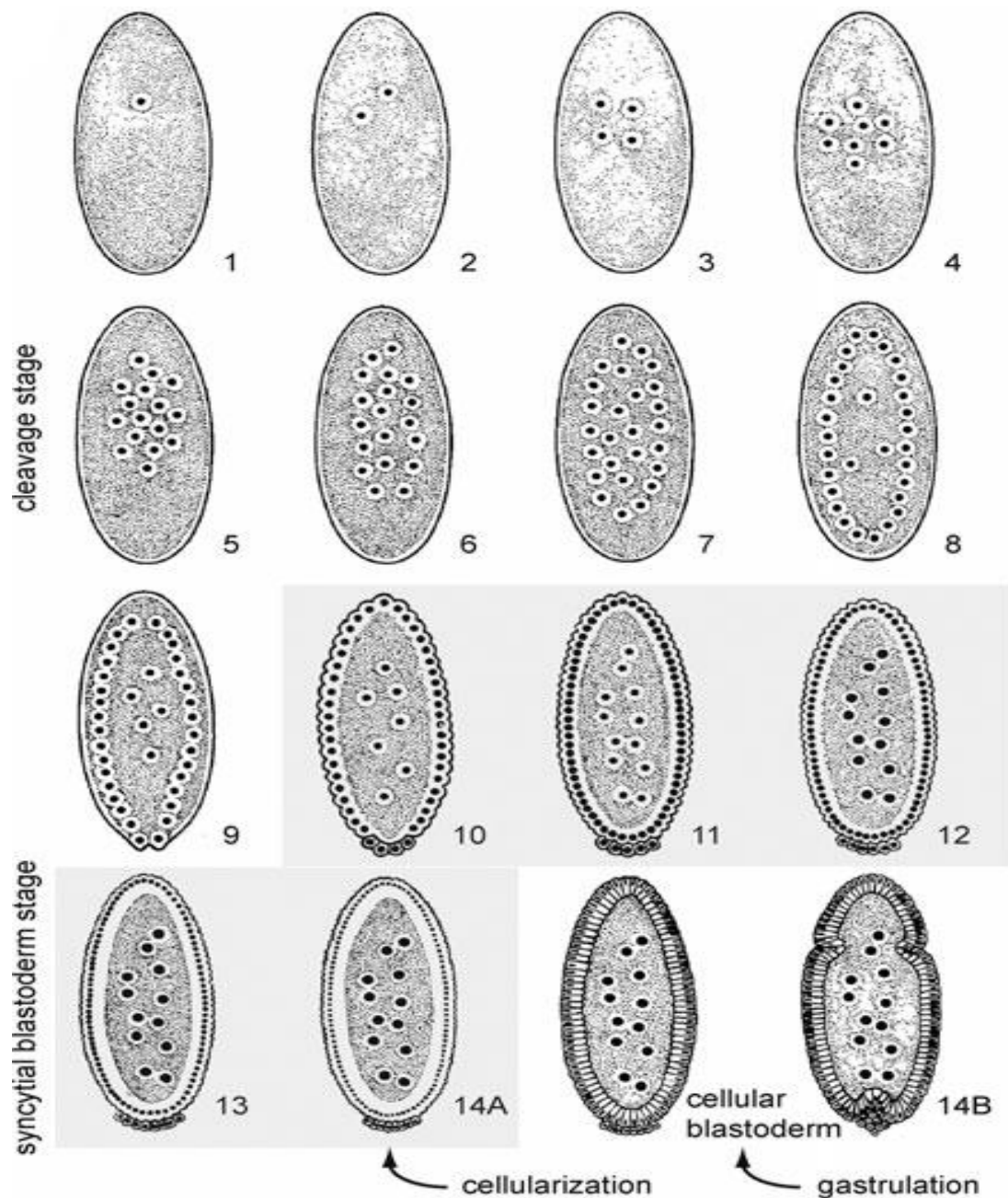


Diagram 2-2. The first 3 hours of embryonic development in *Drosophila*. Reproduced from **Foe and Alberts (1983)**. Numbers indicate mitotic cycle number (not to be confused with embryonic developmental stage). The blastoderm stage lasts from the first minute of cycle 10 to the onset of gastrulation (grey boxed background). The embryo remains syncytial (without membranes between nuclei) until cellularization occurs during cycle 14A. Cycle 14B denotes the part of cycle 14, which occurs after the onset of gastrulation. Embryos are shown with the anterior pole to the top.

approximately 4-5 days ([Diagram 2-4](#)), the hatched larva must thereafter procure nutrients for itself from its environment through constant feeding in order to accommodate its intense growth program where a 1000-fold increase in volume and a 200-fold increase in mass occur in well-fed larvae (**Church 1965, Galloni and Edgar 1999, Thompson 2010**), which results from increases in the cell size of ERTs (in the absence of cell division), and increases in the cell number of proliferating imaginal discs ([Diagram 2-5](#)). Accordingly, as described below, genetic studies conducted throughout the past two decades have highlighted the crucial role of the PI3K/Akt signaling pathway in the hormonal regulation of organismal *growth*, which is promoted systemically through *Drosophila* insulin-like peptides (Dilp1-7), and cell-autonomously through the Dp110/Dakt pathway downstream of *Drosophila* insulin receptor (DIR) activation (reviewed in **Wu and Brown 2006, Shingleton 2010**); and coordinated with the dTOR nutrient sensing apparatus, the genetic inhibition of which, like nutrient deprivation, impinges on organismal growth in a manner that is highly similar to the stereotypical developmental delay and diminished organismal size observed in ISP (insulin signaling pathway) mutants (reviewed in **Neufeld 2004, Hafen 2004, Mirth and Riddiford 2007**).

The transgenic overexpression of any of the seven Dilps results in statistically significant increases in both the weight and size of adults due to increased cell size and cell number; with Dilp2 being: (1) the most broadly and highly expressed isoform in wildtype larvae; (2) the isoform with highest sequence similarity to mammalian insulin; and (3) by far the most potent growth-promoting isoform when overexpressed (**Brogiolo et al 2001, Ikeya et al 2002, Rulifson et al 2002**). Conversely, either (1) the genetic ablation of specialized neurosecretory cells referred to as IPCs (insulin-producing cells), which are located in each brain hemisphere, functionally analogous to the vertebrate pancreatic β cells, and largely responsible for the production of systemically circulated Dilps1-3 and Dilp5; or alternatively, (2) homozygosity for a chromosomal deficiency of Dilps1-5, which are closely clustered together on chromosome III; both result in developmental delay, severe larval growth and metabolic defects, and small adults (**Rulifson et al 2002, Ikeya et al 2002, Zhang et al 2009**). As with the loss or overexpression of circulating Dilps ([Diagram 2-6](#)), the mutant phenotypes of cell-autonomously acting components of the ISP also bear the hallmark defects in the

regulation of organismal size. Although strong mutant alleles of *DIR* cause embryonic lethality in homozygotes (**Fernandez et al 1995**), flies homozygous for a hypomorphic mutation of *DIR* display a proportionate reduction in body size (**Chen et al 1996**, **Brogiolo et al 2001**); and furthermore, this growth deficiency phenotype resembles that seen in *chico* mutants - flies with a mutation in the gene encoding the *Drosophila* IRS1-4 ortholog (**Böhni et al 1999**), in which all aspects of development are normal except for the diminutive size of homozygous *chico* mutant flies (**Diagram 2-6**). Similarly, genetic studies investigating the function of ISP signaling components operating downstream of *DIR* have consistently revealed that throughout post-embryonic development, including the larval, pupal, and/or adult stages, the diminished activity of any of the positive regulators of the pathway, including the catalytic (Dp110) and regulatory (p60) subunits of PI3K (**Weinkove et al 1999**, **Britton et al 2002**), dPDK1 (**Rintelen et al 2001**), dRictor (**Lee and Chung 2007**), and Dakt (**Scanga et al 2000**), results in decreases in organismal size (**Diagram 2-7**). Conversely, the loss of dPTEN function causes larval overgrowth (**Oldham et al 2002**), while its overexpression results in decreased organismal size in larvae and adults (**Gao et al 2000**, **Britton et al 2002**), consistent with its known function as a negative regulator of the ISP (**Diagram 2-7**).

Moreover, the developmental arrest observed in response to the inhibition of PI3K signaling during larval growth (**Britton et al 2002**) was found to phenocopy the cellular effects of nutrient starvation (**Britton and Edgar 1998**); and the constitutive activation of PI3K signaling was demonstrated to bypass the requirement of nutrient availability for growth, causing acute sensitivity to starvation at the organismal level (**Britton et al 2002**). Consistent with the convergence of the Dilp-mediated PI3K/Akt signaling pathway and the parallel-acting nutrient-regulated TOR pathway in the regulation of growth, the characterization of the loss of dRheb, dTOR, and dS6K function (**Saucedo et al 2003**, **Stocker et al 2003**, **Oldham et al 2000**, **Zhang et al 2000**, **Montagne et al 1999**) has recapitulated the defects in organismal growth observed in mutants of the *DIR*/dPI3K/Dakt pathway and in response to starvation (**Diagram 2-8**). Interestingly, as further evidence for coordination and cross talk between nutrient-sensing and Dilp-mediated systemic regulation of growth, the expression of both Dilp3 and Dilp5, as well as the activity of PI3K itself, have been shown to be regulated by nutrient availability

Stage number	Minutes after fertilization	Developmental activity
1*	0-15	Pronuclear fusion
2*	15-70	Preblastoderm (mitotic cycles 1-9) - early cell division - start of cleavage
3*	70-90	Pole bud formation
4*	90-130	Syncytial blastoderm (mitotic cycles 10-13) - end of cleavage divisions
5*	130-180	Cellularization of the blastoderm
6*	180-195	Gastrulation to form mesoderm and endoderm - pole cells included in posterior midgut primordium
7	195-200	Germ band elongation - lengthening of the ventral epidermis
8	200-230	Rapid germ band elongation - start of first postblastoderm mitosis - ends with mesodermal parasegmentation
9	230-260	Slow germ band elongation - segmentation of neuroblasts - end of first and start of second postblastoderm mitosis - cephalic furrow formation
10	260-320	Gnathal and clypeolabral lobe formation (head features) - stomodeal invagination - end of second and start of third postblastoderm mitosis
11	320-440	Epidermal parasegmentation evident - tracheal pits invaginate - mesectodermal cell ingress - end of third postblastoderm mitosis - end of neuroblast formation
12	440-580	Germ band retraction - optic lobe invagination - ventral closure - segment formation - fusion of anterior and posterior midgut
13	580-620	End of germ band retraction - CNS and PNS differentiation
14	620-680	Dorsal closure of midgut and epidermis - head involution begins
15	680-800	End of dorsal closure - head involution - discs invaginate - cuticle deposition begins - dorsal epidermal segmentation
16	800-900	Advanced denticles visible - Shortening of the ventral nerve cord
17	900-hatching	The tracheal tree fills with air - Retraction of the ventral cord continues
Hatchling	1260-1320	Completion of embryogenesis – emergence of 1 st instar larva

Diagram 2-3. Embryonic developmental stages of *Drosophila*. Adapted from **Campos-Ortega and Hartenstein (1985)**. The morphogenic processes that occur from fertilization to larval hatching, with the corresponding Bownes stage number and time frame for each event are listed. Stages 1 through 6 (marked by asterisks) correspond to the mitotic cycles depicted in **Diagram 2-2**.

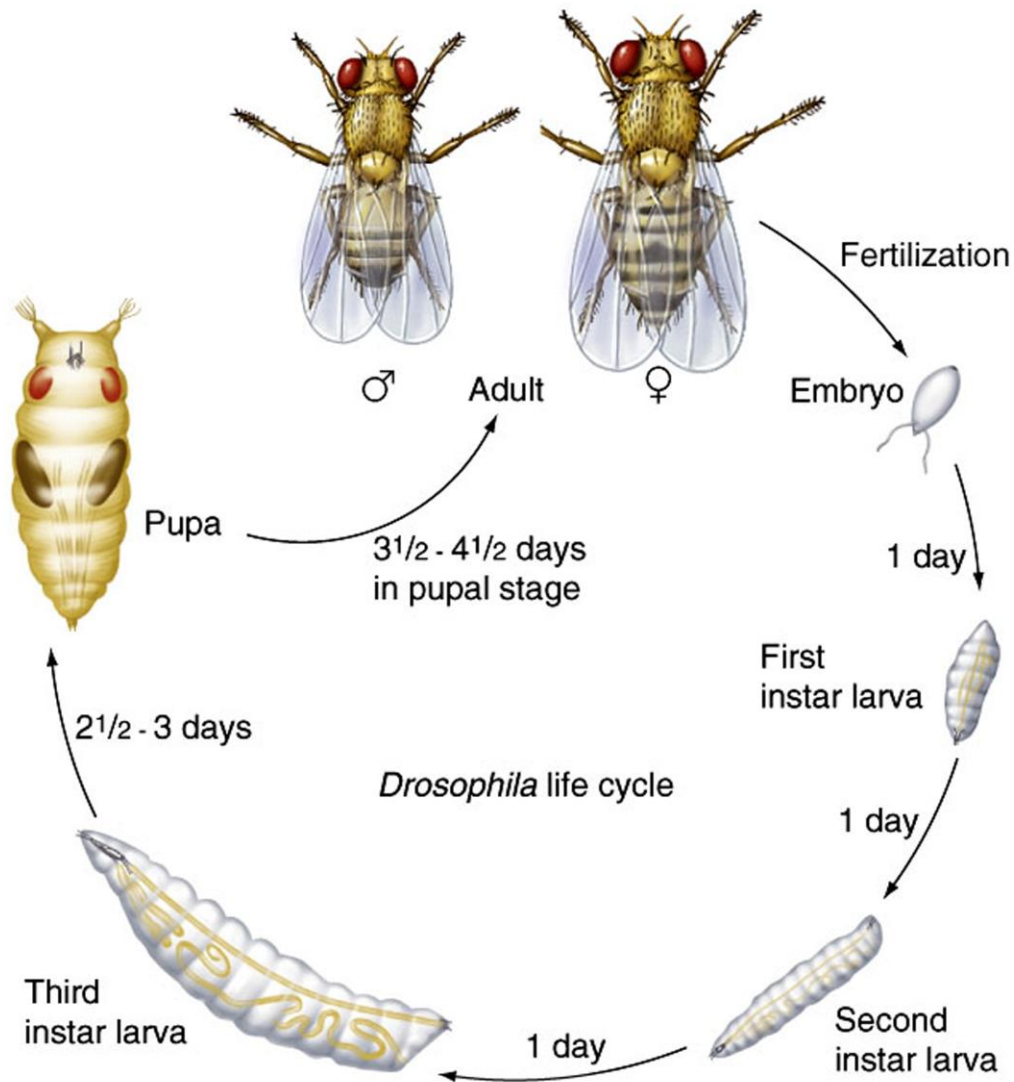


Diagram 2-4. The *Drosophila* life cycle. Following the completion of embryonic development ~24 hours after fertilization, the nascent larva, when reared at ideal environmental and nutritional conditions, proceeds through its larval instars, the 1st and 2nd of which last ~one day each, and thereafter spends 2-3 days in the 3rd instar larval stage, at the end of which pupariation is initiated. The pupal stage itself also spans 4-5 days, at the end of which adults with fully formed appendages eclose. The image above is copyright © of the McGraw-Hill companies, Inc.

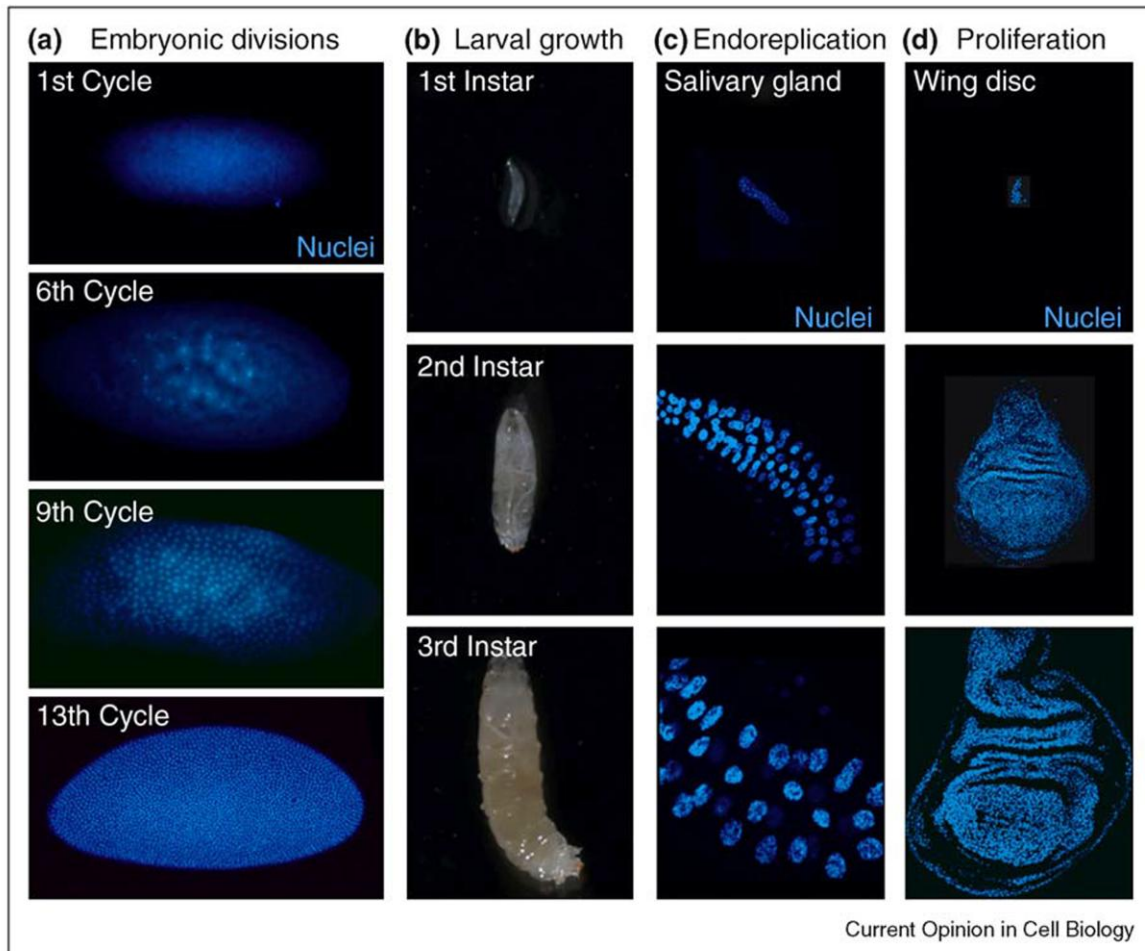


Diagram 2-5. Control of cell growth and division during *Drosophila* development. Reproduced from Thompson (2010). (a) The early syncytial embryo replicates and accumulates nuclei via S-M cell cycles without cell growth or cytokinesis. (b) Embryos hatch as larvae that grow tremendously in size during the three larval instar stages. (c) Larval ERTs, including salivary glands, grow in size and DNA content without mitotic cell division. Images are magnified by approximately 10-fold compared with (b). (d) Some larval tissues, including the imaginal discs that form adult structures in the pupal phase, grow by cell proliferation (cell growth and division) via G1-S-G2-M cycles. Images are magnified by approximately 10-fold compared with (b). The blue signal in (a), (c) and (d) is DAPI fluorescence, commonly used as a marker of nuclei.

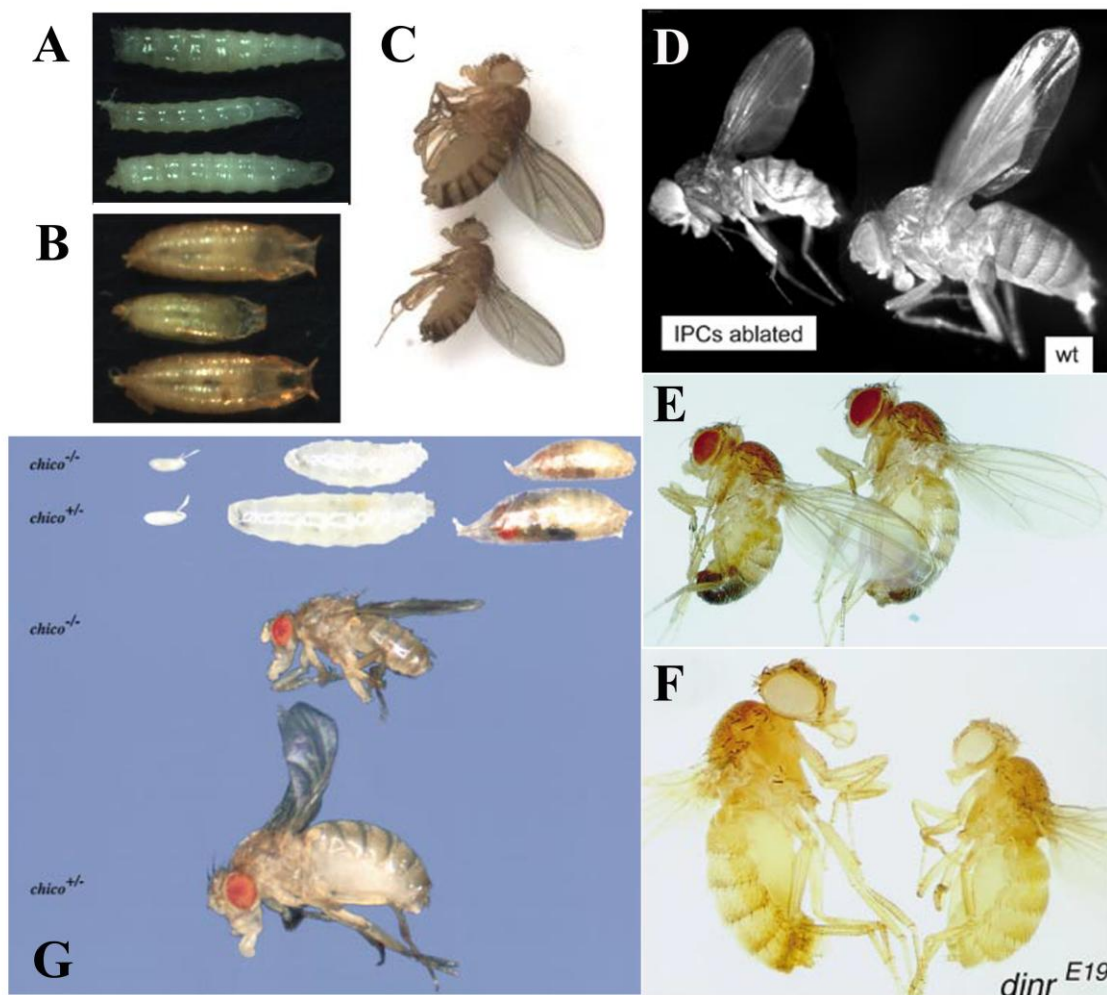


Diagram 2-6. Organismal size defects of Dilp, DIR, and chico mutants. (A-C) Reproduced from Zhang et al (2009). Homozygous *Dilp1-5* deficient mutant larvae and pupae are proportionally diminished in size. For larvae (A) and pupae (B), the parental control lines are on top, the *Dilp*-deficient mutants are in the middle, and transgenically *Dilp2*-expressing rescued *Dilp1-5* deficient mutants are on the bottom. For the adults (C), the wildtype fly is above the *Dilp1-5* deficient viable mutant one day after eclosion. **(D) Reproduced from Rulifson et al (2002).** Adult IPC-ablated mutants (on the left), which do not express *Dilp1-3* and *Dilp5*, are proportionally smaller in size compared to wildtype (on the right). **(E) Reproduced from Brogiolo et al (2001).** Male flies overexpressing *Dilp2* (on the right) are larger than wildtype adults (on the left). **(F) Reproduced from Brogiolo et al (2001).** Flies homozygous for the hypomorphic E19 allele of *DIR* (on the right) show a proportional reduction in body size compared to wildtype (on the left). **(G) Reproduced from Böhni et al (1999).** Homozygous *chico* mutant larvae, pupae, and adults are proportionately smaller than phenotypically wildtype heterozygotes.

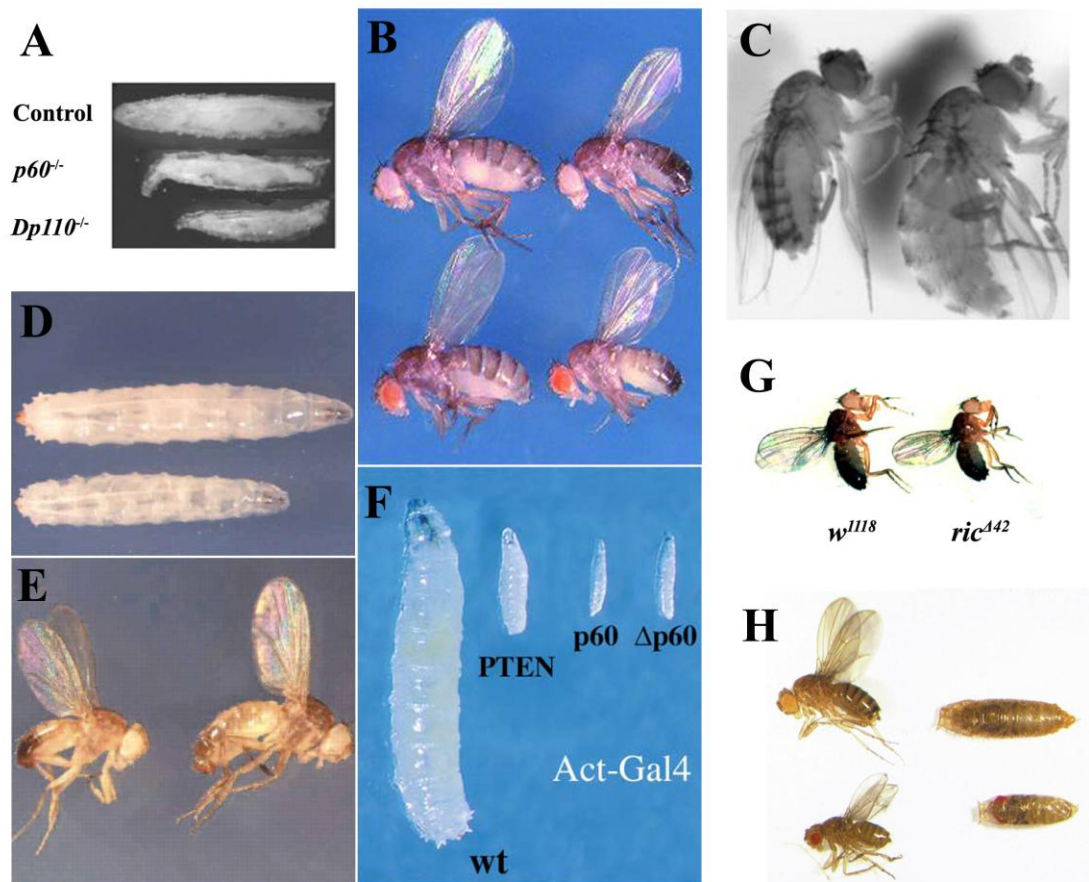


Diagram 2-7. Organismal size defects in PI3K, dPDK1, Dakt, dPTEN, and dRictor mutants. (A) Reproduced from Weinkove et al (1999). Homozygous mutant larvae of the regulatory (p60) and catalytic (Dp110) PI3K subunits are diminished in size in comparison to control animals. (B) Reproduced from Rintelen et al (2001). Heteroallelic male (on the right) and female (on the left) dPDK1 mutants (bottom row) show a proportional decrease in adult size as compared to heterozygous phenotypically wildtype controls (top row). (C) Reproduced from Scanga et al (2000). Homozygous *Dakt*¹ mutant adults (on the left) rescued to viability with intermittent heat-inducible expression of Dakt are proportionally smaller than phenotypically wildtype *Dakt*¹ heterozygotes (on the right). (D,E) Reproduced from Oldham et al (2002). Heteroallelic dPTEN mutant larvae (D, top) and adults (E, right) are proportionally increased in size compared to wildtype. (F) Reproduced from Britton et al (2002). Ubiquitous transgenic expression of dPTEN, as well as p60, or Δp60 (a deletion variant lacking the Dp110-binding domain), whose overexpression has dominant-negative effects on Dp110 signaling result in severely diminished size in L3 larvae as compared to wildtype. (G) Reproduced from Lee and Chung (2007). Adults homozygous for a dRictor loss-of-function mutation (Δ42) are proportionally smaller than wildtype flies. (H) Reproduced from Gao et al (2000). The ectopic overexpression of dPTEN proportionally decreases the size of transgenic mutant pupae and adults (bottom row) in comparison to wildtype (top row).

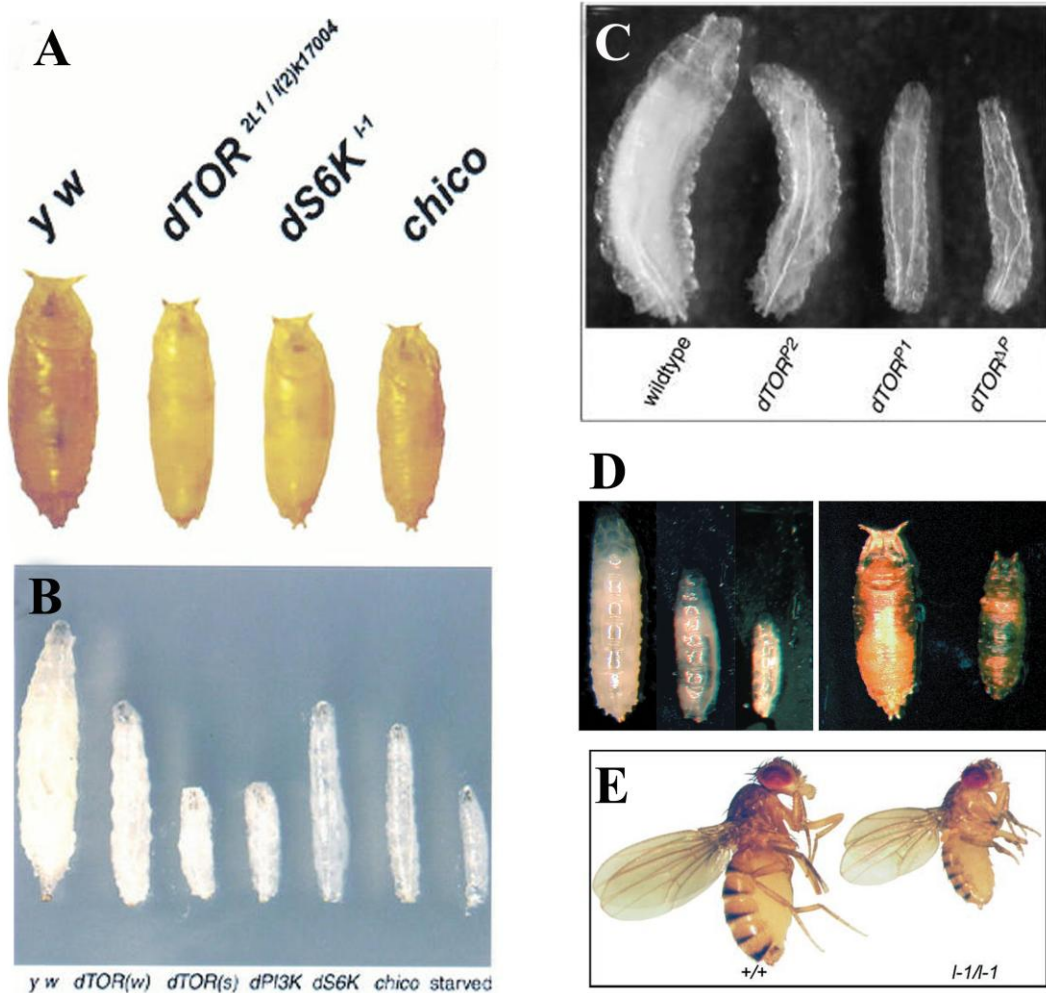


Diagram 2-8. Organismal size defects in dRheb, dTOR, and dS6K mutants. (A,B) Reproduced from Oldham et al (2000). (A) A weak heteroallelic combination of dTOR mutant alleles, like homozygosity for a loss-of-function dS6K allele (l-1), and chico-deficiency, results in a decrease in pupal size as compared to wildtype (yw). (B) Weak (w) and strong (s) heteroallelic combinations of dTOR, the loss of Dp110, dS6K, or chico function, as well as nutrient starvation all result in decreases in larval size of varying severity in comparison to wildtype. (C) **Reproduced from Zhang et al (2000).** dTOR mutants homozygous for either the P1 or the P2 allele, which are P insertion mutants, and ΔP , which is a relatively stronger deletion mutant, all result in diminished larval size in comparison to wildtype. (D) **Reproduced from Stocker et al (2003).** Mutant phenotypes of a pupal lethal heteroallelic combination of dRheb. The range of the larval size defect (left panel) in the mutants (middle and right) is compared to wildtype (left). Whereas smaller mutant larvae (right) die prior to pupariation, larger mutant larvae (middle) are developmentally arrested early in the pupal stage (right panel), forming pupae (right) that are clearly smaller than wildtype (left). (E) **Reproduced from Montagne et al (1999).** In comparison to wildtype adults (left), homozygous dS6K mutant adults (l-1/l-1) are proportionally smaller in size.

(Ikeya et al 2002, Britton et al 2002). This arrangement establishes a relay system whereby under optimal conditions (unlimited food supply), growth is restricted solely by organ-intrinsic program limitations, whereas under sub-optimal conditions, endogenous growth programs are limited by and coordinated with nutrient availability (Slaidina et al 2009). Whereas the growth defects associated with the genetic manipulation of Dilp expression are not cell-autonomous in nature, the examination of growth defects in mutants of intracellular ISP signaling components acting downstream of DIR has revealed context-dependent cell-autonomous roles in their regulation of cell size and/or cell number.

A *Drosophila* larva consists primarily of terminally differentiated endoreplicating tissues (ERTs) which, following a proliferative phase during late embryogenesis, initiate modified cell cycles (endocycles) at the onset of larval growth that lack all visible aspects of mitotic cell division (Smith and Orr-Weaver 1991). Upon the onset of feeding and exit from quiescence following embryonic hatching, the growth of the ERTs accounts for much of the ~200-fold mass increase sustained by the animal during larval stages of development, and occurs as a function of increases in cellular size and DNA ploidy, rather than increases in cell numbers (reviewed in Edgar and Orr-Weaver 2001). During larval development, these ERTs, which include the gut, fat body, salivary glands, malpighian (renal) tubules, trachea, and epidermis, provide a physiologically nurturing environment for undifferentiated proliferating cells of imaginal discs and neuroblasts which generate much of the reproductive adult stage. Most of the biomass accumulated in the ERTs serves as an energy reservoir that is eventually tapped by these progenitor cells as they form their destined adult structures and appendages during pupal metamorphosis. Growth and DNA replication in the ERTs is tightly regulated in response to nutrition, occurring only when animals are fed a protein-rich diet (Britton and Edgar 1998), though interestingly, the rapid cell cycle shutdown that occurs in the ERTs when larvae are protein-starved is due to the loss of required circulating growth factors, rather than amino acid starvation at the cellular level (Britton and Edgar 1998).

Cell size is generally proportional to the amount of nuclear DNA, and this fundamental relationship between ploidy and cell size holds for all organisms that have

been examined (reviewed in **Conlon and Raff 1999**). The polyploidy that results from consecutive endoreplication cycles therefore allows the large and growing differentiated cells of ERTs to increase their mass and/or metabolic output, as required by the developmentally regulated functions of the tissue in question (**Edgar and Orr-Weaver 2001**). The larval salivary glands and the fat body, being the most highly endoreplicative tissues in *Drosophila* larvae, are the ideal setting for the investigation of growth. Since the proliferative program is uncoupled from growth in ERTs, the ISP pathway can be investigated in a signaling context insulated from mitogenic influence, where growth can be assessed from the point of view of nuclear DNA content, without the added signaling dimension of proliferation. Accordingly, in the fat body or the salivary gland, the downregulation of PI3K activity in cells clonally overexpressing p60 (which competes with PI3K for receptor binding and acts as a dominant negative inhibitor) was demonstrated to significantly reduce the size and nuclear DNA content of mutant cells in comparison to neighboring wildtype cells (**Britton et al 2002**). Conversely, the transgenic overexpression of *DIR* or *Dp110* in somatic clones of the fat body was demonstrated to significantly increase the size and DNA content of mutant cells even under nutrient starvation (**Diagram 2-9**), while the clonal overexpression of dPTEN significantly reduced DNA content, and to a lesser extent, cell size (**Britton et al 2002**). Similarly, the clonal overexpression of dRheb in the fat body was also demonstrated to result in the overgrowth and increased DNA content of mutant cells (**Saucedo et al 2003**), while the loss of dS6K, chico, or dTOR function were all found to result in decreases in cellular size and DNA content in mutant salivary glands (**Oldham et al 2000**).

Whereas terminally differentiated ERTs, which make up most of the developing larva, endoreplicate and grow in size through the accumulation of biomass in the absence of cell division, larval imaginal discs - the eventual benefactors of these nutrient and energy stores - are epithelial organs that in contrast to ERTs, organ-autonomously coordinate rates of cell growth with *division* (**Neufeld et al 1998**). This proliferative program controls developmentally-determined organ size and occurs as the result of an increase in the number of cells whose individual size does not vary greatly over time. As such, in the space of a few days between larval hatching and metamorphosis, imaginal

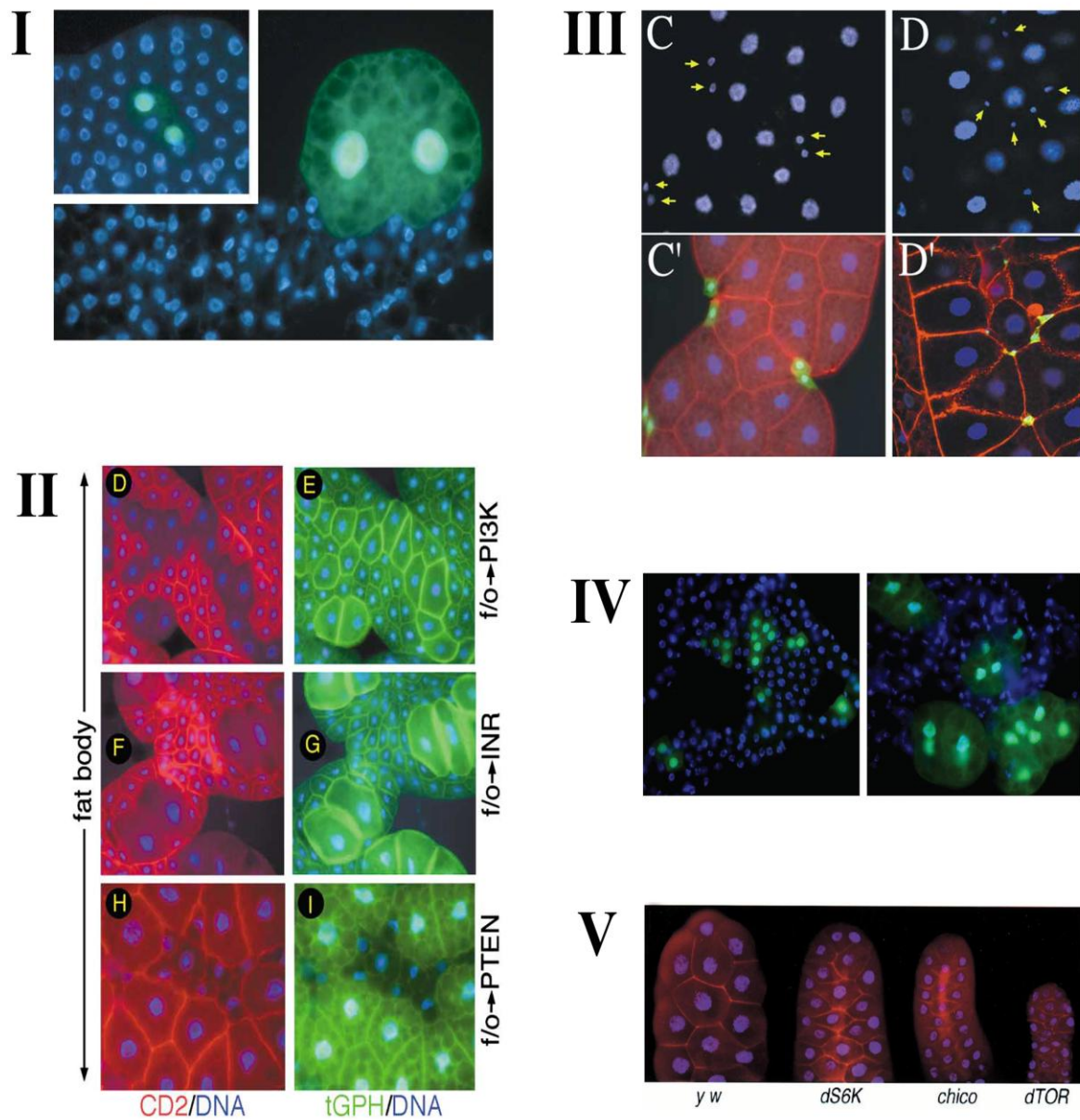


Diagram 2-9. Cell-autonomous growth defects in somatic mutants of ISP components in ERTs. Roman numerals (I-V) were used to designate panels in order to avoid redundancy and maintain the integrity of the depicted images. **(I-III) Reproduced from Britton et al (2002).** (I) Hatchlings clonally overexpressing *Dp110* were starved of dietary protein, and 8 hours after hatching, *Dp110*-expressing clones (marked green with GFP) in the fat body were nearly identical in size to wildtype (inset), but grew dramatically over the following 48 hours despite starvation. (II) Fat body cells from fixed L3 larvae in which the Flp/Gal4 method was used to clonally activate the UAS transgenes (*Dp110*, *DIR*, and *dPTEN*) early in embryonic development. CD2 expression, which is a cell surface marker is shown in red (D, F, H), and is lost upon Gal4 induction in clones. The corresponding tGPH fluorescence is shown in green (E, G, and I). DNA is blue (DAPI) in all panels. *Dp110* or *DIR* overexpression (CD2-negative) results in cell-

autonomous increases in cell size and increased tGPH membrane localization. Conversely, *dPTEN*-overexpressing clones show diminished endoreplication and lack tGPH fluorescence. **(III)** C and C' show an L3 larval fat body in which PI3K activity is suppressed by clonal *p60* overexpression with Flp/Gal4 in GFP-marked cells (indicated by arrows). Red is CD2, and blue is DAPI. As in the case of similarly generated *p60*-overexpressing clones in the salivary glands (red is phalloidin), the inhibition of PI3K activity clonally suppresses cell-autonomous growth. **(IV) Reproduced from Saucedo et al (2003)**. The effect of *dRheb* overexpression in larval fat body tissue prior to (left panel) and at the conclusion (right panel) of three days of dietary protein starvation. DNA is stained with Hoechst 33258. Cells overexpressing *dRheb* are also coexpressing GFP, and show a marked increase in nuclear and cellular size in comparison to the wildtype cells of the starved organism. **(V) Reproduced from Oldham et al (2000)**. DAPI and phalloidin staining of salivary glands from wildtype (yw) and proportionally diminished *dS6K*-, *chico*-, or *dTOR*-mutants examined prior to pupariation (see [Diagram 2-8A](#) for mutant alleles).

discs proliferate, and undergo a dramatic increase in mass, becoming patterned as they grow (reviewed in **Weinkove and Leever 2000, Johnston and Gallant 2002, García-Bellido 2009**). With the added dimension of proliferation in these imaginal tissues, experiments carried out in the eye and the wing have highlighted the role of the DIR/PI3K/dPTEN signaling axis in the regulation of proliferative growth (**Diagram 2-10**), whereby the diminished function in positive regulatory elements such as DIR, chico, Dp110, and Dakt resulted in slowed proliferation and decreased cell size, while their overexpression or constitutive activation resulted in enlarged cells, organs, and adult appendages (**Chen et al 1996, Brogiolo et al 2001, Böhni et al 1999, Weinkove et al 1999, Verdu et al 1999, Scanga et al 2000**). Conversely, the genetic modulation of dPTEN function has been shown to result in the opposite growth phenotypes of those seen in positive components of the ISP (**Huang et al 1999, Goberdhan et al 1999, Gao et al 2000, Scanga et al 2000**).

Loss-of-function mutations in almost all genes are recessive, indicating that 50% of the wildtype level of a protein is sufficient for normal development (reviewed in **St Johnston 2002**). Accordingly, whereas *Dakt*¹ germline clones (which lack both maternal and zygotic Dakt function) perish embryonically, and zygotic *Dakt*¹ mutants (which only retain maternal Dakt function) perish shortly after hatching (**Staveley et al 1998**), *Dakt*¹ heterozygotes (which have half the dosage of maternal and zygotic Dakt) are indistinguishable from wildtype flies, but sensitized to further decreases in Dakt function. As such, any further disruption of the Akt pathway through an additional reduction in a positive regulator of its activity, or an effector of its developmental function during embryogenesis, may reduce Dakt signaling output below the threshold required for embryonic development and survival, thereby *synthetically enhancing* lethality. Therefore, to identify enhancers of Dakt function in *Drosophila* embryogenesis, Dr. Manoukian designed a trans-heterozygous screen (**Diagram 2-11**), whereby *Drosophila* strains bearing P element insertion-induced mutations were individually screened *in vivo* for their ability to synthetically induce embryonic lethality in *Dakt*¹ /+; *P*/+ trans-heterozygotes (**Jin et al 2001**). Since the *Dakt*¹ heterozygotes as well as the P element-induced mutant heterozygotes are both completely viable separately, synthetic

enhancement of embryonic lethality in trans-heterozygotes implies an overlap in signaling processes, and suggests that *Dakt* and the gene represented by the P element-induced mutant allele may function within the same pathway during *Drosophila* embryogenesis (or alternatively, in converging parallel pathways) allowing for the identification of novel putative Dakt regulators, substrates, and developmental functions.

Therefore, with enhanced embryonic lethality as a phenotypic criterion, the *Dakt*¹ screen identified a number of interacting mutations with established roles in Akt signaling, including *Shaggy*, the *Drosophila* homolog of mammalian GSK3; *well-rounded*, the *Drosophila* homolog of the PP2A B' regulatory subunit; and the glucose transporter homolog dGLUT1 (Armen Manoukian, unpublished data). In addition to known regulators or effectors of Akt activity, however, previously uncharacterized *Dakt*-interacting genes were also detected, such as *trachealess* (*trh*), a transcription factor required for tracheal development, which in collaboration with Dr. James Woodgett, was shown by Dr. Manoukian to encode a direct substrate of Dakt (**Jin et al 2001**). One of the more intriguing interacting mutations detected in the screen was *Vha68-2* (**Section 2-3.1**), which as previously mentioned, is the most widely expressed isoform of the three *Drosophila* orthologs of the V-ATPase A subunit (**Allan et al 2005**). In collaboration with Dr. Sam Scanga (then a post-doctoral fellow in Dr. Manoukian's lab), with whom I apprenticed, and whose help and expertise were invaluable in the execution of some of the experiments discussed in this chapter, I undertook the characterization of *Vha68-2* as an enhancer of Dakt activity in the regulation of organismal and cellular growth. Dr. Scanga's contributions to the experiments conducted and described herein are discussed in more detail in this chapter's Materials and Methods (**Section 2-2**).

Study rationale and objectives:

During embryonic *Drosophila* development, the PI3K/Dakt signaling axis is an important determinant of survival, and accordingly, the loss of *Dakt* function in embryos results in widespread ectopic apoptosis (**Staveley et al 1998**). Accordingly, the partial impediment of both Dakt and a putative positive regulator in trans-heterozygotes is manifested by the synthetic enhancement of embryonic lethality, suggesting that *Dakt* and the interacting gene may act within the same pathway, and furthermore, that the gene

in question may normally function as a positive regulator of *Dakt* function. During the larval stage of development however, during which the organism continuously feeds and grows, signaling through the PI3K/Dakt pathway (as described throughout this introductory section) is a central determinant of organismal and cellular *growth*. Since defects in positive regulatory elements of the PI3K/Akt signaling pathway stereotypically result in the diminished organismal size of mutants bearing the defective signaling component in question, we first set out to examine the post embryonic phenotype of *Vha68-2* and *Dakt* mutants during the larval and pupal stages of development (**Section 2-3.2**) in order to test their consistency with the panoply of stereotypical phenotypes observed with mutants of established members of the PI3K signaling axis. These experiments demonstrated that: (1) the diminished function of Dakt resulted in the stereotypical decrease in organismal size (both larval and pupal) expected of positive regulators of growth, and (2) the loss of *Vha68-2* function phenocopied the loss of *Dakt* function with respect to organismal growth.

In addition to the organismal growth phenotypes observed in mutants of the PI3K/Dakt pathway, the intracellularly-acting components of the pathway have also (as previously mentioned herein) been demonstrated to act cell-autonomously in the regulation of cellular growth throughout larval development, and as such, our second objective was to investigate the cell-autonomous phenotypes of *Vha68-2* mutant clones. Following our demonstration of a cell-autonomous role for *Vha68-2* in the growth-factor-dependent intracellular acidification of larval tissues (**Section 2-3.7**), our examination of *Dakt*, *Vha68-2*, and *dPTEN* mitotic clones in the endoreplicating cells of the larval salivary gland, as well as the proliferating epithelial cells of wing imaginal discs revealed that *Dakt* and *Vha68-2* mutant clones exhibit cell-autonomous defects in cellular growth (endoreplicative defects in salivary glands, proliferative defects in cells of the wing disc) consistent with their respectively positive roles in the regulation of growth, and in contrast to *dPTEN* mutant clones, which exhibited a cell-autonomous overgrowth phenotype consistent with its established negative function in PI3K/Dakt signaling (**Section 2-3.4**). Furthermore, our examination of *dPTEN/Vha68-2* double mutant somatic clones in the salivary glands and wing discs of larvae revealed that the loss of *Vha68-2* function effectively suppressed the overgrowth phenotype that results from the loss of

dPTEN function, additionally suggesting that *Vha68-2* acts epistatically downstream of *dPTEN* (**Section 2-3.5**). Moreover, since the resulting growth phenotype in *dPTEN/Vha68-2* double mutant somatic clones phenocopied the loss of *Dakt* function, our findings also suggested that *Vha68-2* function may lie downstream of, or parallel to, *Dakt* function itself.

Our third objective, considering the similarities we observed between the *Dakt* and *Vha68-2* loss-of-function phenotypes, was to correlate the genetic or pharmacological loss of *Vha68-2* function in larval tissues with a corresponding decrease in Akt activity. We biochemically assessed Dakt activity (as measured by HM phosphorylation) in *Vha68-2* mutants during larval development, and consistently demonstrated a downregulation of Akt phosphorylation – an observation that we recapitulated in larval tissues incubated with the V-ATPase inhibitor bafilomycin (**Section 2-3.3**). Our biochemical assessment of Akt activity in response to lost or diminished V-ATPase function was followed by our use of immunofluorescence-based confocal microscopy to examine the state of Akt phosphorylation in larval salivary glands incubated with bafilomycin (**Section 2-3.6**). These immunofluorescence studies confirmed our biochemically observed decrease in Akt phosphorylation, and further demonstrated (1) the insulin-dependent accumulation of phospho-Akt at endomembranes and nuclei of insulin-treated salivary gland cells; (2) the exclusion of phospho-Akt from the nuclei and endomembranes of bafilomycin-treated cells (despite co-treatment with insulin); and (3) the independence of the bafilomycin-induced effect on Akt phosphorylation from possible upstream disruptions in PI3K activity. Combined, our genetic, immunofluorescence-based, and biochemical results vindicate the strong genetic interaction detected between *Vha68-2* and *Dakt*, and suggest that V-ATPase-dependent intracellular acidification may represent a significant regulatory node in the growth factor-dependent activation of Akt downstream of the PI3K/dPTEN junction.

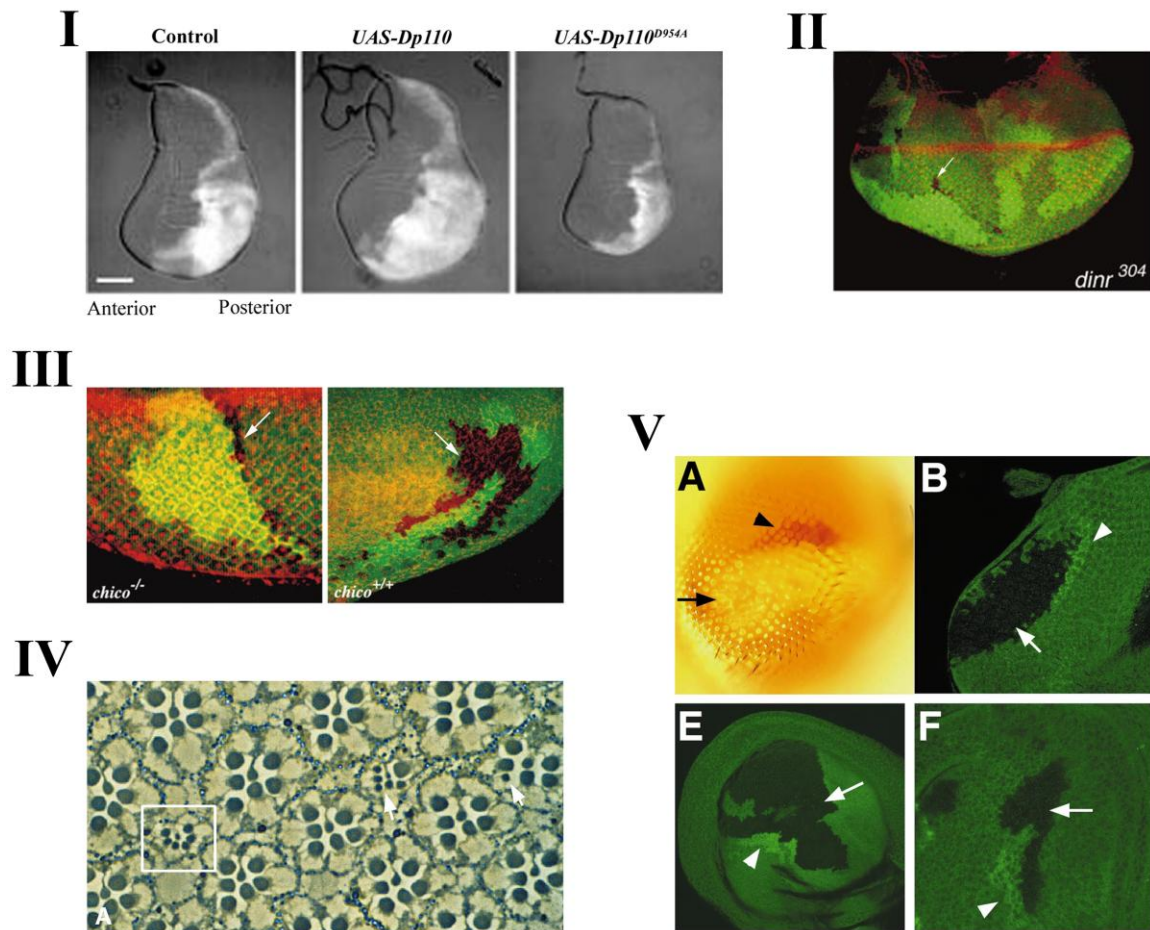
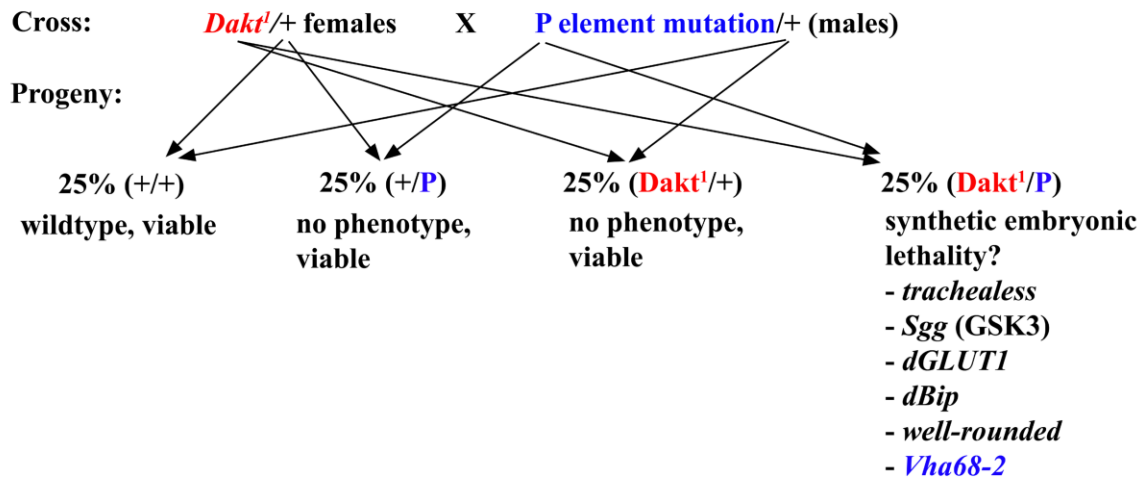


Diagram 2-10. Cell-autonomous proliferative growth defects in somatic mutants of ISP components in imaginal tissues. (I) Reproduced from Weinkove et al (1999). *Engrailed-Gal4* was used to express *UAS-GFP* alone (control) or together with *UAS-Dp110*, or its dominant-negative allele *UAS-Dp110^{D954A}* specifically in the posterior compartment of the L3 larval wing imaginal disc, whose size was significantly increased in *Dp110* expressing mutants, and reduced in *Dp110* dominant-negative mutants. **(II) Reproduced from Brogiolo et al (2001).** Section of an L3 eye imaginal disc containing somatic mitotic clones of *DIR* (*dinr³⁰⁴*) mutant cells (absence of green fluorescence, white arrow), which are significantly smaller (fewer cells) than wildtype (bright green) sister clones. **(III) Reproduced from Böhni et al (1999).** In the panel on the left, somatic clones of *chico* in the eye imaginal disc (white arrow), which also lack GFP, are much smaller than the adjacent wildtype twinspace (bright green) due to a reduced number of ommatidia. By comparison, control somatic clones (right panel) which only lack GFP but express wildtype *chico* contain an equivalent number of cells to twinspace GFP-positive sister clones. **(IV) Reproduced from Verdu et al (1999).** A tangential section through a mosaic eye containing homozygous *Dakt¹* mutant clones. Rhabdomeres lacking *Dakt* (box) are smaller than wildtype neighbors, and coexist with wildtype cells in the same ommatidia (arrows). **(V) Reproduced from Gao et al (2000).** (A) Light microscope image of an adult eye containing a homozygous *dPTEN* mutant clone induced in an L1

larva. The large mutant clone is marked by the absence of yellow pigment (arrow), while the much smaller wildtype twinspace is heavily pigmented. (B,E) Confocal images of L3 eye discs containing homozygous *dPTEN* mutant clones (arrow), which are marked by the absence of green fluorescence, and consistently larger than wildtype twin-spot sister clones (bright green). (F) The overgrowth of *dPTEN*-deficient clones in the eye was similarly extended to the proliferative growth of the wing imaginal disc in L3 larvae.

Diagram 2-11. Trans-heterozygous screen for positive regulators of Dakt function.

See text for details. Both heterozygous combinations (+/P, and *Dakt*¹/+) are viable and phenotypically wildtype, and along with wildtype (+/+) progeny, make up 75% of offspring. Enhanced synthetic lethality in trans-heterozygotic embryos was assessed in unhatched progeny of the parental cross as described in **Section 2-2.1**.



2-2 – Materials and Methods

Fly strains and chemical reagents.

The wildtype (w^{1118}), GFP balancer, and *arm-GAL4* lines were obtained from the Bloomington *Drosophila* Stock Center; as were the P463 ($P\{PZ\}Vha68-2^{EP2364} / CyO$), S4214 ($P\{lacW\}Vha68-2^{S4214} / CyO$), and 11065 ($P\{PZ\}Vha68-2^{01510} / CyO$) mutant alleles of *Vha68-2*. The UAS *Vha68-2*^{RNAi} / *CyO* (ID# 110600, CG3762) line was obtained from the Vienna *Drosophila* RNAi stock center. Previously published lines used herein include the *dPTEN*^{c494} mutant allele (Huang et al 1999), the *Dakt*³ mutant allele (Stocker et al 2002), the UAS-Akt-HA transgenic line (Staveley et al 1998), and the tGPH transgenic line (Britton et al 2002). Bafilomycin A1 (B1793) and Concanamycin A (C9705) were purchased from Sigma Aldrich. Humulin (U-100) was purchased from Eli Lilly.

Evaluation of genetic interaction between *Dakt* and *Vha68-2*.

The percent lethality of *Dakt*¹/*Vha68-2*^{P463} transheterozygous embryos was determined as previously described in Jing et al (2001).

Characterization of lethal phase and organismal size.

For the analysis of survival and organismal size through the progression of larval (and pupal) development, *Vha68-2* mutant (P463 and 11065) and *Dakt* mutant (*Dakt*¹ and *Dakt*³) larvae were reared in collection cups containing apple juice agar plates seeded with yeast paste. Their viability and/or size were scored at each larval instar transition. The mutant alleles are recessive, and in a typical cross of heterozygous siblings (*Vha68-2*^{P463} / *CyO*, GFP [Males] crossed with *Vha68-2*^{P463} / *CyO*, GFP [Females]), the progeny would consist of 25% homozygous GFP-negative mutants, 25% GFP-positive offspring homozygous for the balancer chromosome (which perish as embryos or as young larvae), and 50% GFP-positive heterozygous mutants with genotypes identical to the parental lines. Size and viability was assessed in approximately viable 250 hatchlings (which survived embryogenesis) with lack of GFP fluorescence as a marker for homozygous mutants. For the examination of organismal size in the pupal stage, mutant larvae were identified and isolated from wildtype siblings in the 3rd larval instar, and subsequently examined and compared to phenotypically wildtype heterozygous pupae (not shown), and w^{1118} pupae. **These experiments were carried out by the author of the thesis (SK).**

Immunoblotting of larval tissues.

The phosphorylation of endogenous *Dakt* at S505 was examined in staged homozygous mutant *Vha68-2*^{P463}, *Vha68-2*^{S4214}, and *Vha68-2*¹¹⁰⁶⁵ whole larvae, as well as *Vha68-2*^{RNAi} transgenic whole larvae at the indicated stages of larval development. Homozygous *Vha68-2* mutant progeny were generated and identified based on GFP fluorescence as described above. In order to isolate transgenic mutants expressing the *Vha68-2* RNAi construct, UAS-*Vha68-2*^{RNAi} / *CyO*, GFP males were crossed with *arm-Gal4/arm-Gal4* females, and the GFP-negative (UAS *Vha68-2*^{RNAi} / *arm-Gal4*) larvae were analyzed by Western blotting. Whole larvae were homogenized in 1x RIPA lysis buffer [10mM sodium phosphate pH 7.2, 150mM NaCl, 2mM EDTA, 50nM NaF, 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 5.4mg/mL β -glycerophosphate, 1mM benzamidine,

0.1mM sodium orthovanadate, protease inhibitor cocktail tablet (Roche 11873580001)], first in a Dounce homogenizer (10-15 strokes) on ice, and then by passage (~15-20 times) through a 23G needle (Becton Dickinson 305194Z). The lysate was centrifuged at 10,000g (10 min, 4°C) to clear debris, and after quantitation (Bradford assay), the supernatant was prepared for Western blotting by the addition of 4x sample buffer (40% glycerol, 250mM Tris-HCl pH 6.8, 8% SDS, 10% β -mercaptoethanol, 0.04% bromophenol blue), and the denaturation of the samples in a 95°C water bath for approximately 2 minutes. Equal amounts (by mass) of the samples were loaded onto Novex® 6-18% gradient Tris-glycine polyacrilamide gels (Invitrogen) and separated by SDS-PAGE electrophoresis. Overnight wet transfer (4°C, constant current of 15mA, Tris/Glycine/methanol transfer buffer) onto a PVDF membrane (Millipore), was followed by blocking of the membrane and incubation with primary antibodies against Akt phospho-S505 (Cell Signaling Technologies #4054), total Akt (CST #9272), β -tubulin (Developmental Studies Hybridoma Bank #E7), and the A subunit of V-ATPase, ATP6V1A (Abcam ab137574). The immunoblots were developed following HRP-conjugated secondary antibody incubation (CST #7074, or #7076), and ECL AdvancedTM-based chemiluminescence (GE Healthcare Amersham RPN2135). Akt phosphorylation was also examined in larval heads treated with bafilomycin and concanamycin, or in Vha68-2 RNAi-expressing transgenic mutants. Briefly, the tissues of 3rd instar larvae transgenically expressing HA-tagged Akt (*UAS-Akt-HA*) using an armadillo-driven Gal4 expression line (*arm-Gal4*) were dissected in BSS [Balance salt solution, see *Drosophila* Laboratory Manual, CSH Press, Ashburner (1989), page 376], incubated in M3 insect medium (Shields and Sang 1977) containing 5% FBS and 0.2U/mL insulin; and treated with either Bafilomycin A1, Concanamycin A, or the DMSO vehicle for 12 hrs at 25°C. Following the incubation period, the culture media was aspirated and discarded, while the larval tissues were homogenized in RIPA buffer as described above for the experiments conducted with whole larvae. Protein extracts were Western blotted for the HA epitope (CST #2367) as a loading control for transgenically expressed Akt-HA; phospho-Akt at S473 (CST #4060, also recognizes endogenous Dakt phosphorylated at S505); and phospho-Akt at T308 (CST #9275). **These experiments were carried out by the author of the thesis (SK).**

Generation of recombinant somatic clones in imaginal wing discs.

The *dPTEN* and *Vha68-2* genes are located on chromosome 2L. Parental crosses were set up whereby females homozygous for the heat shock-driven yeast FRT-specific recombinase (*hs-FLP22*) and the FRT insertion site (FRT40A) were mated with heterozygous mutant (or double mutant) males as follows:

y w *hs-flp22*; FRT40A Ubi-GFP / y w *hs-flp22*; FRT40A Ubi-GFP [Females]

X

(A) y w; *Vha68-2*^{P463} FRT40A / CyO [Males] OR

(B) y w; *dPTEN*^{c494} FRT40A / CyO [Males] OR

(C) y w; *dPTEN*^{c494}, *Vha68-2*^{P463} FRT40A / CyO [Males]

F1 embryos from the parental cross were collected for ~6 hours and aged at 25°C until ~48 hours after egg laying (AEL), at which point recombinant clones were induced in the developing larvae by heat shock (1 hour at 36°C). Following heat shock, larvae were allowed to recover at 25°C until the 3rd instar was reached, at which point the wing discs were dissected, fixed, stained, and examined by confocal microscopy as described below.

Homozygous somatic mutant clones lack GFP fluorescence (no GFP transgene), homozygous wildtype twin-spot sister clones are GFP positive (2 copies), while the heterozygous germline is GFP positive, but only bears a single copy of the GFP transgene, and therefore fluoresces with less intensity in comparison to twin-spot wildtype clones encoding two GFP transgenes (see [Diagram 2-12](#)). **The maintenance of *Drosophila* lines mentioned above and the setting of the appropriate crosses were assisted by Dr. Scanga.**

Generation of recombinant somatic clones in larval salivary glands.

The *Dakt* gene is located on chromosome 3R. For the generation of somatic Dakt clones, the parental cross was as follows:

y w hs-flp12; FRT82B Ubi-GFP / y w hs-flp12; FRT82B Ubi-GFP [Females]
X
y w; Dakt¹ FRT82B / CyO [Males]

For the generation of dPTEN and Vha68-2 single or double mutant somatic clones, the parental crosses were as follows:

y w hs-flp22; FRT40A Ubi-GFP / y w hs-flp22; FRT40A Ubi-GFP [Females]
X
(A) y w; Vha68-2^{P463} FRT40A / CyO [Males]
(B) y w; dPTEN^{c494} FRT40A / CyO [Males]
(C) y w; dPTEN^{c494}, Vha68-2^{P463} FRT40A / CyO [Males]

F1 embryos from the parental cross were collected for ~6 hours and aged at 25°C until ~24 hours after egg laying (AEL), at which point recombinant clones were induced in the developing larvae by heat shock (1 hour at 36°C). Following heat shock, larvae were allowed to recover at 25°C until the 3rd instar was reached, at which point the salivary glands were dissected, fixed, stained, and examined by confocal microscopy as described below. **The maintenance of *Drosophila* lines mentioned above and the setting of the appropriate crosses were assisted by Dr. Scanga.**

Fixation, staining, and confocal analysis of imaginal discs.

Wing imaginal discs were fixed and stained as described by Struhl and Basler (1993). Briefly, wing discs were fixed in 4% formaldehyde in PEM buffer (0.1 M PIPES pH 7.0, 1 mM MgSO₄) for 20 min, rinsed in BSS, and then transferred to a PBSBT blocking solution (1% bovine serum albumin, 0.1% Triton X-100, in phosphate-buffered saline). The discs were then incubated with the rabbit anti-GFP primary antibody (Abcam ab290) overnight at 4°C. Following incubation with the primary antibody, the salivary glands were washed for 2 hours in PBST at room temperature, and incubated with biotinylated anti-rabbit secondary antibody (Vector BA-1000) and streptavidin FITC (Vector SA-5001) overnight at 4°C. Following the incubation period, wing discs were washed once more in PBST at room temperature for 2 hours, after which the discs were additionally stained with phalloidin-Texas Red (Sigma P1951), 5µl of which were included in the fluorescent mounting media containing DAPI (Molecular probes D3571) and antifade (Vectashield from Vector). The discs were examined using a Zeiss 2-Photon confocal microscope. The confocal microscope settings and length of exposure were identical in

all analyses. **The fixation, staining, and confocal analysis of imaginal discs conducted by the author of the thesis (SK) was logistically assisted by Dr. Scanga.**

Fixation, staining, and confocal analysis of salivary glands.

Salivary glands were fixed and stained as described in Martin and Baehrecke (2004). Briefly, salivary glands were dissected from late 3rd instar larvae, fixed in 4% formaldehyde in PEM buffer for 30 minutes at room temperature, blocked in phosphate buffered saline containing 1% BSA and 0.1% Triton-X (PBSBT), and incubated with the rabbit anti-GFP and/or mouse anti-PIP₃ (Echelon Z-A345) primary antibodies overnight at 4°C. Following incubation with primary antibodies, salivary glands were washed for 2 hours in PBSBT at room temperature, and incubated with the appropriate secondary antibodies overnight at 4°C. To detect GFP, we used a chicken secondary anti-rabbit antibody conjugated with FITC (Abcam ab6825), and for PIP₃ we used a goat anti-mouse biotinylated secondary antibody (Vector BA-9200). Following incubation with the secondary antibodies, the salivary glands were once again washed in PBSBT for 2 hours at room temperature, and in experiments involving a biotinylated secondary antibody (as in the case of PIP₃ detection), an additional streptavidin-Texas Red (Vector SA-5006) incubation or streptavidin-FITC incubation was carried out for 1.5 hours at room temperature, followed by a final 2 hour wash in PBSBT at room temperature. In the case of phospho-Akt (S473) detection by confocal microscopy, the Akt antibody (CST #4060) was detected with biotinylated anti-rabbit secondary antibody (Vector BA-1000) and streptavidin-FITC (Vector SA-5001). Filamentous actin was stained with phalloidin-Texas Red (Sigma P1951) in a 20 minute incubation at room temperature following antibody staining and prior to mounting for confocal analysis. Salivary glands were mounted in Vectashield and examined using a Zeiss 2-Photon confocal microscope. The confocal microscope settings and length of exposure were identical in all analyses. In the assessment of intracellular acidification, dissected salivary glands were incubated with LysoTracker® Red DND-99 (Molecular Probes L7528) and analyzed as recommended by the manufacturer. **The fixation, staining, and confocal analysis of imaginal discs conducted by the author of the thesis (SK) was logistically assisted by Dr. Scanga.**

2-3 – Results

2-3.1 – Identification of a genetic interaction between *dVha68-2* and *Dakt*

The P463 line ($P\{lacW\}Vha68-2^{s4214}$) is a P element-induced larval lethal mutant allele of the *Vha68-2* gene, which as a trans-heterozygote with *Dakt*¹ ($Vha68-2^{P463}/+;$ *Dakt*¹ /+) significantly enhanced synthetic embryonic lethality. The dramatic increase in percent lethality observed in these trans-heterozygotes was ~20% (**Table 2-1**) out of the possible 25% trans-heterozygous progeny (see **Diagram 2-11**). By comparison, Dr. Manoukian's previous investigation of the *Dakt/trh* interaction (**Jin et al 2001**) had revealed that P1747 ($P\{PZ\}trh^{10512}$), a loss of function mutant allele of the *tracheless* gene, produced a more modest enhanced lethality rate of 6.5% (out of a possible 25%) as a trans-heterozygote with *Dakt*¹ ($trh^{P1747}/+;$ *Dakt*¹ /+). Moreover, a relatively intermediate level of enhanced lethality (12.5%) was detected in the trans-heterozygous combination of a mutation in the *Drosophila* PI3K regulatory subunit *p60* and *Dakt*¹ ($p60A/+;$ *Dakt*¹ /+, **Jin et al., 2001**). Considering the relative strength of the *Dakt*¹ interaction with *Vha68-2*^{P463} with respect to the severity of enhanced lethality, the screen data suggests that *Dakt* and *Vha68-2* may be intimately involved in overlapping signaling processes during embryonic development.

The systematic mutational characterization of the entire V-ATPase gene family in *Drosophila* (**Allan et al 2005**) had revealed that the loss of function of most V-ATPase components resulted in lethality, whose onset, depending on the gene in question, ranged from late embryonic to pupal stages of development. Their survey included P67 and *vha67*(S6), two P element-insertion mutant alleles of *Vha68-2*. Homozygosity for either *Vh68-2* mutant allele was found to result in 1st instar larval lethality. Our own investigation of *Vha68-2* was undertaken with zygotic homozygotes of the P463 allele, as well as those of 11065 ($P\{PZ\}Vha68-2^{01510}$), an additional P-element insertion-induced mutant allele of *Vha68-2*. Of the two mutant alleles, homozygosity for the stronger P463 allele resulted in early (1st instar) larval lethality, similar to both the P67 and *vha67*(S6) mutant alleles of *Vha68-2* (**Allan et al 2005**), as well as the aforementioned early larval lethality of *Dakt*¹ mutant homozygotes (**Staveley et al 1998**). In contrast, homozygotes of

the weaker allele, 11065, displayed a late larval lethal phase, with some 3rd instar larvae escaping into the pupal stage, but without any eclosion of adults (**Table 2-2**). Having detected a strong genetic interaction between *Vha68-2* and *Dakt*, we set out to examine the mutant phenotypes of *Dakt*¹, P463 and 11065 in the context of organismal growth during larval and/or pupal development prior to the onset of lethality.

2-3.2 – *Dakt* and *Vha68-2* mutants have similar post embryonic growth phenotypes

After embryonic hatching (AH), *Drosophila* larval development spans approximately 4 days at 25°C with ample nutrition, and includes three larval instars (**Diagram 2-4**). The 1st (L1) and 2nd (L2) instar stages last one day each, while the 3rd instar stage lasts 2 days, at which point larvae reach their wandering stage, and prepare to pupate. Although P463 mutants, much like *Dakt*¹ mutants, perish early in larval development, a significant number of both homozygous mutant strains are viable as late 1st instar larvae. At 24hr AH, at which point the 1st larval instar stage is normally concluded, comparison of *Dakt*¹ and *Vha68-2*^{P463} mutant homozygous larvae (GFP-negative) to phenotypically wildtype heterozygous mutant siblings revealed a sizeable and comparable growth defect in homozygotes of either mutant allele (**Figure 2-1A**).

As mentioned in this Chapter's introduction, nutrient starvation, much like hypomorphic mutations in positive components of the ISP, not only causes growth defects, but also results in developmental delays of varying severity, and in some cases, the arrest of development and death (reviewed in **Oldham and Hafen 2003, Hietakangas and Cohen 2009**). This delay in development reflects the flexibility of the larval growth program, where in cases of nutritional starvation, the organism can temporarily idle at its current stage of larval development until nutrient availability is restored. Despite their delay in growth, provided that the nutrient source is restored prior to lethality, the once-starved larvae begin feeding again, and can eventually reach their mature size. With respect to the execution of cellular growth programs, the consequences of external nutrient deprivation are mimicked by deleterious *genetic* modifications of intracellular growth promoting factors. However, due to the genetic nature of the developmental delay, mutant larvae that are not viable arrest at various stages of

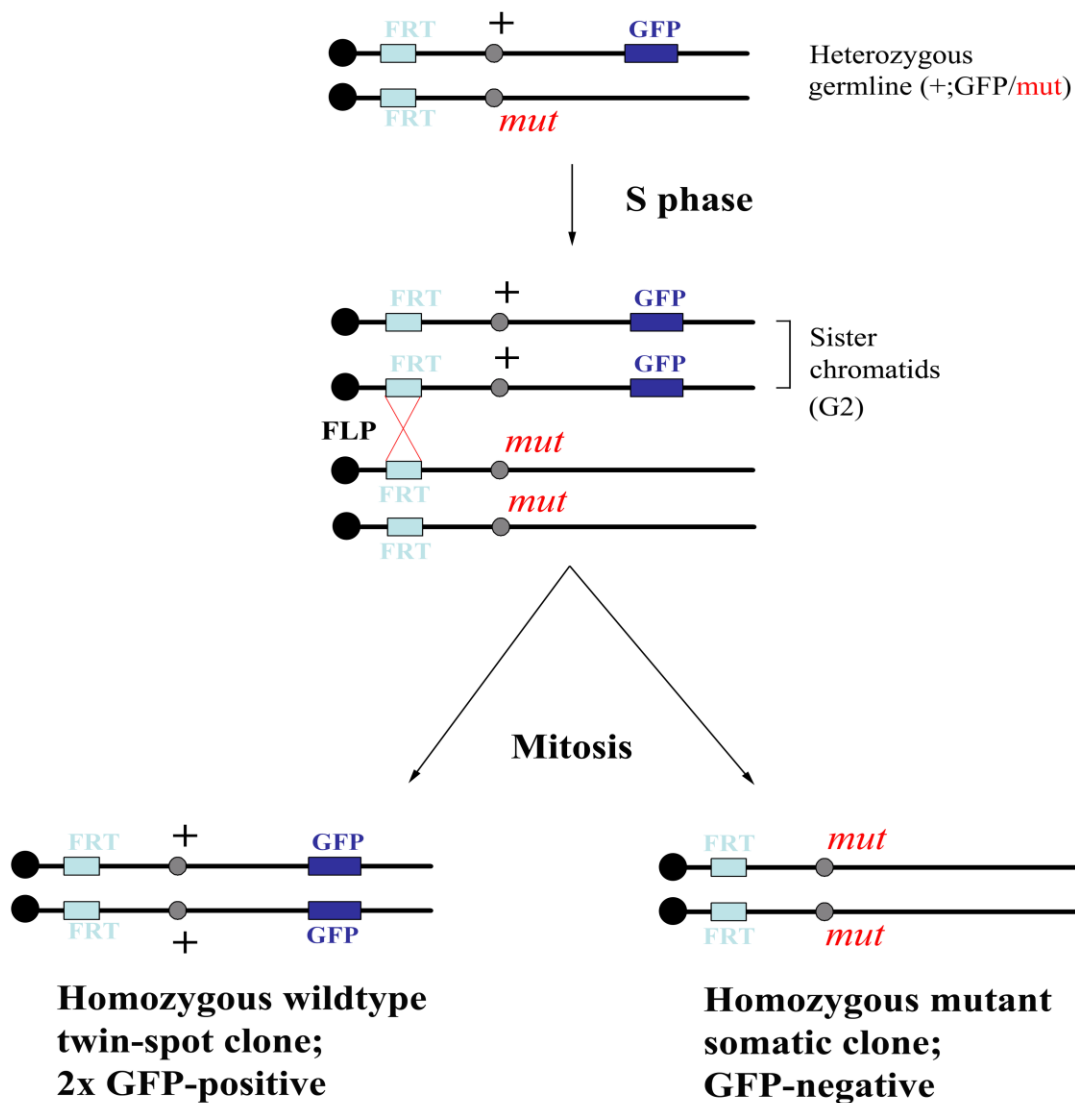


Diagram 2-12. Using the FLP/FRT system to generate mitotic clones in a heterozygous germline. FLP recombinase mediates site-specific recombination between FRT (Flp recombinase target) sites during replication in yeast, and works efficiently when expressed in flies (**Golic and Lindquist 1989**). After DNA replication, if the site-specific recombination between homologs occurs at the FRT sites (which are near the centromere), the portion of the chromosomal arm that lies distal to the FRT site, including the gene(s) of interest, will be made homozygous in the daughter cells, with each cell inheriting two copies of this region from one of the parental chromosomes following segregation. The resulting mosaic tissue consists mostly of the non-recombinant heterozygous germline (+;GFP/*mut*, intermediate level of GFP fluorescence), with a small fraction of somatic clones consisting of sporadically induced recombinant homozygous mutants of wildtype (+;GFP/+;GFP, “twin-spot”, high GFP fluorescence) or mutant (*mut/mut*, no GFP) genotypes. In the case of mutations located on the same chromosomal arm (such as *dPTEN* and *Vha68-2* on 2L), this technique can also be exploited to generate somatic clones of double mutants.

Cross Genotype	% embryonic lethality	Embryos assayed (n)
Wildtype (+)	1.0%	n=298
<i>Dakt</i> ¹ /+ x <i>Dakt</i> ¹ /+	1.5%	n=395
<i>Vha68-2</i> ^{P463} /+ x <i>Vha68-2</i> ^{P463} /+	1.4%	n=324
<i>Dakt</i> ¹ /+ x <i>Vha68-2</i> ^{P463} /+	19.8%	n=386
<i>Dakt</i> ¹ /+ x <i>trh</i> ^{P1747} /+ *	6.5%	n=418
<i>Dakt</i> ¹ /+ x <i>p60</i> ^A /+ *	12.5%	n=272

Table 2-1. Genetic interaction between *Dakt* and *Vha68-2*. The observed lethality of control and trans-heterozygous embryos is expressed as a percentage of the total number of embryos assayed from each cross (n). The baseline % lethality in control heterozygous crosses (*Dakt*¹/+ x *Dakt*¹/+ and *Vha68-2*^{P463}/+ x *Vha68-2*^{P463}/+) was comparable to that seen in the wildtype cross. The increased lethality in the progeny of transheterozygous crosses, assuming random and nonbiased segregation of chromosomes, reflects the severity of the compromised viability of the transheterozygous progeny (25% of total embryos from the cross). Previously published % lethality values for *Dakt/trh* and *Dakt/p60* mutant transheterozygotes (*) are for comparative purposes (Jin et al 2001).

Gene	Mutant allele	Mutation	Lethal phase
<i>Vha68-2</i>	<i>P463</i>	P{LacW} insertion	1 st larval instar
	<i>11065</i>	P{LacZ} insertion	3 rd larval instar/pupal
<i>Dakt</i>	<i>Dakt</i> ¹	Point mutation F327I	1 st larval instar
	<i>Dakt</i> ³	Point mutation G99S	Viable

Table 2-2. Lethal phase of examined mutant alleles of *Vha68-2* and *Dakt*. *P463* homozygotes die shortly after larval hatching as 1st instars, whereas *11065* homozygotes survive into late larval development, and perish in the late stages of the 3rd larval instar. A small number of *11065* mutants escape into pupariation, but without the eclosion of any adults. The characterization of the *Dakt* mutant alleles *Dakt*¹ and *Dakt*³ is described and discussed in detail in Staveley et al (1998) and Stocker et al (2002), respectively.

development at reduced sizes, and perish irrespective of nutrient availability. As such, the growth defect observed in 1st larval instars of *Dakt* and *Vha68-2* loss-of-function mutants may be a reflection of developmental delay following hatching. That is, whereas the wildtype 1st instar stage lasts approximately 24hr, the mutants may spend a considerably longer time as 1st instars prior to their impending lethality. Consequently, despite proper staging, at 24hr AH, *late* 1st instar wildtype larvae would be compared to delayed early 1st instar mutant larvae, potentially accounting, at least in part, for the size disparity observed in the mutants as compared to wildtype counterparts.

In order to neutralize the contribution of developmental delay to the growth defect phenotype, size comparisons can be carried out after completion of the larval growth program – in pupae, and surviving adults. Accordingly, periodic ectopic expression of *Dakt* through a heat shock-inducible *Dakt* transgene (*hsDakt*) was previously used to rescue early larval lethality of *Dakt*¹ homozygotes (Scanga et al 2000). In this manner, homozygous *Dakt*¹ mutant animals were generated at all subsequent stages of development for the assessment of size defects, and in all cases tested, *Dakt*¹ larvae, pupae, or adult flies were found to be significantly smaller in size (Scanga et al 2000). Mutants of *Vha68-2* bearing the weaker 10065 allele survive as late as the 3rd larval instar, with a small fraction of mutant larvae escaping into pupae. Although no 11065 adults could ever be recovered, the size of 11065 pupae was assessed in comparison to wildtype pupae (Figure 2-1B). A clear disparity in size was once again detected; however the size difference with this weaker allele was much subtler at the pupal stage than the drastic effect observed 24hr AH in 1st instar larvae of the stronger larval lethal mutant allele *Vha68-2*^{P463}. These results suggest that although developmental delay may contribute significantly to the growth defect phenotype in early larval development, the persistence of the size phenotype observed in the terminal stages of development recapitulates the role of *Vha68-2* as a possible positive regulator of growth in *Drosophila*, in a capacity similar to that of ISP components that manifest similar organismal growth and developmental delay phenotypes. In fact, the size deficiency observed between wildtype and 10065 mutant pupae was nearly identical to the size defect observed between wildtype and *Dakt*³ mutant pupae (Figure 2-1B). Unlike *Dakt*¹ which is early larval lethal, *Dakt*³ is a viable hypomorphic mutant allele of *Dakt* (Table 2-2) that results

in flies with severely reduced body size throughout development (Stocker et al 2002), and as such, the size of homozygous mutant pupae can be readily compared to pupae of 11065, the weaker of the *Vha68-2* mutant alleles.

2-3.3 – Genetic or pharmacological inhibition of V-ATPase downregulates Akt

Considering the striking similarity observed in the organismal growth phenotype resulting from the loss of either V-ATPase or Akt function in post-embryonic *Drosophila* development, and the importance of HM and T-loop phosphorylation in the activation of Akt, the levels of Akt phosphorylation at these two crucial residues were examined in both *Vha68-2* mutants and in cultured larval tissues incubated with pharmacological inhibitors of V-ATPase activity. Western blots of whole-larval lysates extracted from L1 *Vha68-2*^{P463} and *Vha68-2*^{S4214} (a similarly strong P element-induced larval lethal mutant allele of *Vha68-2*) mutant larvae showed a severe decrease in T-loop phosphorylation at S505 compared to staged wildtype larvae despite normal levels of Akt protein (**Figure 2-2A**). Similarly, S505 phosphorylation was found to be diminished in L3 larvae of a knockdown mutant ubiquitously expressing a *Vha68-2* RNAi transgene (*arm-GAL4/UAS-Vha68-2*^{RNAi}) in comparison to wildtype L3 larvae (**Figure 2-2B**). Furthermore, whole-larval lysates of the weaker *Vha68-2* mutant 11065 were analyzed as both L1 larvae and as L3 survivors, and found to consistently exhibit diminished levels of S505 phosphorylation compared to staged wildtype counterparts (**Figure 2-2C**).

Although a large selection of antibody-based reagents are available for the biochemical analysis of Akt phosphorylation at S473 and T308 in mammals, and to a lesser degree, S505 in *Drosophila*, there remains a near-complete dearth of similar reagents for the biochemical assessment of Dakt phosphorylation at T342. Lacking an antibody against phospho-T342, the effect of V-ATPase inhibition in *Drosophila* on TORC2 and PDK1-mediated Akt phosphorylation was investigated in *phenotypically wildtype* larvae transgenically expressing hemagglutinin (HA)-tagged bovine Akt, using a *Gal4* transgene driven by the *armadillo* promoter, allowing expression of the Akt-HA construct in a fairly ubiquitous pattern using an upstream activating sequence (UAS) transgene. The transgenic expression of bovine Akt in this fashion was found to partially

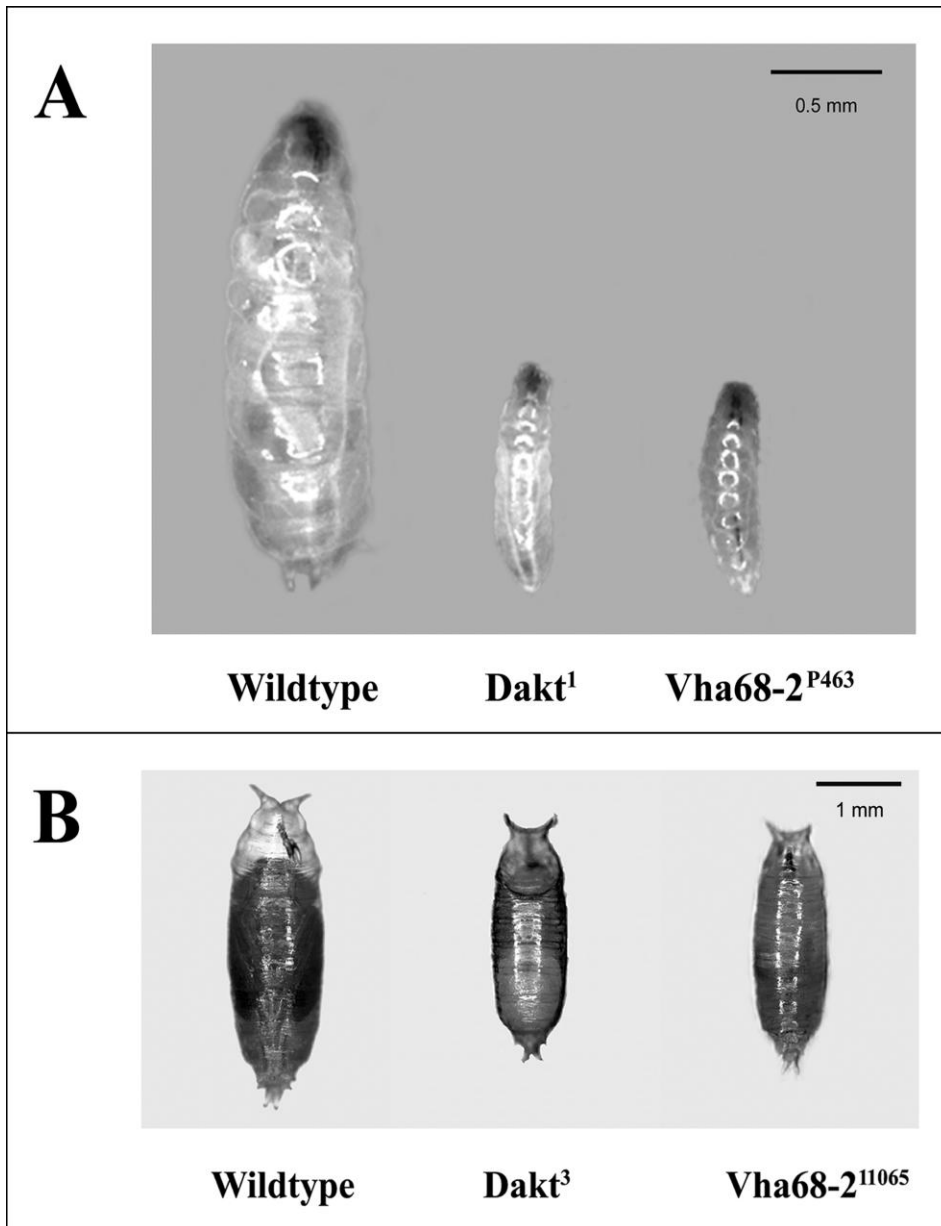


Figure 2-1. Larval and pupal size phenotypes of *Dakt* and *Vha68-2* mutants. The larvae and pupae shown above are representative of ~20 larvae/pupae examined for each genotype. (A) The sizes of wildtype, *Dakt*¹, and *Vha68-2*^{P463} homozygous mutant larvae isolated from a staged collection were compared 24 hours after larval hatching. As in the determination of lethal phase (Table 1-2), homozygous mutant larvae were distinguished by the absence of GFP fluorescence. The significant size defect exhibited by *Dakt*¹, a kinase-dead allele of *Dakt*, is comparable to that observed for *Vha68-2*^{P463} in early larval development (late 1st larval instar). (B) The pupae of phenotypically wildtype, *Dakt*³ (a viable hypomorphic allele of *Dakt*) and *Vha68-2*¹¹⁰⁶⁵ homozygous mutants were collected shortly after puparium formation, and their sizes were compared. The mutant pupae were clearly smaller than wildtype, and the terminal developmental size defect associated with both mutant alleles was comparable.

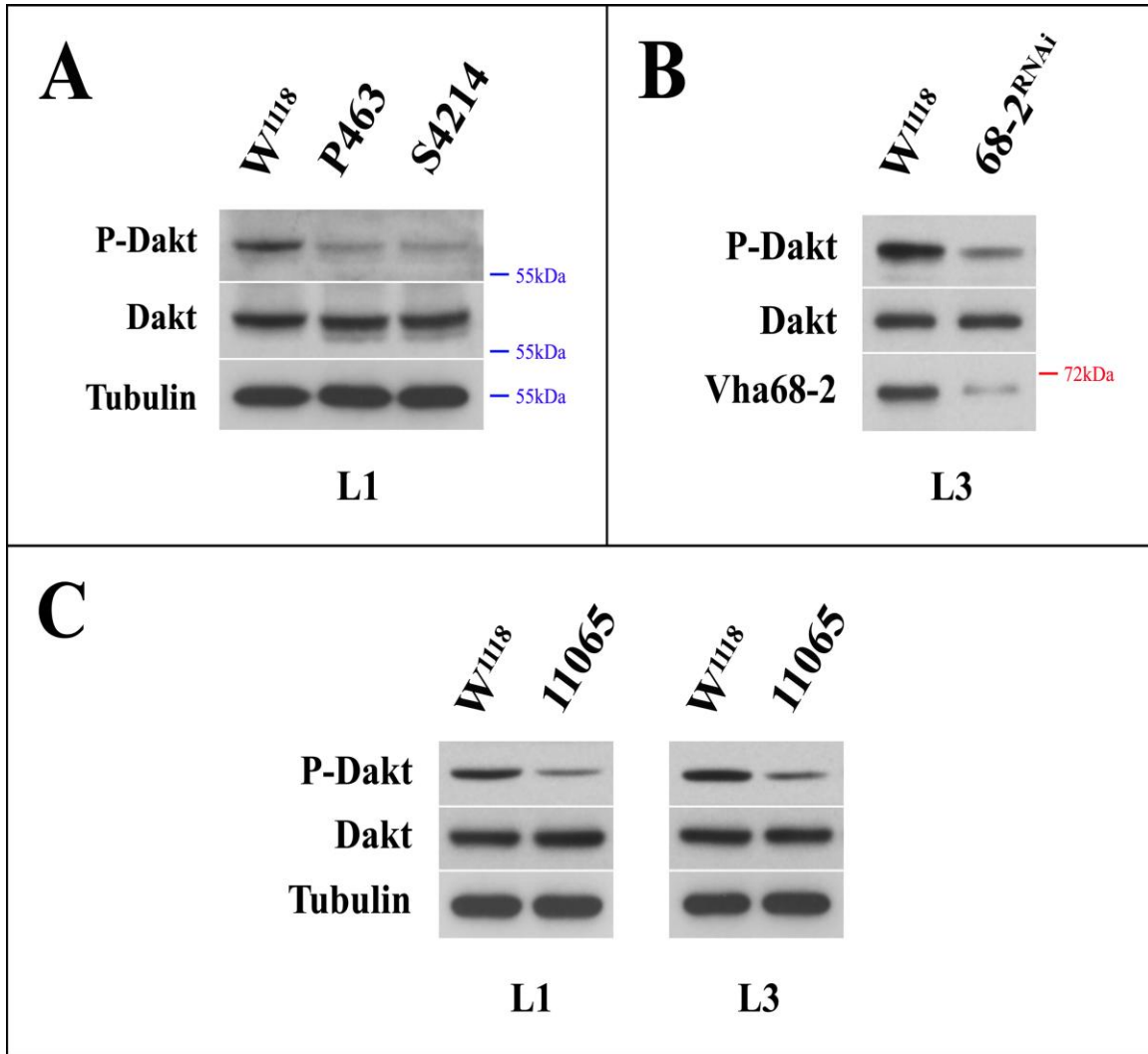


Figure 2-2. Dakt phosphorylation at S505 is downregulated in *Vha68-2* mutants. Western-blot analyses demonstrate a decrease in Dakt phosphorylation at S505 in: **(A)** whole-cell lysates of mutant 1st instar larvae (L1) as compared to L1 wildtype (*w¹¹¹⁸*) larvae; **(B)** whole-cell lysates of *Vha68-2* knockdown mutant (*arm-GAL4/UAS-Vha68-2^{RNAi}*) L3 larvae as compared to L3 wildtype (*w¹¹¹⁸*) larvae, in which *Vha68-2* blotting confirmed the knockdown efficiency of the RNAi transgene; and **(C)** whole-cell lysates of weak *Vha68-2* hypomorphic mutant (*11065*) L1 larvae, and surviving L3 larvae as compared to their wildtype (*w¹¹¹⁸*) staged counterparts. Total Dakt protein and β -tubulin were used as loading controls. Immunoblots are representative of multiple repetitions (at least three in all cases) of the experiments in question. The molecular weight values in the right-hand margins are based on the PageRulerTM (Fermentas) prestained protein ladder.

rescue the *Dakt*¹ mutant phenotype, demonstrating the conservation of the regulatory mechanisms and activity of mammalian Akt in a *Drosophila* signaling context (**Staveley et al 1998**). In order to investigate the effects of V-ATPase inhibition on the phosphorylation of the transgenic Akt-HA molecule, the dissected tissues of wildtype L3 larvae transgenically expressing Akt-HA were incubated in insect medium supplemented with the V-ATPase inhibitor bafilomycin A1. Bafilomycins (A1, B, C, and D) are macrolide antibiotics that were originally isolated from the mycelium of the gram-positive bacterium *Streptomyces griseus*, and found to inhibit the growth of yeast, gram-negative bacteria, and fungi (**Werner et al 1984**), which was followed thereafter by their functional definition as inhibitors of V-ATPase (**Bowman et al 1988**).

Whole cell lysates generated from Akt-HA-expressing larval tissues were purified after a twelve hour time course of treatment with 500nM bafilomycin (see **Section 3-3.1** for rationale behind the concentration chosen), and analyzed by Western-blotting, which revealed a significant time-dependent decrease in the insulin-induced phosphorylation of bovine HA-tagged Akt at S473, an effect that was detectable at the earliest examined time point after treatment (2 hours), and maximal after 12 hours of treatment (**Figure 2-3A**). Based on this timeline, dissected Akt-HA-expressing larval tissues were incubated with either 500nM bafilomycin or 500nM concanamycin A (another highly potent specific inhibitor of V-ATPase) and harvested following twelve hours of treatment, after which purified protein samples from whole-cell lysates were analyzed by Western-blotting. In both bafilomycin- and concanamycin-treated samples, a comparable decrease in the phosphorylation of both S473 and T308 was evident (**Figure 2-3B**), suggesting that V-ATPase inhibition downregulates both PDK1-mediated T-loop, and TORC2-mediated HM phosphorylation of the Akt protein. Incidentally, the S473-specific antibody also weakly cross-reacts with endogenous *Drosophila* Akt phosphorylated at S505, whose signal intensity also diminishes concomitantly with the phosphorylation of transgenically expressed bovine Akt at S473 (**Figure 2-3A,B**), providing a useful intrinsic control for the phosphorylation status of the endogenous protein.

2-3.4 – Vha68-2 mutant clones cell-autonomously phenocopy *Dakt* deficiency

The organismal growth defects observed with mutant alleles of *Vha68-2* phenocopy those observed in *Dakt* mutants, which is consistent with our biochemical demonstration that the inhibition of V-ATPase function itself results in the diminished phosphorylation and activation of Akt. However, these defects in organismal growth during the larval development of *Vha68-2* mutants could be due to non cell-autonomous functions, such as defects in the production of Dilps in IPCs, or their efficient processing, release, and circulation. After all, ablation of these IPCs, which are the main systemic supply of Dilp2 during larval growth, is sufficient to cause developmental delays and growth defects in the resulting organism (**Rulifson et al 2002**) in a manner similar to that stereotypically observed in all mutants of the ISP ([Diagram 2-6](#)). In fact, V-ATPase itself has been demonstrated to have a regulatory function in the exocytosis and secretion of insulin in mice (**Sun-Wada et al 2006**). Therefore, in order to determine whether V-ATPase, as with other known and characterized intracellular ISP components, also functions in growth control in a cell-autonomous manner, mosaic analyses of homozygous *Vha68-2*, *Dakt*, or *dPTEN* mitotic clones were undertaken in the endoreplicating cells of the salivary glands, in which growth defects could be assessed independently of proliferation. Somatic mutant clones of *Dakt*¹ and *Vha68-2*^{P463} were randomly generated by FRT-mediated, heat shock FLP-driven mitotic recombination (**Xu and Rubin 1993**) in a heterozygous (+;GFP / -) phenotypically wildtype background of the mutant allele in question, resulting in a mosaic animal containing a small percentage of sporadically induced mutant homozygous clones (-/-) marked by the absence of GFP expression, as well as their sister wildtype (+;GFP / +;GFP) twin-spot clones in a heterozygous (+;GFP / -) phenotypically wildtype germ-line ([Diagram 2-12](#)). Inherent advantages of genetic mosaics, as demonstrated herein, include the possibility of examining tissue-specific mutant phenotypes of otherwise lethal mutations at any stage in development, and the ability to juxtapose wildtype and mutant cells - a highly useful attribute that allows immediate comparison to normal cells and the comparative assessment of cell-autonomy for the mutant phenotype in question (**Xu and Rubin 1993**).

Somatic clones of mutant cells generated by heat shock induced recombination during late embryogenesis were thereafter examined for cell autonomous growth defects

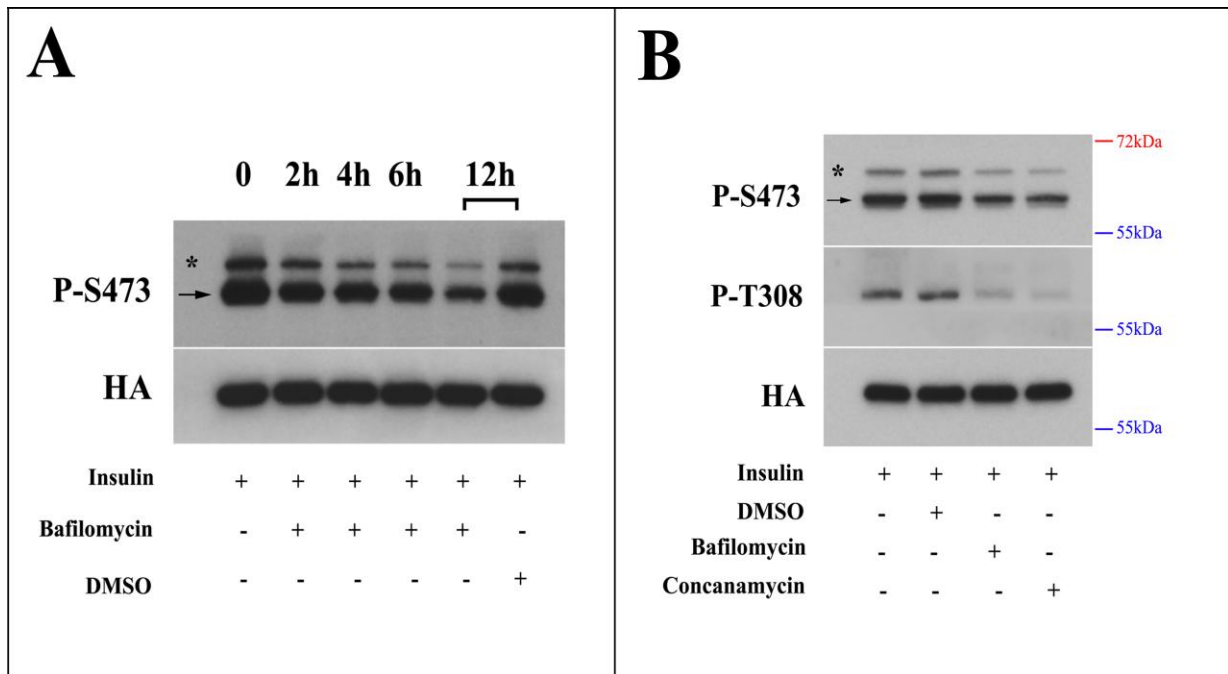


Figure 2-3. Pharmacological inhibition of V-ATPase activity downregulates both endogenous and transgenically expressed Akt phosphorylation. (A) Dissected tissues of L3 larvae expressing haemagglutinin (HA)-tagged bovine Akt under the control of an *armadillo* driver (*arm-GAL4/UAS-Akt-HA*) were incubated in insect media (5% FBS and 0.2U/mL insulin) supplemented with either 500nM bafilomycin (Sigma), 500nM concanamycin A (Sigma), or the vehicle (DMSO) control for 12 hours. Whole-cell lysates of each sample were generated from the dissected tissues at the end of the incubation period, and Western-blotted to examine the levels of Akt-HA phosphorylation with antibodies against either P-S473 or P-T308. The P-S473 antibody detects phosphorylated S473 residues (indicated by the black arrow) on transgenic bovine Akt, as well as endogenous Dakt phosphorylated at S505 (marked by black asterisk), though with lower binding efficiency in comparison to the *Drosophila*-specific S505-directed antibody (Cell Signaling Technology #4054). (B) 12 hour time course of HA-tagged bovine Akt-expressing L3 larval tissues dissected and incubated in insect media (5% FBS and 0.2U/mL insulin) supplemented with 500nM bafilomycin. Tissues were harvested after the indicated lengths of incubation time and Western-blotted to examine the levels of transgenic bovine Akt phosphorylation at S473 (indicated by black arrow). In both (A) and (B), levels of transgenically expressed HA-tagged bovine Akt were distinguished from endogenous Dakt by blotting against the HA epitope, which also served as a protein loading control. Immunoblots are representative of multiple (at least three) repetitions of the experiments in question. The molecular weight values in the right-hand margin of (A) are based on the PageRuler™ (Fermentas) prestained protein ladder.

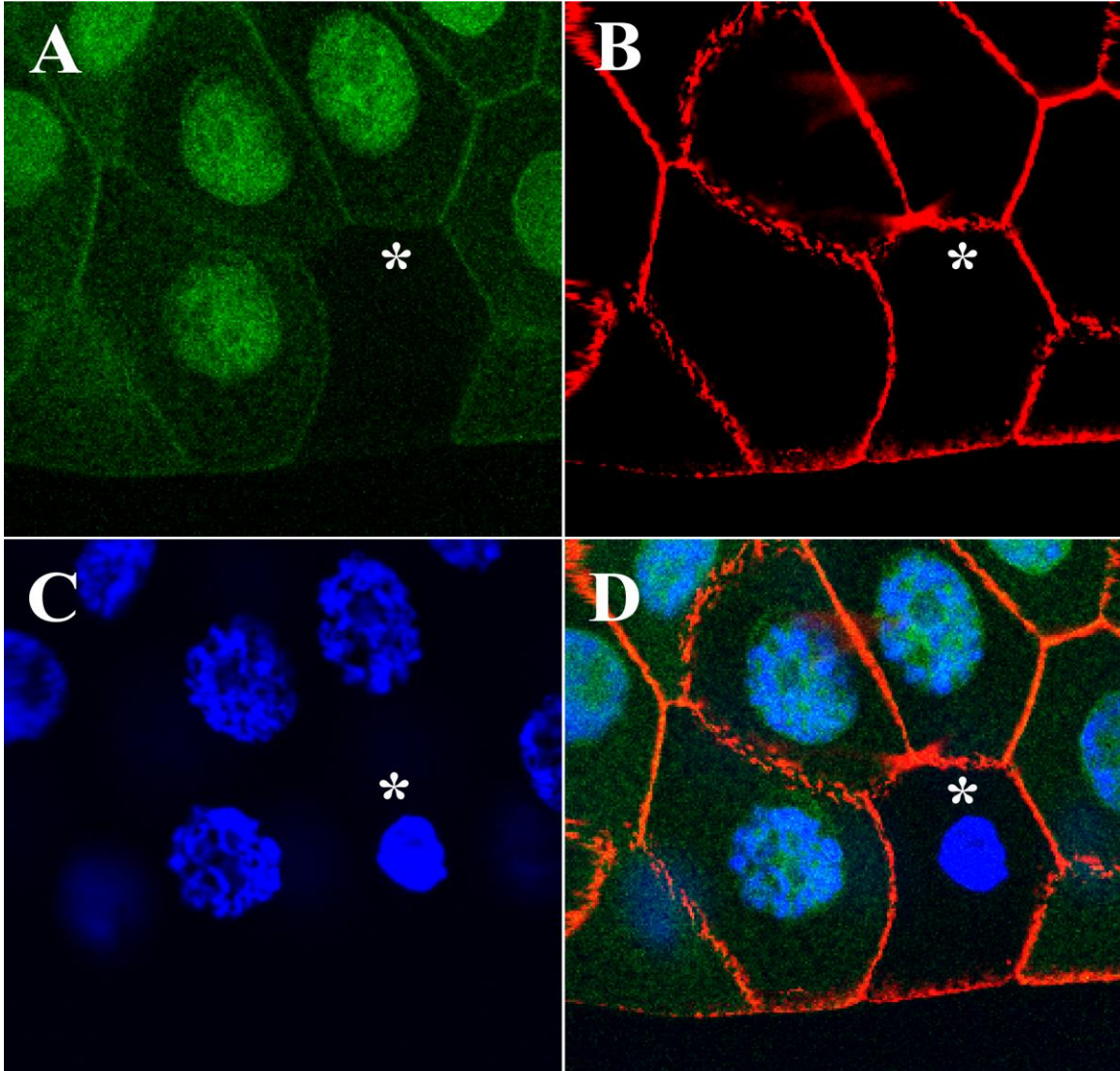


Figure 2-4. Cell-autonomous growth phenotype of *Dakt* mutant clones in cells of the salivary gland. Somatic clones of *Dakt*¹ mutant cells were induced embryonically by heat shock, and examined for cell-autonomous growth defects in L3 salivary glands. The results shown here are representative of >20 somatic clones examined over the course of five experimental repetitions. The mitotic recombination mutants were sporadically induced in a heterozygous germline (*Dakt*^{1/+};GFP), resulting in GFP-negative somatic mutant clones (*Dakt*¹/*Dakt*¹) that lack Dakt function in a phenotypically wildtype GFP-positive background including germline heterozygotes (*Dakt*^{1/+};GFP), and/or “twin spot” wildtype clones (+;GFP/+;GFP), which are the mitotic sisters of homozygous mutant clones. (A) GFP fluorescence of salivary gland cells. The GFP-negative mutant clone is marked (*), and is surrounded by GFP-expressing phenotypically-wildtype neighboring cells. (B) The red signal is phalloidin fluorescence, which binds F-actin and demarcates the boundaries of individual cells. (C) DNA is stained blue with the nuclear marker DAPI. The *Dakt* mutant clone (*) showed a clear reduction in nuclear size compared to phenotypically wildtype neighbors. (D) Merged view of all three signals.

in 3rd instar larval salivary glands, which were fixed and stained with DAPI, a DNA-binding fluorescent label commonly used as a marker for nuclei. In endoreplicating salivary glands, the comparison of the DAPI stain of *Dakt*¹ mutant clones (GFP-negative) to that of wildtype neighbors (GFP-positive) revealed a distinct decrease in nuclear size (**Figure 2-4, 2-5**), consistent with the positive role attributed to Dakt function in ISP-mediated growth control. Similarly generated somatic clones of *Vha68-2*^{P463} homozygous mutant cells were examined and found to phenocopy the cell-autonomous growth defect observed in *Dakt*¹ mutant clones with respect to nuclear size (**Figure 2-6**), whereby both *P463* and *Dakt*¹ mutant nuclei were consistently smaller in size as compared to the normal-sized nuclei of wildtype cells. These results suggest that *Vha68-2*, like Dakt, acts cell-autonomously to positively regulate cellular growth, either as a component of the ISP, or alternatively, in a parallel-acting cellular pathway or process that converges with the PI3K/Akt pathway in the modulation of size control.

In contrast to the clonal overexpression of positive regulatory components of the ISP such as DIR or Dp110, which causes cell-autonomous *overgrowth* in ERTs (**Diagram 2-9**), clones of cells overexpressing the negative regulator *dPTEN* have been found to contain *smaller* nuclei than adjacent control cells (**Britton et al 2002**), resulting in a growth phenotype highly similar to the one we have demonstrated to occur cell-autonomously following the loss of Dakt or *Vha68-2* function in mutant clones of cells in the salivary gland. In order to assess the cell-autonomous growth phenotype of *dPTEN*-deficiency, we employed the c494 allele of *dPTEN*, which encodes a protein containing a point mutation (G135E) in an invariant glycine residue required for phosphatase activity (**Denu and Dixon 1998**), rendering the mutant molecule incapable of converting PIP₃ back to PIP₂. Flies homozygous for the c494 allele of *dPTEN* (**Huang et al 1999**) die early in larval development (late 1st instar), which is similar to the lethal phase observed with *Dakt*¹ and *Vha68-2*^{P463} embryos. In contrast to the reduction in nuclear size observed in *Dakt* and *Vha68-2* mutant clones, as well as clones overexpressing wildtype *dPTEN* (**Britton et al 2002**), c494 mutant clones lacking PIP₃ phosphatase activity were found to result in cells with enlarged nuclei when compared to phenotypically wildtype neighbors (**Figure 2-7**), an effect consistent with the established role of *dPTEN* as a negative regulator of the ISP (**Goberdhan et al 1999, Huang et al 1999, Gao et al 2000, Scanga**

et al 2000), and the crucial function of PIP₃ in the DIR/Dp110-dependent activation of Dakt (**Oldham et al 2002**, **Stocker et al 2002**). Accordingly, whereas the overexpression of *PTEN* *in vitro* has been demonstrated to diminish insulin-induced PIP₃ production without impinging on the insulin-dependent activation of PI3K (**Maehama and Dixon 1998**), our examination of c494 mutant clones, which conversely lack dPTEN function, demonstrated a substantial cell-autonomous accumulation of PIP₃ concomitantly with the aforementioned increases in nuclear size (**Figure 2-7**).

2-3.5 – *Vha68-2^{P463}* suppresses the overgrowth phenotype of *dPTEN* deficiency

Many of the studies discussed in this chapter that investigated and characterized the mutant phenotypes of ISP signaling components also explored the relationships between the various components of the ISP in genetic epistasis experiments, through which the capacity of the components in question to enhance or suppress the growth phenotypes associated with alterations in the activity of other components with putative roles in the same pathway were assessed. For example, the function of dPTEN was placed epistatically *downstream* of DIR activity based on genetic interactions demonstrating that the loss *dPTEN* function in *DIR*-deficient heads was capable of suppressing the growth and proliferation defects (a so-called “pin-head” phenotype) associated with the loss of *DIR* function in the same organ (**Oldham et al 2002**); and similarly, the loss of *dPTEN* function in a growth defective heteroallelic *DIR* mutant genetic background was shown to suppress the defective growth associated with the loss of *DIR* function in the wing (**Gao et al 2000**). Moreover, the lethality of a heteroallelic *DIR* combination which arrests development at the 2nd instar larval stage, was rescued by the additional reduction of PTEN function, which allowed larval development to proceed to the pupal stage, even producing some pharate adult escapers (**Oldham et al 2002**), further demonstrating that decreased dPTEN function can compensate to a significant degree for the absence of *DIR* function in growth signaling during development. Conversely, the overexpression of *dPTEN* was found to completely rescue the lethality and overproliferation phenotypes of *DIR*-overexpressing cells in the eye (**Huang et al**

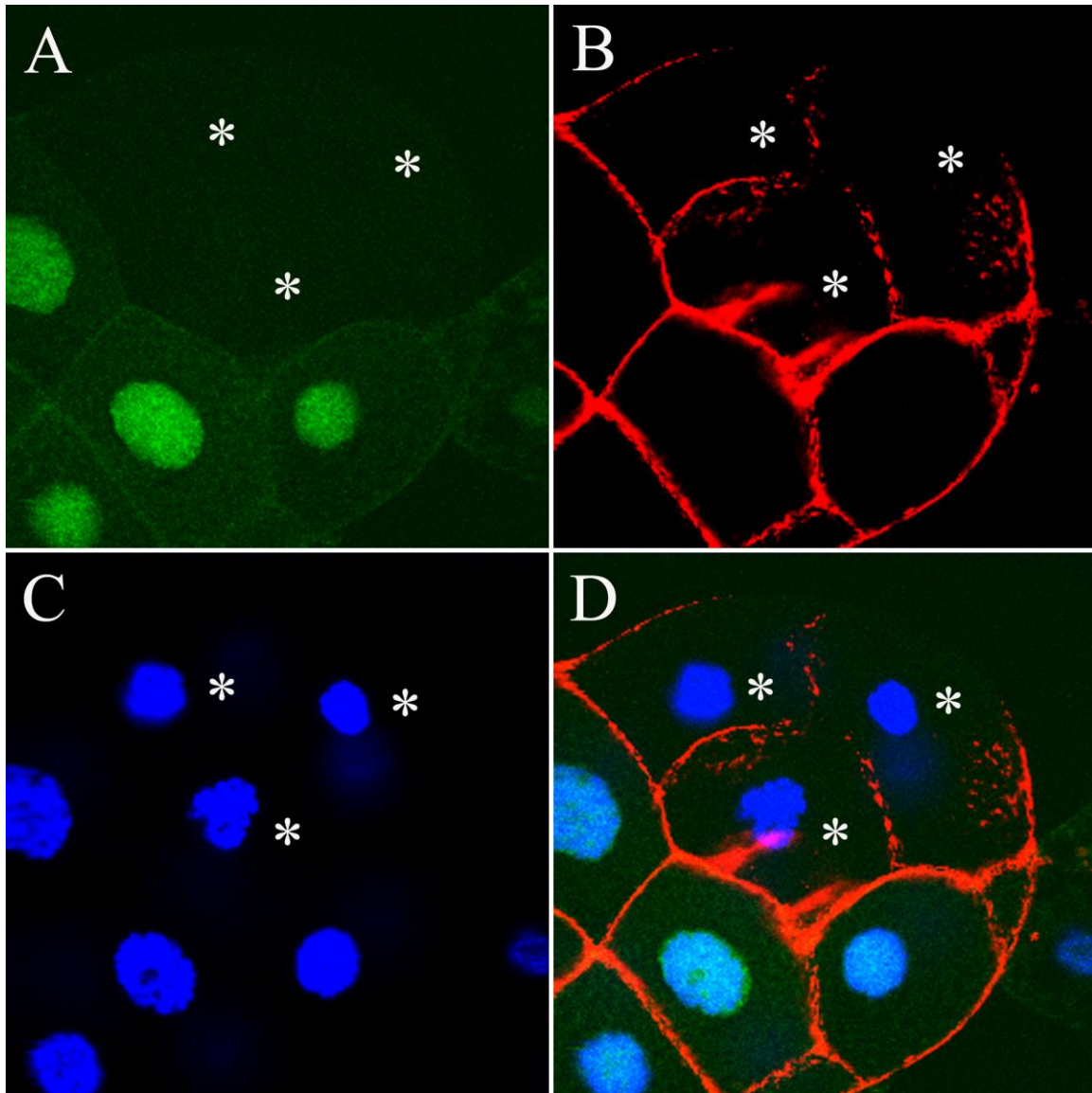


Figure 2-5. Cell-autonomous growth phenotype of *Dakt* mutant clones in cells of the salivary gland. As in **Figure 2-4**, (A) GFP fluorescence of salivary gland cells. The GFP-negative mutant clones are marked (*), and are situated above a patch of GFP-expressing phenotypically-wildtype cells. (B) The red signal is phalloidin fluorescence, which binds F-actin and demarcates the boundaries of individual cells. (C) DNA is stained blue with DAPI, and serves as a measure of nuclear size. The *Dakt* mutant clones (*) consistently showed a drastic reduction in nuclear size compared to phenotypically wildtype neighbors. (D) Merged view of all three signals.

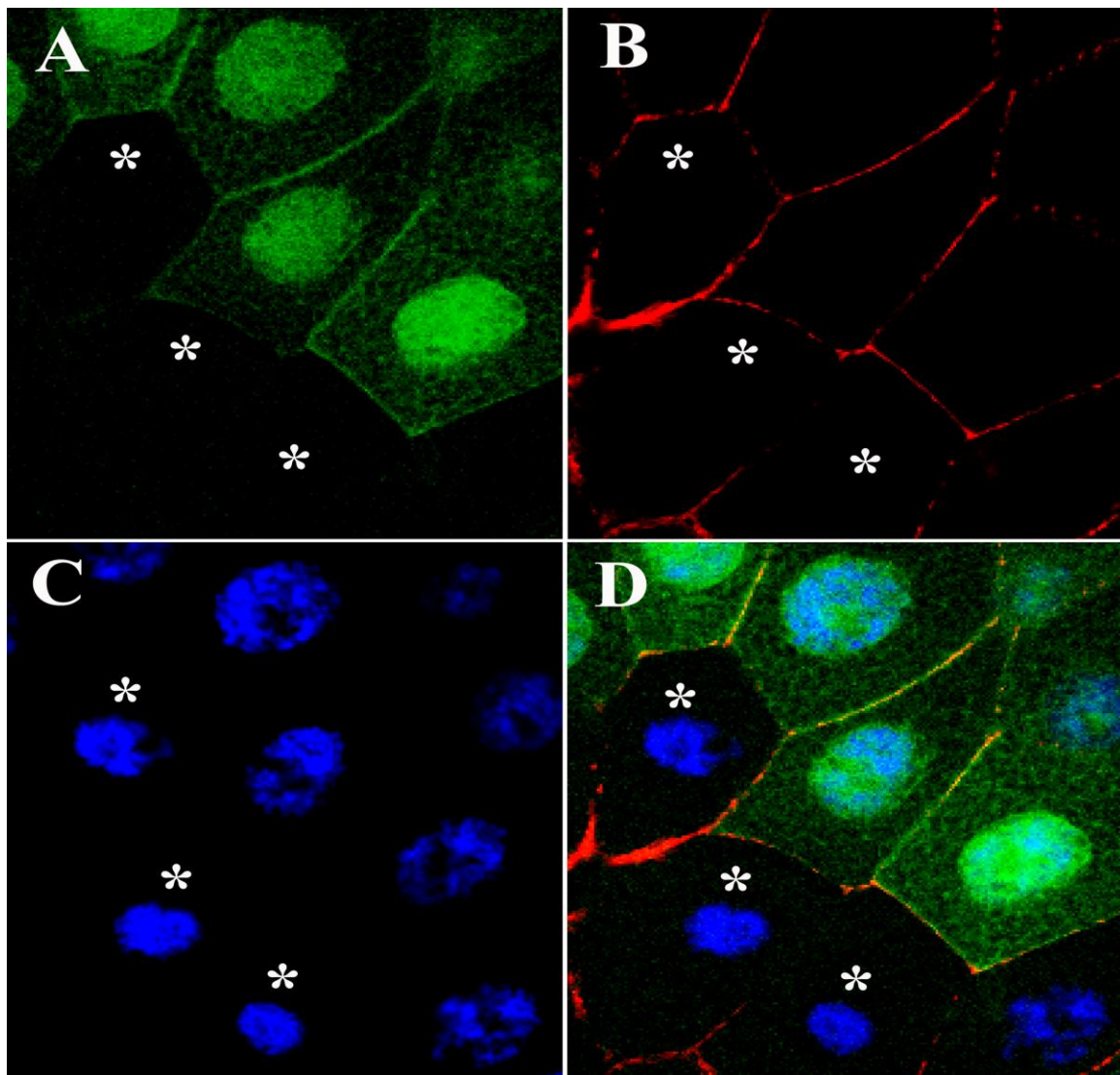


Figure 2-6. Cell-autonomous growth phenotype of *Vha68-2* mutant clones in cells of the salivary gland. The results shown here are representative of >20 somatic clones examined over the course of five experimental repetitions. As in the case of *Dakt*¹ somatic clones, mitotic recombination mutants were sporadically induced in a heterozygous germline (*Vha68-2*^{P463} /+;GFP), resulting in tissues containing GFP-negative somatic mutant clones (*Vha68-2*^{P463} / *Vha68-2*^{P463}) that lack *Vha68-2* function, in a phenotypically wildtype GFP-positive background including germline heterozygotes (*Vha68-2*^{P463} /+;GFP) and/or “twin spot” wildtype clones (+;GFP/+;GFP). (A) GFP fluorescence of salivary gland cells. The GFP-negative mutant clones are marked (*), and are adjacent to GFP-expressing phenotypically-wildtype neighboring cells. (B) The red signal is phalloidin fluorescence (C) DNA is stained blue with DAPI in all cells, and serves as a measure of nuclear size. The *Vha68-2* mutant clones (*), like *Dakt* mutant clones (Figure 2-4, 2-5) consistently showed a clear reduction in nuclear size compared to phenotypically wildtype neighbors. (D) Merged view of all three signals.

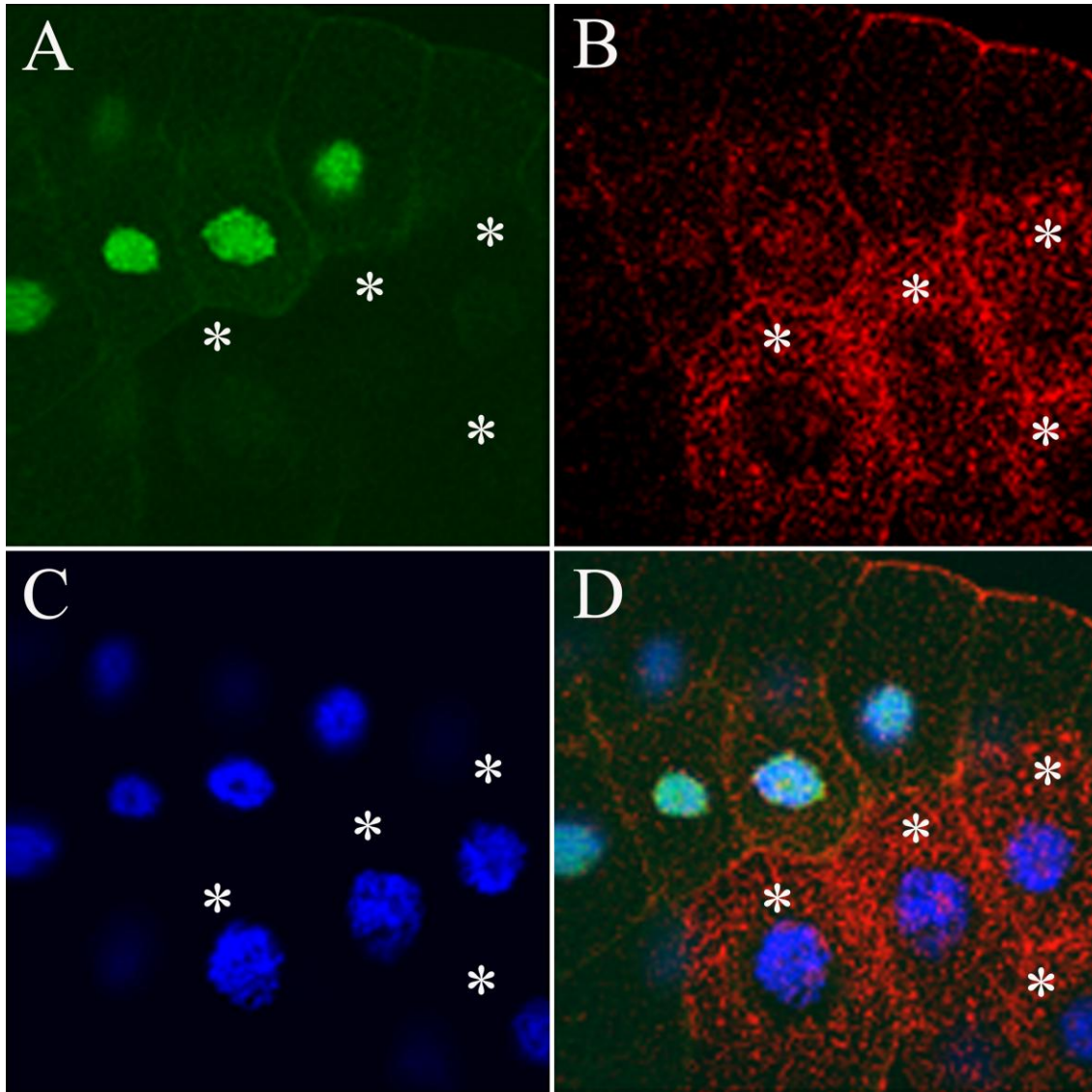


Figure 2-7. Cell-autonomous overgrowth and elevation of PIP₃ levels in *dPTEN* mutant clones of salivary gland cells. The results shown here are representative of >20 somatic clones examined over the course of three experimental repetitions. As in the case of *Dakt*¹ or *Vha68-2*^{P463} somatic clones, mitotic recombination mutants were sporadically induced in a heterozygous germline (*dPTEN*^{c494} /+; *GFP*), resulting in tissues containing GFP-negative somatic mutant clones (*dPTEN*^{c494} / *dPTEN*^{c494}) that lack dPTEN function in a phenotypically wildtype GFP-positive background including germline heterozygotes (*dPTEN*^{c494} /+; *GFP*), and “twin spot” wildtype clones (+; *GFP* /+; *GFP*). (A) GFP fluorescence of salivary gland cells. The GFP-negative mutant clones are marked (*), and are located below a row of GFP-expressing phenotypically-wildtype cells. The abundance and intensity of the punctate red signal in (B) corresponds to PIP₃ immunofluorescence, which is significantly elevated in *dPTEN* mutant clones (*). (C) DNA is stained blue with DAPI in all cells, and *dPTEN* mutant clones (*) consistently showed an increase in nuclear size compared to phenotypically wildtype neighbors. (D) Merged view of all three signals.

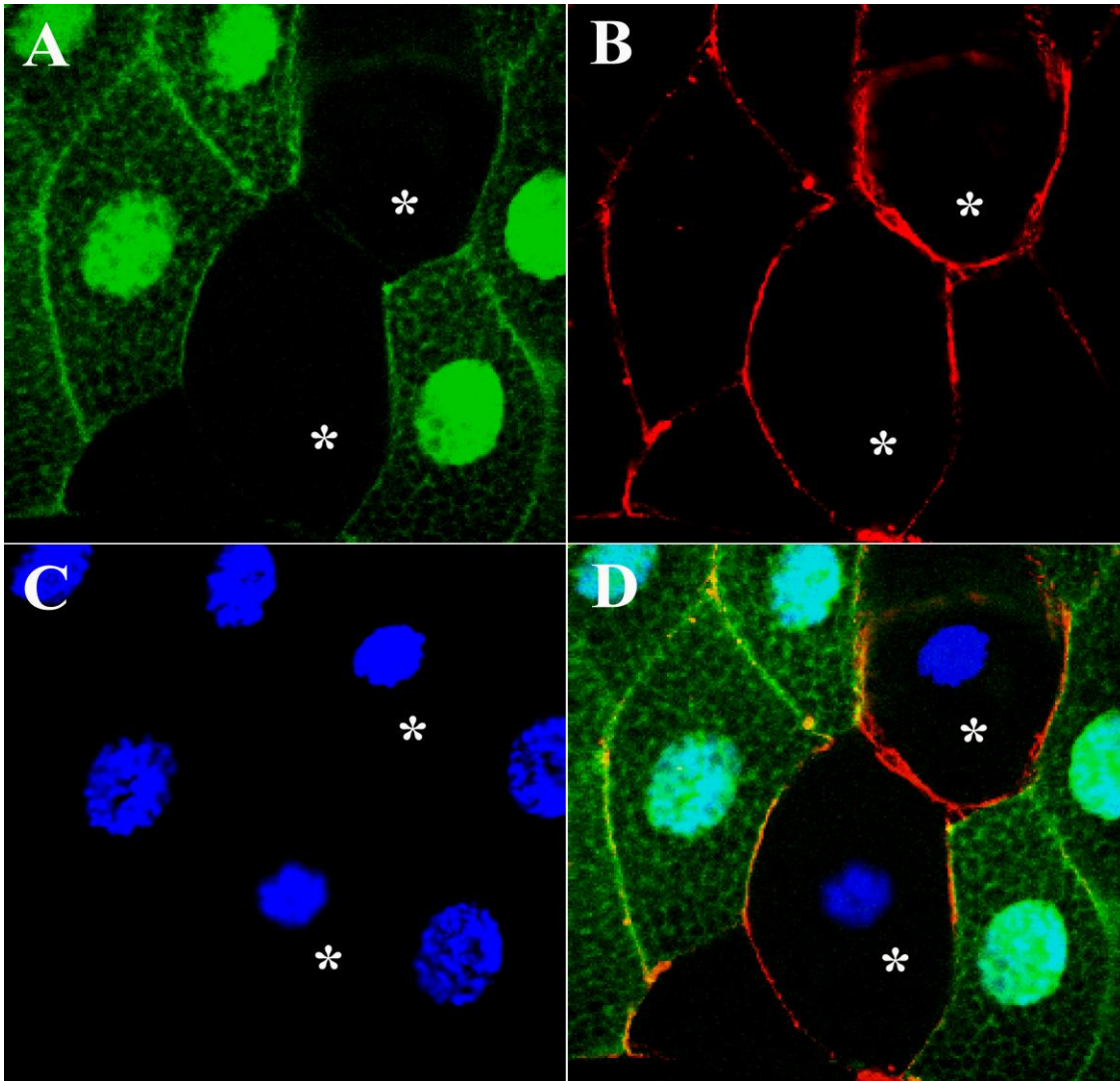


Figure 2-8. Suppression of the $dPTEN^{c494}$ growth phenotype in $dPTEN/Vha68-2$ double mutant clones in the salivary gland. The results shown here are representative of >20 somatic clones examined over the course of five experimental repetitions. As in the case of single *Dakt*, *dPTEN* or *Vha68-2* mutant clones, the recombinant double mutants were sporadically induced in a germline heterozygous for both mutant alleles ($dPTEN^{c494};Vha68-2^{P463}/+;GFP$), resulting in tissues containing GFP-negative somatic double mutant clones ($dPTEN^{c494};Vha68-2^{P463}/dPTEN^{c494};Vha68-2^{P463}$) that lack both *dPTEN* and *Vha68-2* function in a phenotypically wildtype GFP-positive background that includes germline heterozygotes ($dPTEN^{c494};Vha68-2^{P463}/+;GFP$) and “twin spot” wildtype clones ($+;GFP/+;GFP$). (A) GFP fluorescence of salivary gland cells. The GFP-negative double mutant clones are marked (*), and are adjacent to two rows of GFP expressing phenotypically-wildtype neighboring cells. (B) The red signal is phalloidin fluorescence. (C) DNA is stained blue with DAPI in all cells, and in the $dPTEN/Vha68-2$ double mutant clones (*), the *dPTEN* mutant size phenotype was suppressed, resulting instead in a reduction in nuclear size resembling the phenotype seen in *Dakt* or *Vha68-2* single mutant clones. (D) Merged view of all three signals.

1999), suggesting that all signals originating from DIR activation in diverse tissues can be antagonized by elevating downstream dPTEN function.

Downstream of DIR activation, flies overexpressing both *dPTEN* and *Dp110* were found to have much higher viability rates than those overexpressing either gene alone (Goberdhan et al 1999). Conversely, the lethality associated with complete loss of PI3K function could not be rescued by the concomitant loss of dPTEN function (Oldham et al 2002) due to the latter molecule's catalytic activity being rendered obsolete in the absence of PI3K-dependent PIP₃ production. In flies co-overexpressing *Dp110* and *dPTEN*, the targeted compartment of the wing was found to be nearly as reduced in area as animals overexpressing *dPTEN* alone, resulting in complete suppression of the growth promoting effects of *Dp110* overexpression in this system (Goberdhan et al 1999). Similarly, wing cells co-overexpressing *dPTEN* and a dominant negative (DN) allele of *Dp110* resulted in an enhancement of the *Dp110* DN-associated reduction in wing size (Gao et al 2000). In the eye, the lethality associated with *Dp110* overexpression was found to be rescued by co-expression of *dPTEN*, while the small eye phenotype of *dPTEN* overexpression was effectively suppressed by *Dp110* overexpression, and enhanced by the overexpression of DN *Dp110* (Huang et al 1999). These observations are consistent with a growth signaling paradigm where both molecules act rheostatically downstream of DIR activation to modulate PIP₃ abundance and PIP₃-dependent signaling events. As for *Dakt*, in flies homozygous for the semi-lethal 04226 allele (Spradling et al 1999), survivors show reduced body and cell size, and the examination of *dPTEN* mutant clones induced in 04226 mutant animals demonstrated that 04226 completely suppressed the increase of cell size associated with the *dPTEN* mutation in the wing (Gao et al 2000), suggesting that *Dakt* may function downstream of dPTEN in the cell-autonomous regulation of growth. This conclusion was recapitulated in Dr. Manoukian's previous study (Scanga et al 2000) examining the efficacy of ectopically expressed *Dakt* in suppressing the eye phenotypes associated with the overexpression of *dPTEN* and DN *Dp110* (decrease in cell number, smaller eyes). Accordingly, the ectopic expression of *Dakt* was found to significantly suppress the growth phenotypes associated with the co-expressed *dPTEN* and *Dp110* DN transgenes. These genetic epistasis experiments indicated that *Dakt* acts downstream of both *Dp110* and *dPTEN*.

In the endoreplicating cells of the larval salivary glands, we have demonstrated opposite roles for Dakt (**Figure 2-4, 2-5**) and dPTEN (**Figure 2-7**) in the cell-autonomous regulation of growth. Furthermore, the similarity of the phenotypes associated with *Dakt* and *Vha68-2* mutants, including both organismal and cell-autonomous roles in the positive regulation of growth, presents the possibility that *Vha68-2*, like Dakt, may act downstream of the Dp110/dPTEN signaling junction. As such, considering the ability of decreased Dakt function to suppress growth phenotypes associated with the loss of dPTEN function (**Gao et al 2000**), we examined the growth phenotype of somatic clones doubly mutant for both *dPTEN*^{c494} and *Vha68-2*^{P463} (both located on chromosome 2L), which we induced in the salivary glands of developing heterozygous animals in a manner identical to that described for the single mutants in the previous section. In these *dPTEN/Vha68-2* double mutant cells (**Figure 2-8, 2-9**), the increase in nuclear size observed in *dPTEN* mutant cells (**Figure 2-7**) was effectively suppressed by the concomitant loss of *Vha68-2* function, suggesting that V-ATPase functions epistatically downstream of dPTEN. Furthermore, not only did the concomitant loss of *Vha68-2* function suppress the *dPTEN* deficiency-associated growth phenotype, it further resulted in a growth phenotype resembling that obtained in mutant clones of *Vha68-2* alone, itself a phenotype identical to that observed in *Dakt* mutant clones.

With respect to the dimension of proliferation in the growth of imaginal tissues, experiments carried out in the eye and the wing have uncovered a dPTEN-associated proliferation phenotype in addition to the demonstrated function of dPTEN in the control of cellular size. In the eye, in addition to increased ommatidial cell size, mutant clones of *dPTEN* were found to contain at least twice as many ommatidia as neighboring wildtype twin spots, suggesting that in the absence of dPTEN activity, mutant cells overproliferate (**Goberdhan et al 1999**). Conversely, the decrease in eye size resulting from the overexpression of *dPTEN* has been suggested to result from a proliferation defect in cells of the imaginal eye disc (**Huang et al 1999, Scanga et al 2000**). Furthermore, *dPTEN* mutant clones induced during the 1st instar larval stage and examined in the 3rd instar eye and wing imaginal discs were found to be several times larger than their wildtype twin spots, to contain larger cells, and to contain more cells than their twin spots (**Gao et al 2000**). Importantly, ommatidial composition and identity was not found to be altered in

dPTEN mutant clones in the eye (Goberdhan et al 1999), and differentiation and pattern formation was undisturbed in eyes overexpressing dPTEN (Huang et al 1999). Combined, these experiments suggest that dPTEN cell-autonomously controls cell number as well as cell size in imaginal discs independently of cell differentiation.

In addition to the demonstrated cell-autonomous ability of *Vha68-2*^{P463} to suppress the *dPTEN*^{c494} loss of function-associated growth phenotype in *Vha68-2/dPTEN* mutant clones with respect to nuclear size in endoreplicating cells of the larval salivary gland, we further investigated the ability of *Vha68-2*^{P463} to suppress the *dPTEN* deficiency-associated cell-autonomous proliferation phenotype in *Vha68-2/dPTEN* double mutant clones of the imaginal wing disc. In the proliferating cells of wing discs, cell-autonomous growth defects would be expected to give rise to mutant cells that are unable to proliferate, which are eliminated and replaced by wildtype cells; or to clones that grow more slowly than normal, and therefore contain fewer cells. Conversely, cell autonomous mutations leading to the enhanced proliferative capability of mutant cells would result in clones that grow more rapidly than normal, and contain more cells. As such, somatic *Vha68-2/dPTEN* clones were induced in 2nd instar larvae, and examined in early 3rd larval instar wing imaginal discs. We found that in the double mutant clones (Figure 2-10C), the concomitant loss of *Vha68-2* function completely suppressed the dramatic increase in proliferation resulting from the loss of dPTEN function observed in similarly generated *dPTEN*^{c494} clones (Figure 2-10B). Furthermore, the resulting double mutant proliferation phenotype was identical to that seen in wing discs containing single mutant clones of *Vha68-2*^{P463} (Figure 2-10A). Combined, these results suggest that *Vha68-2*, like *Dakt*, positively and cell-autonomously regulates growth, genetically interacts with dPTEN in growth signaling, and acts epistatically downstream of dPTEN activity in both endoreplicating tissues of the salivary glands as well as in proliferating cells of the imaginal wing discs of the developing *Drosophila* larva.

2-3.6 – Bafilomycin inhibits *Dakt* phosphorylation independently of PI3K activity

Whole-cell lysates of phenotypically wildtype larvae transgenically expressing HA-tagged bovine Akt allowed us to examine the phosphorylation status of the Akt T-

loop (T308 in Akt1, T342 in Dakt), for which a *Drosophila*-specific antibody is not commercially available (**Section 2-3.3**). Our demonstration that both T-loop and HM phosphorylation of Akt is downregulated in *Vha68-2* mutants or in larval tissues of Akt-HA-expressing transgenic mutants was consistent with our genetic experiments examining the loss *Vha68-2* function in cells of the salivary gland or the wing disc, which revealed a similarity between Dakt and *Vha68-2* deficiency phenotypes with respect to cell-autonomous endoreplicative or mitotic growth, and detected a genetic interaction that placed *Vha68-2*, like *Dakt*, epistatically downstream of *dPTEN* in ISP-dependent growth signaling. Having the Akt-HA-expressing line at our disposal also presented us with the opportunity to examine the status of HM phosphorylation (S473 in Akt1) by immunofluorescence confocal microscopy, for which (unlike S505 of Dakt) a monoclonal antibody optimized for microscopy is commercially available.

As described for the experiments in **Section 2-3.3**, Akt-HA-expressing larval tissues were incubated for 12 hours in insect media containing 5% fetal bovine serum (FBS) as either vehicle-treated controls, or samples supplemented with bafilomycin, insulin, or a combination of both molecules. In vehicle-treated (5% FBS) samples (**Figure 2-11A,B,C**), phospho-S473 fluorescence (in green) is most clearly visible in the cellular boundaries delineating the plasma membrane and its associated structures, and intracellular areas corresponding to the nucleus and perinuclear region; but also clearly detectable throughout the cytoplasm in a punctuate pattern. The subcellular enrichment of phospho-S473 at the membrane and nuclei of vehicle-treated cells is recapitulated in an exaggerated form in response to insulin supplementation (**Figure 2-11D,E,F**), resulting in the enhanced fluorescence of membrane-associated and nuclear pools of phospho-S473, as well as an increase in the intensity of the cytoplasmic punctate pattern. Conversely, supplementation with bafilomycin over the same period of time results in the opposite effect (**Figure 2-11G,H,I**), whereby a wholesale decrease in phospho-S473 fluorescence was observed, including the loss of phospho-S473 immunofluorescence at the cellular boundaries, and its complete absence in nuclei, resulting in “pits” devoid of fluorescence. When these opposing effects are combined through the simultaneous treatment of the larval tissues with both bafilomycin *and* insulin (**Figure 2-11J,K,L**), the cytoplasmic signal was not noticeably altered in comparison to vehicle-treated or insulin-

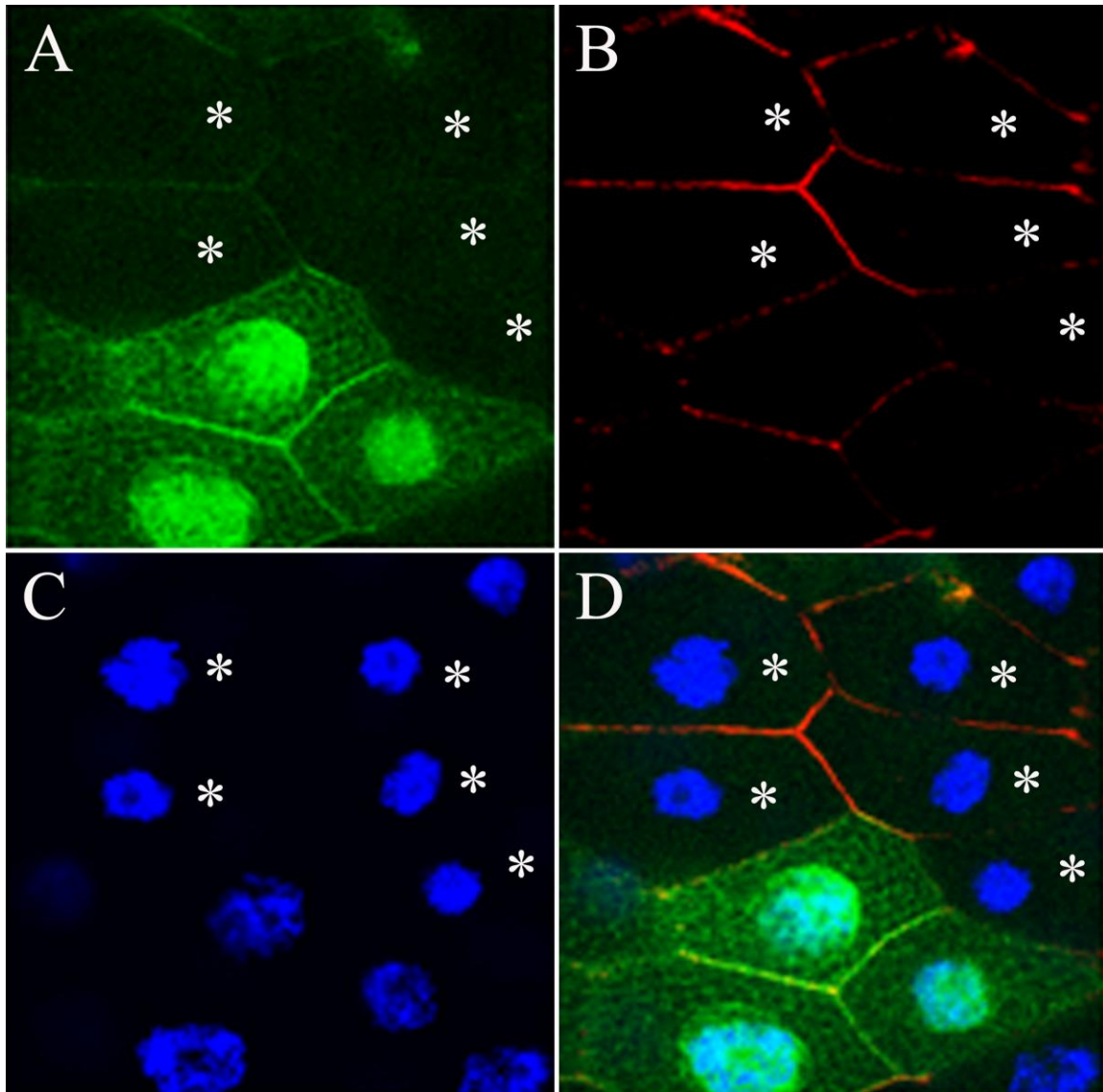


Figure 2-9. Suppression of the *dPTEN*^{c494} growth phenotype in *dPTEN/Vha68-2* double mutant clones in the salivary gland. As in **Figure 2-8**, (A) is the GFP fluorescence of salivary gland cells. The GFP-negative double mutant clones are marked (*), and are located above a patch of GFP-expressing phenotypically-wildtype neighboring cells. (B) The red signal is phalloidin fluorescence. (C) DNA is stained blue with DAPI in all cells, and in *dPTEN/Vha68-2* double mutant clones (*), the *dPTEN* mutant size phenotype was consistently suppressed, resulting instead in a reduction in nuclear size resembling the phenotype seen in *Dakt* or *Vha68-2* single mutant clones. (D) Merged view of all three signals.

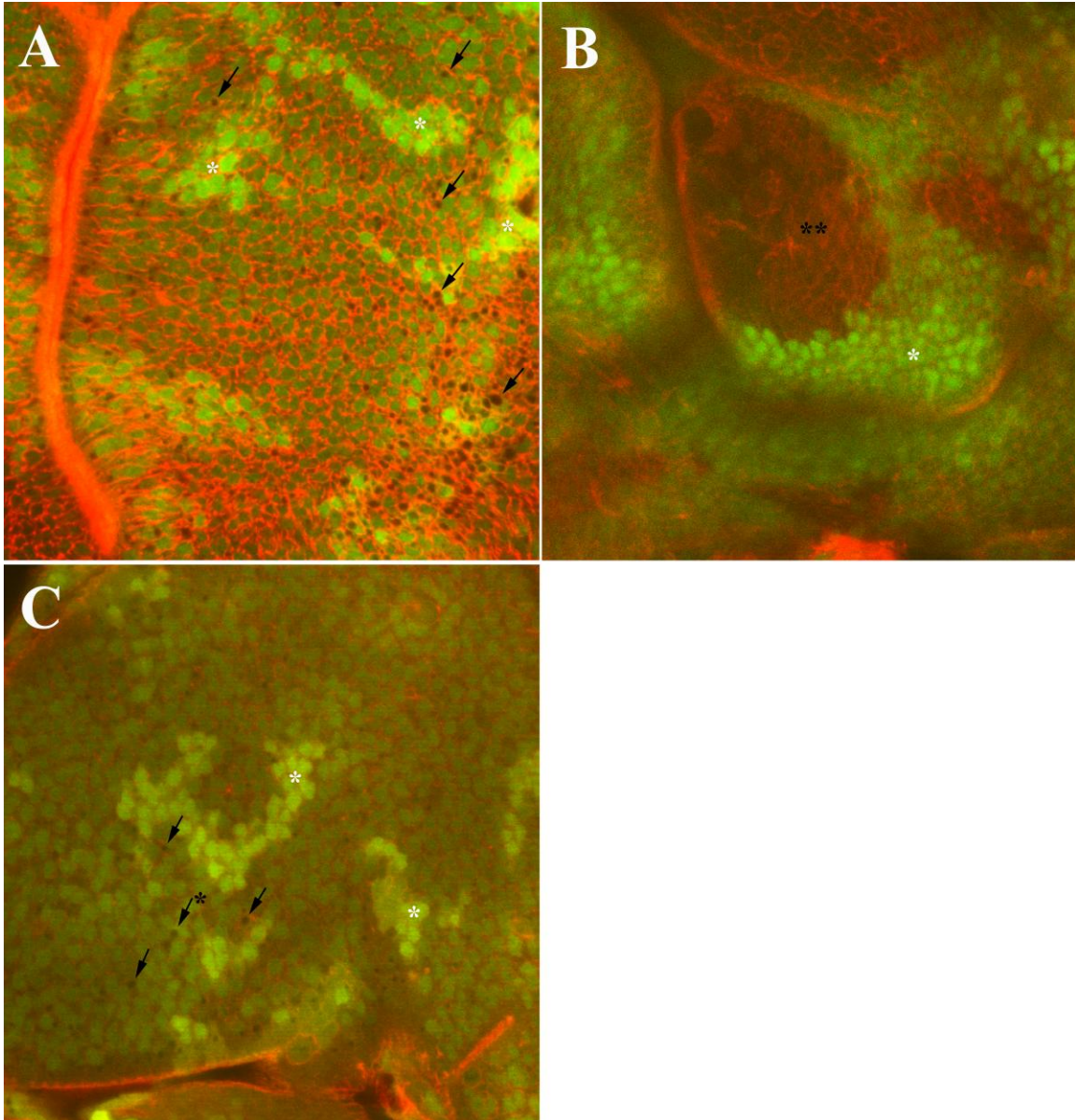


Figure 2-10. Suppression of the *dPTEN*^{c494} proliferation phenotype in double mutant *dPTEN/Vha68-2* clones in the imaginal wing disc. The results shown here are representative of >20 somatic clones examined over the course of four experimental repetitions. Somatic clones of homozygous (A) *Vha68-2*^{P463} and (B) *dPTEN*^{c494} single mutants, or (C) *dPTEN*^{c494}/*Vha68-2*^{P463} double mutants, were induced in late L1 heterozygous larvae by heat shock, and the resulting cell-autonomous proliferation phenotype was examined in early L3 imaginal wing discs. In (A), examples of *Vha68-2*^{P463} homozygous mutant clones (GFP-negative) are indicated by the black arrows. The mutant clones were generally smaller than GFP-positive wildtype neighbors, and failed to proliferate in comparison to patches of wildtype twinspot mitotic sister clones (+;GFP/+;GFP), examples of which are designated by white asterisks. Conversely, GFP-negative *dPTEN*^{c494} homozygous mutant clones shown in (B) were hyperproliferated. In particular, the large patch of highly proliferative mutant clones

marked with two black asterisks can be directly compared to clones of normally proliferating wildtype twinspots directly adjacent and marked with a white asterisk. This *dPTEN* loss of function-associated proliferative phenotype was suppressed in *dPTEN*^{c494}/*Vha68-2*^{P463} double mutants (C), as homozygous double mutant clones (marked by black arrows), similar to the *Vha68-2*^{P463} single mutants in (A), failed to proliferate. In contrast to single *dPTEN* mutant clones which hyperproliferate to occupy a sizeable portion of the wing disc, most detectable *dPTEN*^{c494}/*Vha68-2*^{P463} double mutant clones were single cells, with the exception of the clone marked by a black asterisk which may have undergone a single round of cell division.

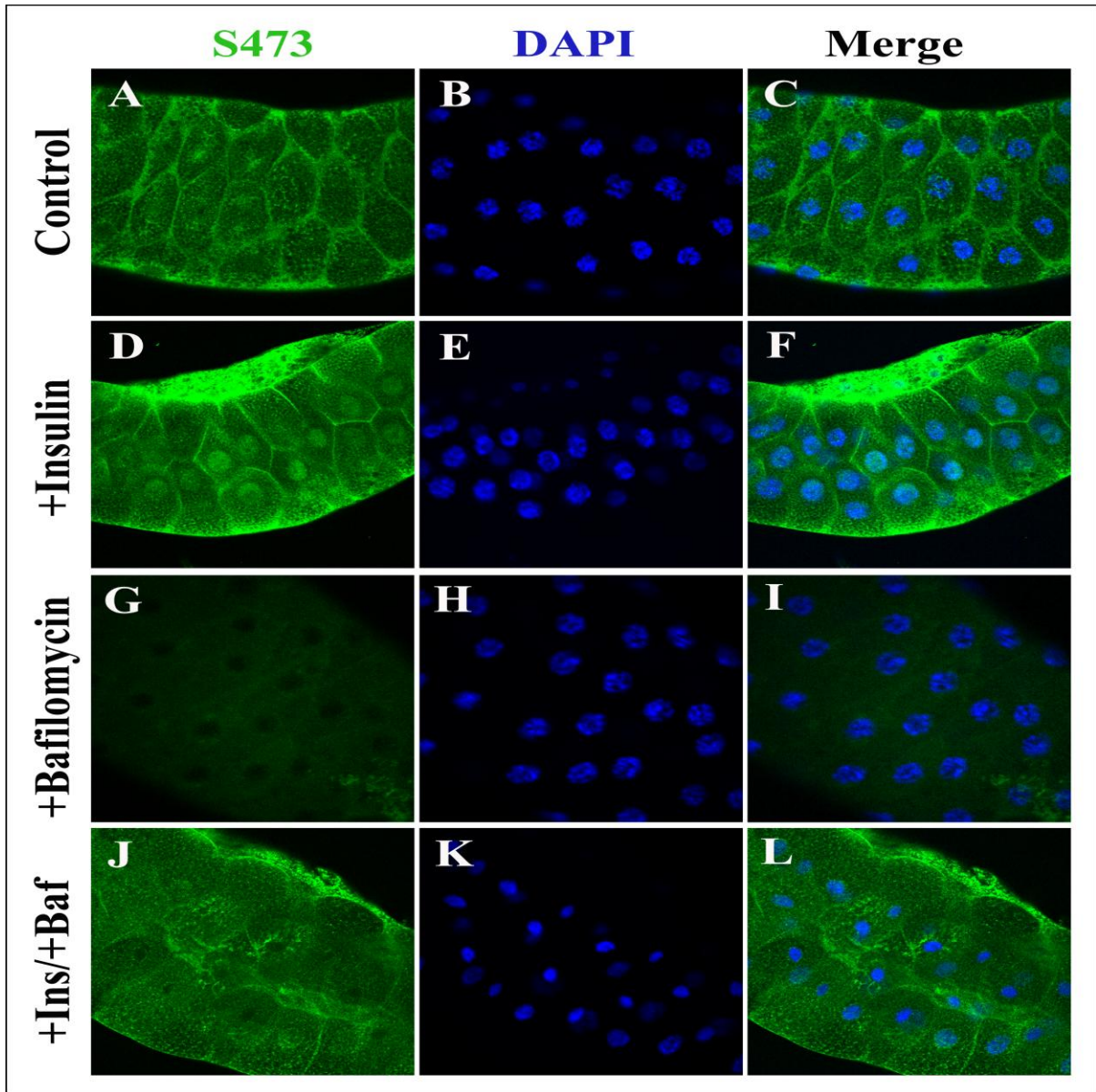


Figure 2-11. Opposite effects of insulin and bafilomycin treatment on the intracellular abundance and distribution of Akt phosphorylated at S473. The results shown here are representative of three experimental repetitions. Dissected tissues of L3 larvae transgenically expressing haemagglutinin (HA)-tagged bovine Akt under the control of an *armadillo* driver (*arm-GAL4/UAS-Akt-HA*) were incubated in insect media (5% FBS) supplemented with either 0.2U/mL insulin, 500nM bafilomycin, or a combination of both, for 12 hours. Following the incubation period, the salivary glands (SG) were dissected and analyzed by confocal microscopy for mammalian phospho-Akt (S473) immunofluorescence (green) in control vehicle-treated SGs (**A-C**), 0.2U/mL insulin-treated SGs (**D-F**), 500nM bafilomycin-treated SGs (**G-I**), and (**J-L**) SGs treated with a combination of both molecules (0.2U/mL insulin/500nM bafilomycin). Panels **B**, **E**, **H**, and **K** are the DAPI nuclear signals (blue) for each treatment. The merged signals from both S473 and DAPI fluorescence are shown in **C**, **F**, **I**, and **L**.

treated cells. However, the effects of the combined treatment with insulin and bafilomycin on the abundance and localization of phospho-S473 with respect to the membrane and nuclei, where the opposite effects of insulin and bafilomycin are most evident, mimics the fluorescence pattern seen in response to bafilomycin treatment as suggested by the diminished abundance of phospho-S473 at the membrane boundaries, and the absence of phospho-S473 immunofluorescence in the “pitted” nuclei of the doubly-treated cells. These immunofluorescence effects suggest that the downregulation of Akt phosphorylation in response to V-ATPase inhibition occurs predominantly at signaling membranes (the established sites of PI3K-dependent activation), and that the loss of V-ATPase function prevents the insulin-induced nuclear localization of activated Akt.

Considering our demonstration that Vha68-2 acts downstream of dPTEN in the ISP, and that the loss of its function mimics Akt-deficiency irrespective of dPTEN function in larval tissues, we set out to determine whether the effect on phospho-S473 observed in salivary glands in response to V-ATPase inhibition was similarly independent of PI3K-mediated PIP₃ production in response to DIR activation. As such, Akt-HA expressing larval tissues were once again incubated with insulin alone, or with a combination of insulin and either bafilomycin or concanamycin A, and the salivary glands were dissected and analyzed by confocal microscopy for total Akt-HA, phospho-S473 (as controls for the occurrence of the previously demonstrated effect of V-ATPase inhibition on Akt phosphorylation and distribution) and PIP₃ immunofluorescence. In insulin-treated samples, Akt immunofluorescence (detected with an anti-HA primary antibody) was most prominent at the cellular boundaries delineating the plasma membranes, and throughout the cytoplasm in a punctate pattern (**Figure 2-12A**). In response to bafilomycin or concanamycin treatment, the fluorescence pattern of total Akt at the plasma membranes which clearly delineates the cellular perimeter in control samples was somewhat obfuscated (boundaries remained clearly discernable but in a more fragmented pattern), although overall levels of Akt were undiminished (**Figure 2-12B,C**). Immunofluorescence of phospho-S473 in insulin-treated samples resembled that of HA (Akt), with clear enrichment at the plasma membrane (**Figure 2-12D**). Treatment with either bafilomycin or concanamycin, as previously described (**Section 2-3.3**),

resulted in a decrease in S473 immunofluorescence at the plasma membrane (**Figure 8E,F**). In contrast to the effect on Akt localization and phospho-S473 phosphorylation levels, the abundance and subcellular distribution of PIP₃ immunofluorescence was unchanged in bafilomycin or concanamycin-treated samples (**Figure 2-12H,I**) when compared to insulin-treated controls (**Figure 2-12G**), suggesting that insulin-induced PI3K activation is unaffected by V-ATPase inhibition, and that the contribution of V-ATPase activity to the regulation of Akt occurs downstream of *both* PI3K and PTEN.

Furthermore, the independence of the bafilomycin-mediated inhibition of Akt activation from PIP₃ abundance was recapitulated in the confocal fluorescence microscopy analysis of the salivary glands of larvae transgenically and ubiquitously expressing tGPH, a GFP/PH-domain fusion protein under the control of the *tubulin* promoter (**Britton et al 2002**), which can be used as an indicator of PH domain-mediated PIP₃ binding. In these cells, neither tGPH fluorescence intensity nor tGPH subcellular localization were altered by bafilomycin treatment (**Figure 2-13A,B**), as a strong fluorescence signal could be observed at the plasma membrane, the cytoplasmic surface of endomembranes, and the perinuclear surface, further suggesting that the observed effect on Akt phosphorylation in response to bafilomycin-induced V-ATPase inhibition cannot be accounted for by defects in the PH domain-mediated association of Akt (or PDK1, and other PH domain-encoding lipid-binding molecules) with PIP₃ at the membrane during its translocation-dependent activation process. Interestingly however, the examination of tGPH expressing salivary glands did clearly reveal a significant alteration in the intracellular organization of endomembranes. In samples treated solely with insulin, a densely-packed honeycomb pattern of endomembranes was observed throughout the cellular interior stretching from the inner surface of the plasma membrane to the perinuclear surface (**Figure 2-13A,A'**). Conversely, salivary glands treated with a combination of insulin and bafilomycin appeared “degranulated” due to the diminished abundance and organization of transitory endomembranes, coupled with the conspicuous swelling of what are likely lysosomes or autophagosomes (**Figure 2-13B, B'**, see Discussion). These results suggest that the mechanism of bafilomycin-dependent inhibition of Akt phosphorylation and activity may involve the formation, maintenance, and maturation of various intracellular endomembranes (such as endosomes, MVBs, and

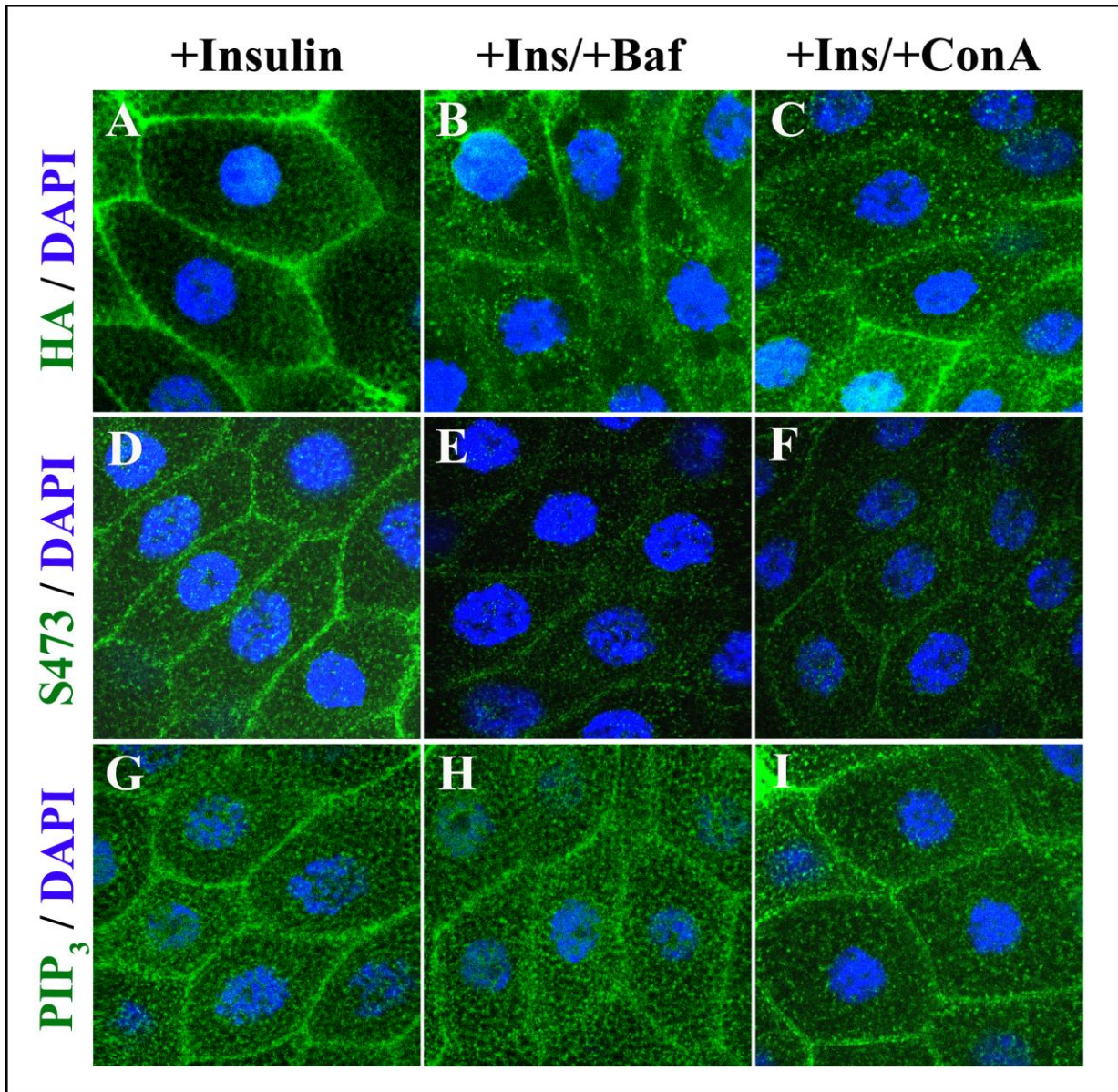


Figure 2-12. Pharmacological inhibition of V-ATPase activity downregulates Akt phosphorylation independently of PI3K activity. The results shown here are representative of three experimental repetitions. Dissected tissues of L3 larvae transgenically expressing haemagglutinin (HA)-tagged bovine Akt under the control of an *armadillo* driver (*arm-GAL4/UAS-Akt-HA*) were incubated in insect media (5% FBS and 0.2U/mL insulin) in control samples (**A**, **D**, and **G**), and supplemented with either 500nM bafilomycin (**B**, **E**, and **H**) or 500nM concanamycin A (**C**, **F**, and **I**), for 12 hours. Following the incubation period, the salivary glands were analyzed by confocal microscopy for: (**A-C**) the distribution of total Akt-HA detected by HA immunofluorescence (green); (**D-F**) the distribution and abundance of mammalian phosphorylated (S473) Akt immunofluorescence (green); and (**G-I**) the distribution and abundance of PIP₃ immunofluorescence (green). Nuclei are stained blue with DAPI in all panels.

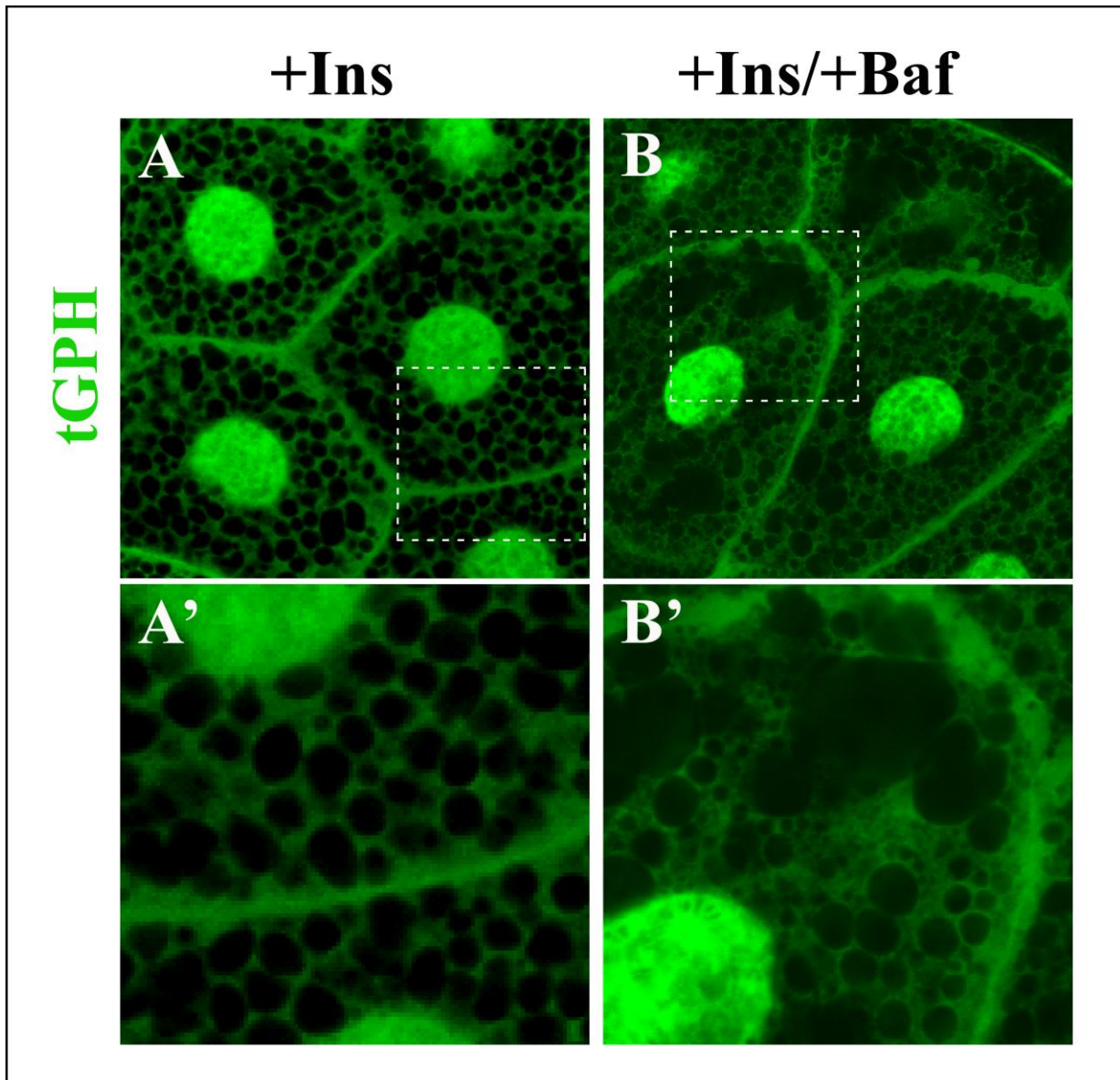


Figure 2-13. Inhibition of V-ATPase activity with bafilomycin causes degranulation and lysosomal/autophagosomal swelling in cells of the salivary gland. The results shown here are representative of two experimental repetitions. Dissected tissues of wildtype 3rd instar larvae transgenically expressing a GFP-PH domain fusion protein (GPH) under the control of a β -*tubulin* driver (tubulin-GPH, or tGPH) were incubated in insect media (5% FBS and 0.2U/mL insulin) for control samples (**A**, **A'**), or supplemented with 500nM bafilomycin (**B**, **B'**), for 12 hours. Following the incubation period, the salivary glands were analyzed by confocal microscopy for tGPH fluorescence. The areas demarcated by the dashed boxes in **A** and **B** are shown at higher magnification in **A'** and **B'** respectively.

lysosomes) which serve crucial roles in ISP transduction (see Discussion), and that the V-ATPase-dependent acidification of these endomembranes may be required for the execution of cellular processes requiring Akt activation and/or nuclear translocation.

2-3.7 – V-ATPase activity cell-autonomously promotes intracellular acidification

The experiments described in the previous section suggest that whereas insulin *stimulates* PI3K-dependent Akt activation (which can be opposed by PTEN), the *downregulation* of Akt activation in response to bafilomycin treatment appears to be independent of PI3K activity judging by the unchanged abundance and distribution of PIP₃, and the maintenance of PH domain-mediated membrane recruitment observed in tGPH-expressing cells. However, based on our detection of defects in endomembrane integrity, we speculated that the primary function of V-ATPase as a proton pump, whose activity is crucial for endomembrane-dependent processes, may serve an implicit role in ISP-dependent cellular responses. As such, we set out to extend the seemingly reciprocal effects of insulin stimulation and bafilomycin treatment on Akt activation to the V-ATPase-dependent acidification of endomembranes. First we examined the effects of *Vha68-2* deficiency on intracellular acidification genetically in somatic clones of *Vha68-2*^{P463} mutants which, as previously described (**Section 2-3.4**), were induced embryonically and analyzed in the developing salivary glands of L3 larvae for their ability to incorporate the vital fluorescent acidotropic dye LysoTracker (Molecular Probes), which preferentially accumulates on acidified endomembranes of live cells. We found that *Vha68-2*^{P463} mutant cells incorporate very low levels of the LysoTracker dye in comparison to immediately adjacent phenotypically wildtype cells which display a very strong accumulation of acidified intracellular structures (**Figure 2-14**), indicating a cell-autonomous impairment of *Vha68-2* mutants in the ability to acidify endomembranes.

To test the reciprocity of insulin and bafilomycin in the V-ATPase-dependent process of subcellular acidification, we tested the cellular response of larval salivary glands to either insulin or bafilomycin treatment. Wildtype L3 larvae were dissected, and the harvested larval tissues were incubated overnight (12 hours) in insect medium (5%

FBS) supplemented with either insulin (0.5U/mL) or bafilomycin (500nM). Immediately following the incubation period, the salivary glands were stained with LysoTracker dye, and analyzed by confocal fluorescence microscopy. Insulin treatment was found to result in a significant incorporation of the LysoTracker dye (**Figure 2-15C**) in comparison to moderately fluorescing control (5% FBS) samples (**Figure 2-15A**), suggesting a dramatic induction of subcellular acidification in response to insulin stimulation. Conversely, bafilomycin treatment was found to severely inhibit the acidification of endomembranes, resulting in salivary glands with very low levels of dye incorporation (**Figure 2-15B**), thereby suggesting a correlation between insulin-mediated stimulation of intracellular acidification and concomitant Akt activation, as well as conversely, between the V-ATPase inhibition-dependent loss of endomembrane acidification and the concomitant downregulation of Akt phosphorylation.

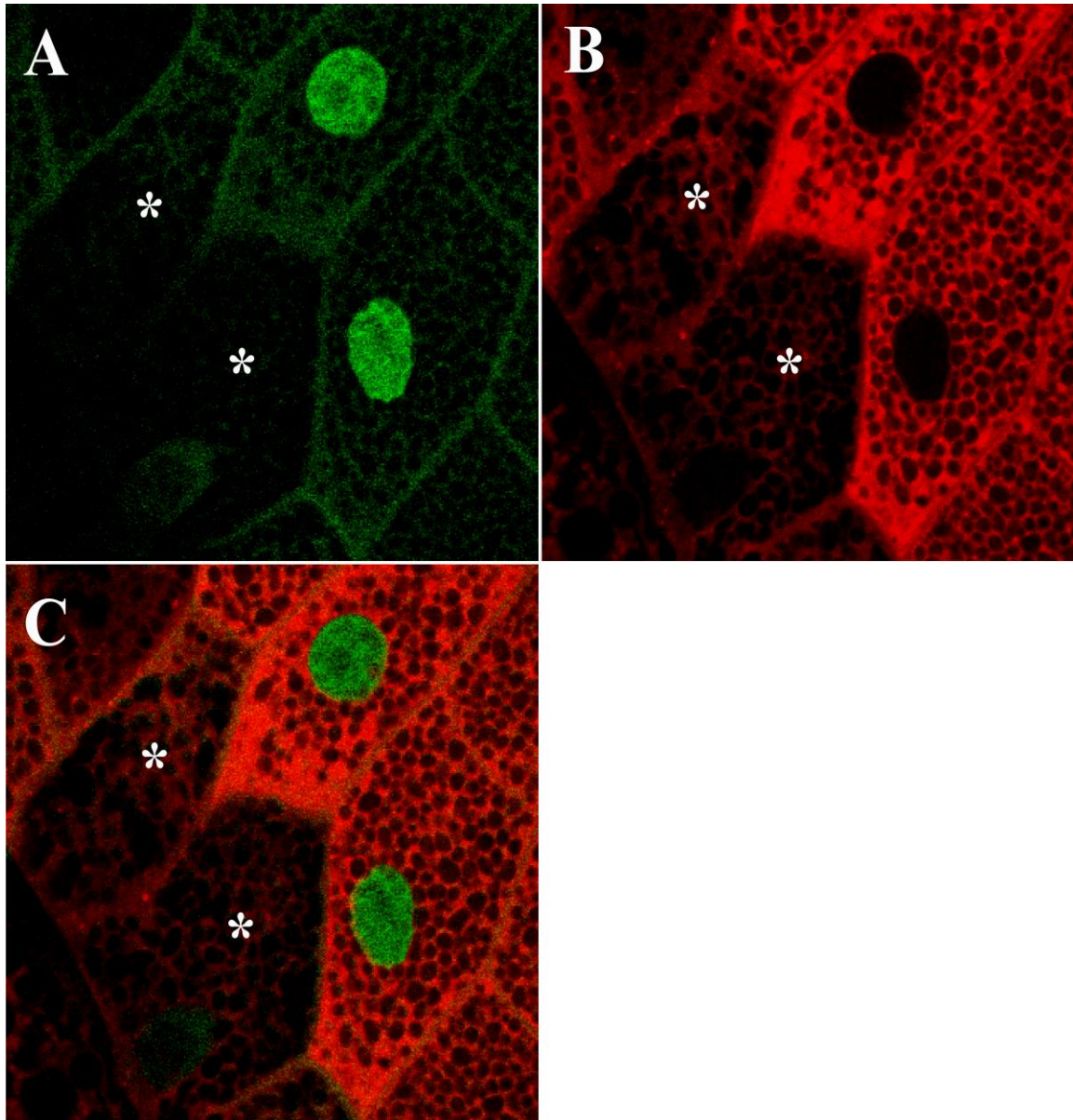


Figure 2-14. Vha68-2 cell autonomously regulates intracellular acidification. The results shown here are representative of >20 somatic clones examined over the course of five experimental repetitions. Somatic clones of *Vha68-2*^{P463} mutant cells (*) were induced embryonically by heat shock, and examined for cell-autonomous intracellular acidification defects in live L3 salivary glands. (A) GFP fluorescence indicated the presence of two somatic mutant GFP-negative cells, with phenotypically wildtype neighbors (GFP positive) directly adjacent. LysoTracker dyes are cell membrane permeable fluorescent acidotropic probes used for the labeling of acidic organelles in live cells. In (B), with relative fluorescence from the incorporation of LysoTracker Red (Molecular Probes) dye as a measure of acidification, *Vha68-2*^{P463} mutant clones showed a distinct cell-autonomous defect in the intracellular acidification of their endomembranes in comparison to brightly stained GFP-positive wildtype neighboring cells. (C) merged view of both GFP and LysoTracker Red signals.

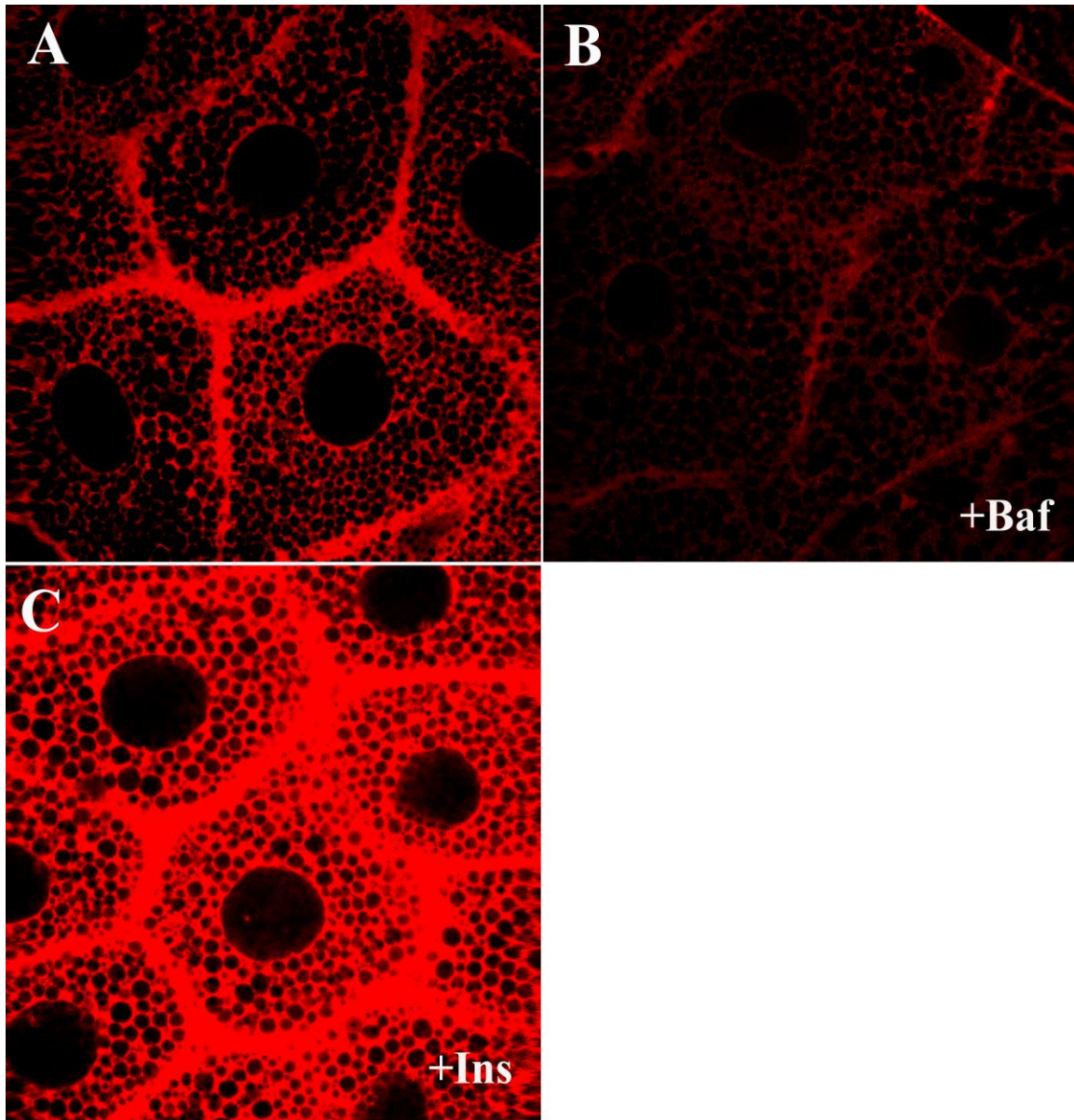


Figure 2-15. Reciprocal effects of insulin stimulation and V-ATPase inhibition on the intracellular acidification of larval salivary glands. The results shown here are representative of three experimental repetitions. Live L3 salivary glands were incubated in insect medium supplemented with 5% FBS and treated for 12 hours with 500nM Bafilomycin, 0.2U/mL insulin, or vehicle control. (A) Staining of vehicle-treated salivary glands with LysoTracker Red (Molecular Probes) was used as a standard for the basal level of cellular acidification, and showed a moderate level of dye incorporation as it correlates to endomembrane acidification. (B) Treatment of salivary glands with bafilomycin resulted in a drastic decrease in LysoTracker dye incorporation and fluorescence, indicative of a decrease in intracellular endomembrane acidification in comparison to vehicle treated controls. Conversely, incubation of salivary glands with insulin (C) resulted in a marked increase in the abundance of acidified endomembranes compared to vehicle-treated control samples.

2-4 – Discussion

In *Drosophila*, the genetic relationship between Dakt and the upstream modulators of its activity was genetically investigated in previously published studies (Huang et al 1999, Gao et al 2000), including Dr. Manoukian's (Scanga et al 2000). These experiments placed the Dakt molecule epistatically downstream of the combined antagonistic activities of Dp110 and dPTEN. The similarity of the organismal (Figure 2-1) and cell-autonomous (Figures 2-4, 2-5, 2-6) phenotypes observed in *Dakt* and *Vha68-2* mutants prompted our investigation of a possible genetic interaction between *Vha68-2* and *dPTEN*, and the P463 mutant allele of *Vha68-2* was demonstrated to suppress the dPTEN-deficiency overgrowth phenotype associated with c494 mutant clones induced in both endoreplicating (salivary gland) or proliferative (wing disc) larval cells (Figures 2-7, 2-8, 2-9, 2-10), suggesting that *Vha68-2*, like *Dakt*, may also act epistatically downstream of *dPTEN*. The ISP-dependent activation of Dakt in response to PI3K activation and increased plasma membrane PIP₃ abundance is mediated by its PH domain-dependent membrane localization. This process is enhanced or prolonged when dPTEN function is lost and the catalytic activity of Dp110 is unopposed, resulting in constitutively elevated levels of PIP₃, and consequently, ectopic Dakt activation. Importantly, the suppression of the cell-autonomous *dPTEN* mutant phenotype we observe in *Vha68-2/dPTEN* double mutant clones, in which the growth defect is reminiscent of that observed in *Dakt* mutants, occurs despite the presence of endogenous Dakt at normal levels. This suggests that sustained high levels of PIP₃ at the membrane caused by the loss of dPTEN function, which typically hyperactivates Dakt, is incapable of doing so in the concomitant absence of V-ATPase activity irrespective of the stimulatory effect of PIP₃ accumulation in dPTEN-deficient cells.

A signaling paradigm in which *Vha68-2* acts downstream of dPTEN to suppress dPTEN loss of function-dependent phenotypes, and upstream of (or parallel to) Dakt, whereby the loss of *Vha68-2* function mimics the loss of Dakt function in its ability to suppress cell-autonomous *dPTEN* mutant growth phenotypes implies that ectopic activation of Dakt is the main pathological consequence of the loss of dPTEN function. This was proven to be the case in an elegant series of experiments conducted by Ernst

Hafen and his colleagues (Stocker et al 2002), who demonstrated that early larval lethal *dPTEN*-null mutants (*dPTEN*^{dj189} and *dPTEN*¹¹⁷) could be rescued to viability by any allelic combination that included the viable hypomorphic *Dakt*³ allele (Stocker et al 2002, see Table 2-2 and Figure 2-1). In their study, neither a reduction in *Dakt* expression using the *Dakt*⁴²²⁶ allele (Spradling et al 1999), which contains a P-element insertion upstream of the *Dakt* gene that results in the reduced transcription of wildtype *Dakt* (Gao et al 2000), nor catalytic inactivation of Dakt activity using the loss-of-function *Dakt*¹ allele (Staveley et al 1998) was capable of rescuing *dPTEN* loss of function-associated lethality. The viable 4226 allele was incapable of rescuing the *dPTEN* mutant lethality due to maintained hyperactivation of the reduced pool of wildtype Dakt, whereas the kinase dead *Dakt* allele (*Dakt*¹) did not rescue lethality in *dPTEN* mutants because Dakt function is itself indispensable for organismal survival (Staveley et al 1998), even in the absence of *dPTEN* function. As mentioned above however, the viable *Dakt*³ allele, which results in mutant flies with severely reduced body size, can rescue the lethality of *dPTEN* loss of function mutants. This hypomorphic allele of *Dakt* bears a point mutation (G99S) in its PH domain, selectively impairing the membrane recruitment of Dakt in response to increased PIP₃ concentrations. In contrast, with both 4226 and *Dakt*¹ mutants, the endogenous Dakt molecule (wildtype in the 4226 mutant, kinase-dead in *Akt*¹) was found to be effectively recruited to the plasma membrane in response to insulin stimulation. The rescued *dPTEN/Dakt*³ mutant flies did not display any morphological defects associated with clones of *dPTEN* mutant cells, and furthermore, no abnormalities were detected in the structure of adult eyes and wings, despite elevated PIP₃ concentrations (Stocker et al 2002). Combined these experiments demonstrated that during *Drosophila* larval development, which requires ISP signaling, Dakt is the most critical target of PI3K-dependent increases of PIP₃ at the membrane, and that the developmental lethality associated with the ectopic activation of Dakt in *dPTEN*-deficient larvae can be remedied by diminishing PH domain-dependent Dakt membrane recruitment, whereas in contrast, neither the loss of Akt function nor the diminished expression of the molecule can similarly rescue *dPTEN* mutants to viability. Therefore, if the activation of Dakt is the only crucial outcome of the loss of *dPTEN* function in the regulation of growth and survival, constitutive Dakt activation would presumably mimic

dPTEN deficiency phenotypes. Accordingly, constitutively activated membrane-anchored Dakt (**Andjelković et al 1997**) expressed in the developing eye resulted in increased size due to enlarged ommatidia, a phenotype nearly identical to the overgrowth associated with clones of *dPTEN* mutant cells (**Stocker et al 2002**).

Whereas the capacity of *Dakt*³ to rescue phenotypes associated with *dPTEN*-deficiency can be directly correlated to an intrinsic defect in the mutant molecule's capability to bind PIP₃ at the membrane, the ability of *Vha68-2*^{P463} to (1) suppress the overgrowth phenotype of *dPTEN* mutant clones; and furthermore, (2) to mimic the loss of Dakt function despite elevated levels of PIP₃ and the presence of wildtype Dakt at normal levels, likely results from the disruption of multiple signaling processes required for both (1) the efficient activation of Dakt at endomembranes and its translocation to important subcellular locales such as the nucleus, as well as (2) the proper function of signaling molecules acting downstream of (or parallel to) Dakt activation in the cell-autonomous regulation of cellular growth, including the dTOR-regulated nutrient sensing module. As previously mentioned, dTOR is required for normal growth in *Drosophila* (**Oldham et al 2000, Zhang et al 2000**), and the loss of its function phenocopies the growth phenotypes of ISP mutants as well as nutrient starvation (**Zhang et al 2000**). Furthermore, the expression of both Dilp3 and Dilp5, as well as the activity of PI3K itself, have been shown to be regulated by nutrient availability (**Ikeya et al 2002, Britton et al 2002**); and moreover, as described in the introductory chapter (**Section 1-4.5**), V-ATPase was recently identified as a positive regulator of nutrient-dependent mTORC1 activation (**Zoncu et al 2011a**), whereby its inhibition in cultured HEK-293T cells acutely treated with high concentrations of concanamycin A was found to concentration-dependently diminish *nutrient*-mediated mTORC1-dependent S6K1 phosphorylation, at concentrations (>2μM) and over a treatment period (<1 hour) that did not alter Akt activity. These observations suggest that the diminished TORC1 activity that results from V-ATPase inhibition, which acutely mimics nutrient starvation, may combine with the starvation-induced downregulation of PI3K signaling and contribute to the growth phenotype associated with V-ATPase deficiency. However, our own experiments demonstrate that in intact larval salivary glands incubated for a longer period of time (12

hours) in the presence of both insulin and bafilomycin (or concanamycin), the phosphorylation of Akt is diminished in comparison to tissues treated with insulin alone, and importantly, that the abundance and distribution of PIP₃ was unchanged in the presence of the V-ATPase inhibitors (**Figure 2-12**). Furthermore, whereas the treatment of cultured HEK-293T cells with relatively high concentrations (>2μM) of concanamycin over a relatively short treatment period (<1 hour) resulted in mTORC1 inhibition without impinging on Akt activity (**Zoncu et al 2011a**), our prolonged (12 hour) treatment of salivary glands with concentrations of concanamycin A (500nM) that do not appreciably alter mTORC1 (or Akt) activity over a short (12-50min) treatment period (**Zoncu et al 2011a**) resulted in a clear decrease in Akt activation (**Figure 2-12**). Moreover, the downregulation of Akt phosphorylation was also demonstrated to occur in *Vha68-2* mutants, as well as whole-cell lysates of larval tissues incubated with V-ATPase inhibitors (**Figures 2-2, 2-3**).

Although our findings do not conflict with the proposed role for V-ATPase as a nutrient-dependent activator of TORC1 in the regulation of cell-autonomous growth, they strongly suggest that V-ATPase may also serve an important function in the promotion of Akt activation in the ISP independently of its requirement for nutrient based TORC1 activation (**Diagram 2-13**), which is consistent with the demonstration in *Drosophila* that ERTs overexpressing *DIR*, *Dp110* (**Britton et al 2002**), or even *dRheb* (**Saucedo et al 2003**), continue to endoreplicate and grow *even in the absence of nutrients* (**Diagram 2-9**). Our genetic epistasis experiments did not include an examination of *dPTEN/dTOR* double mutant clones, whose examination in the salivary gland could clarify the contribution of V-ATPase activity in the regulation of cell-autonomous growth. Like *dPTEN* and *Vha68-2*, *dTOR* is also located on the left arm of chromosome 2 (L2), making it possible to generate mitotic recombinant *dPTEN/dTOR* double mutants in the same manner as that used to generate *dPTEN/Vha68-2* double mutant clones in the salivary glands and the wing discs of developing larvae. If the loss of V-ATPase function suppresses the dPTEN deficiency-associated overgrowth phenotype by predominantly inhibiting the AA-dependent lysosomal recruitment of colocalization of TORC1 with dRheb, then the loss of dTOR function in the same dPTEN-deficient backgrounds should be similarly capable of suppressing the dPTEN overgrowth phenotype. However, if on

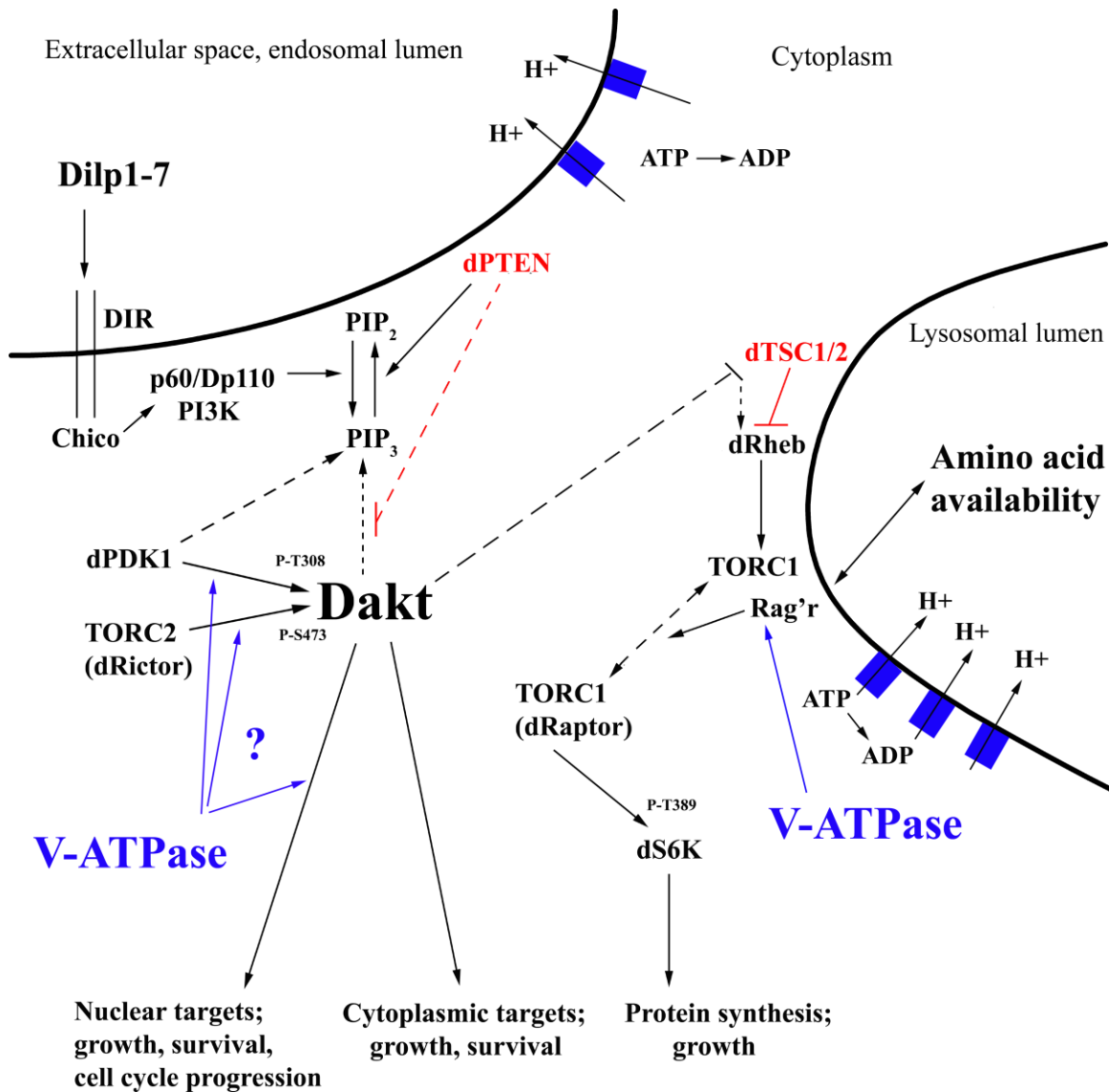


Diagram 2-13. Role of V-ATPase activity in the promotion of ISP-mediated growth signaling. V-ATPase activity participates in TORC1 activation by promoting Ragulator-mediated TORC1 recruitment to the lysosomal surface when amino acid abundance is plentiful. This nutrient-dependent role in Ragulator activation only requires ATPase activity, and is independent of V-ATPase-mediated luminal acidification. Our results suggest that V-ATPase activity also promotes growth factor-mediated Akt activation and nuclear localization, and this effect may correlate with endomembrane acidification in endosomes and MVBs. See text for details.

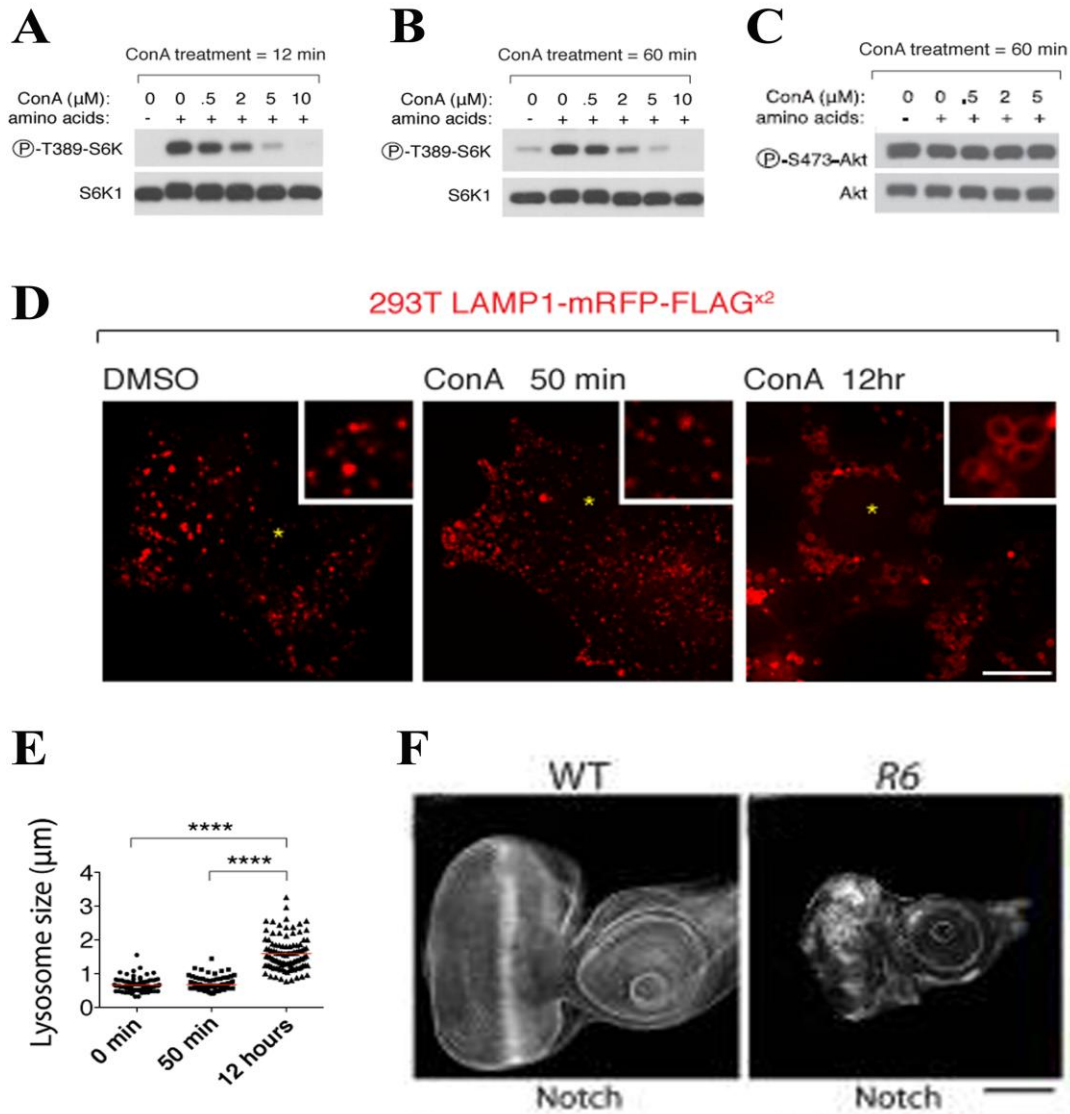


Diagram 2-14. (A-E) Reproduced from Zoncu et al (2011a). Immunoblots of S6K1 (T389) and Akt (S473) HM phosphorylation in cultured HEK-293T cells deprived of amino acids for 50 min and then stimulated for 10 min with amino acids. During the starvation/stimulation period, cells were treated with the indicated concentrations of concanamycin A (ConA) for 12 minutes (A) or 60 minutes (B,C). (D) Representative images from LAMP1-mRFP-FLAGX2-expressing HEK-293T treated with 2.5 μM ConA for the indicated times. Yellow asterisks indicate the position of cell nuclei. Inset shows a higher magnification of a selected field. Red LAMP1 fluorescence is unchanged after 50 minutes of ConA treatment. By 12 hours, the punctate LAMP1 fluorescence pattern is lost, and remaining LAMP1-positive lysosomal structures (inset) are swollen and perinuclear. (E) Measurement of lysosomal diameter in LAMP1-mRFP-FLAGX2 expressing HEK-293T cells that were treated with 2.5 μM ConA for 0 min, 50 min or 12 hrs. (F) Reproduced from Vaccari et al (2010). Eye imaginal discs stained to detect Notch. Compared with wildtype, eye discs consisting predominantly of R6 mutant cells are smaller and show high levels of mislocalized Notch.

the other hand, the loss of V-ATPase function impinges on another important aspect of growth signaling, such as the promotion of Akt activity, the *dPTEN*-deficiency overgrowth phenotype would not be suppressed (or only partially suppressed, depending on the strength of the “secondary” interaction), much as starvation fails to inhibit the overgrowth of *DIR*- or *Dp110*-overexpressing mutant clones in ERTs (**Britton et al 2002**).

The experiments of Zoncu et al (**2011a**) suggested that V-ATPase activity at the lysosomal membrane acted as an AA sensor, promoting Ragulator activation and the recruitment of mTORC1 to the lysosomal surface for Rheb-dependent activation, which is itself under the control of RTK signal transduction. In their study, the inhibition of mTORC1 activity (based on S6K1 HM phosphorylation at T389) was shown to occur (1) following acute concanamycin treatment (12 minutes) in a concentration dependent fashion over a range of 2 μ M to 10 μ M (**Diagram 2-14A**); (2) to be independent of treatment duration over the course of the 12-60 minute time course (**Diagram 2-14B**); and (3) to occur within this time frame without concomitant changes in the levels of Akt HM phosphorylation (**Diagram 2-14C**) or lysosomal morphology (**Diagram 2-14D,E**). Furthermore, its capacity to promote mTORC1 activation was shown to specifically require the ATPase driven rotation of the proton pump *without* a concomitant requirement for lysosomal acidification and the establishment of the proton gradient – accordingly, whereas the inhibition of V-ATPase activity can gradually raise lysosomal pH and reduce lysosomal degradative capacity, lysosomal acidification was dispensable for the effects of acutely administered high concentration of concanamycin on the nutrient-stimulated activation of mTORC1 *in vitro* (**Zoncu et al 2011a**). Importantly however, using a concentration of concanamycin (2.5 μ M) that significantly inhibits mTORC1-dependent S6K1 phosphorylation within 12 min of administration, their investigation of lysosomal morphology following the chronic (12 hour) administration of 2.5 μ M concanamycin – their only experiment examining chronic treatment – revealed that whereas lysosomal morphology is unchanged within the first hour of treatment, a nearly threefold increase in lysosomal size is shown to occur by the twelfth hour of treatment (**Diagram 2-14D,E**), including immunofluorescence data (using LAMP1 as a

lysosomal marker) that is highly reminiscent of our confocal microscopic analysis of tGPH-expressing salivary glands treated with 500nM bafilomycin ($1/5^{\text{th}}$ of the concanamycin concentration used in the study above, **Figure 2-13**), in which a considerable degree of swelling in what are presumably lysosomal (or autophagosomal, see below) structures was detected concomitantly with a cell-wide subversion in the organization and abundance of endomembranes. Therefore, (1) the independence of the activation of mTORC1 from the endomembrane-acidifying function of V-ATPase, (2) the late onset of acidification-dependent endomembrane defects, and (3) the correlation of the GF-mediated role of V-ATPase function in the activation of Akt with the latent loss of endomembrane acidification and integrity; combine to suggest that whereas the maximal inhibition of V-ATPase activity (short treatment window, high inhibitor concentrations) acutely inhibits nutrient-dependent mTORC1 activation, transitory endomembrane compartments that are vulnerable to the chronic inhibition of V-ATPase-dependent acidification (which is time-dependent irrespective of inhibitor concentration) may serve as important locales of ISP-mediated Akt activation.

As alluded to above and in **Section 2-3.6**, the enlarged endomembranes detected in bafilomycin-treated L3 salivary glands (**Figure 2-13**) could also correspond to autophagosomes. Following its discovery and identification as a potent inhibitor of V-ATPase function, bafilomycin A1 (10nM-1 μ M) was shown to inhibit acidification and protein degradation in lysosomes within an hour of treatment in cultured cells (**Yoshimori et al 1991**); to prevent the fusion of autophagosomes and lysosomes in rodent hepatoma cells (**Yamamoto et al 1998**); and at a concentration of 100nM, to result in autophagosome accumulation concomitantly with apoptotic death after 24 hours of treatment in cultured HeLa cells (**Boya et al 2005**). Whereas the larval feeding stages (during which *Drosophila* ERTs grow in size and accumulate biomass while epithelial tissues of imaginal discs proliferate) last 4-5 days, their metamorphosis into adults occurs following the cessation of larval feeding and the initiation of the pupal stage, which like larval development, also occurs over the span of approximately 4 days (**Diagram 2-4**). The biomass (nutrients, biomolecules, and energy) required for successful metamorphosis is appropriated through the programmed autophagic degradation of ERTs such as the fat

body and salivary glands, and the steroid hormone ecdysone defines the length of the developmental period in *Drosophila*, as peaks in its expression initiate larval molting and metamorphosis (reviewed in **Juhász and Neufeld 2008, McPhee and Baehrecke 2009**). At the concluding stages of larval development, autophagy is induced in a number of tissues including the fat body (a major site of nutrient storage akin to the mammalian liver) as a normal physiological response to hormonally communicated cues such as ecdysone, which additionally triggers cell death in the obsolescent larval midgut and salivary glands (**Lee and Baehrecke 2001, Lee et al 2002**). In response to either starvation, or the rise in ecdysone that triggers metamorphosis in late 3rd instar larvae, Dp110 signaling is downregulated in the fat body, and results in autophagy induction (**Rusten et al 2004**). Similarly, growth arrest through the downregulation of Dp110-dependent processes has been demonstrated to be required for the induction of autophagy in salivary gland degradation, while the activation of positive regulators of growth including Dp110 or Dakt inhibit autophagy and the degradation of the salivary glands in a dTOR-dependent manner (**Berry and Baehrecke 2007**), suggesting a role for the TOR-dependent signaling pathway downstream of Dakt in the prevention of autophagy in this context.

V-ATPase activity cell-autonomously regulates intracellular acidification (**Figure 2-14**). In the developmental context of our experiments demonstrating the endomembrane swelling and the loss of acidification in response to V-ATPase inhibition (**Figures 2-13, 2-15**), which were carried out in mid-L3 larvae, the bafilomycin-dependent inhibition of Dakt signaling may in fact mimic the ecdysone-mediated downregulation of Dp110-dependent processes, resulting in the induction of autophagy. Future experiments using lysosomal-specific markers such as LAMP1 (lysosomal-associated membrane protein 1) and autophagosome markers such as LC3 (microtubule-associated protein 1-light chain 3) would allow the distinction between lysosomal swelling and autophagosome generation. Nonetheless, although the induction of autophagy in this developmental context may prove to be an important consequence of V-ATPase inhibition, it is unlikely to be a causal event in the cell-autonomous or organismal regulation of growth, since the induction of autophagy during *Drosophila* larval development is itself a consequence of Dp110/Dakt downregulation.

Lastly, the endosomal compartments of eukaryotic cells have been implicated in the induction of the cellular responses elicited by diverse classes of receptors, and contrary to their initial perception as transitory structures predominantly employed for the termination of receptor-borne signals, they have gained considerable acceptance as hubs of receptor activation-derived signal transduction (reviewed in **Murphy et al 2009, Platta and Stenmark 2011**). In *Drosophila* for example, recent genetic experiments using mutant alleles of V-ATPase components (including the R6 allele of *Vha68-2*) suggest that V-ATPase, through its acidification of early endosomes, is required for Notch receptor signaling (**Vaccari et al 2010, Yan et al 2009**). Notch activation following ligand binding requires two consecutive proteolytic cleavage events, the second of which is catalyzed by γ -secretase, and occurs in endosomes allowing the release of the intracellular domain of the Notch receptor, which then translocates to the nucleus, where it induces its transcriptional responses (**Struhl et al 1993, Rebay et al 1993**, reviewed in **Lai 2004, Schweisguth 2004**). The R6 allele of *Vha68-2* was identified in a screen for altered notch localization in mutant imaginal eye discs, and significantly, in addition to aberrant morphology and compromised epithelial polarity, R6 mutant eye discs were consistently found to be smaller than wildtype discs (**Diagram 2-14F**), suggesting that the R6 mutation disrupts a process that not only regulates Notch trafficking, but also the growth of imaginal disc tissue (**Vaccari et al 2010**). Although the size defect in the R6 mutant eye disc was not addressed in this study, their demonstration of diminished size in the R6 eye discs is consistent with the size defects we have demonstrated at the organismal and cellular levels (**Figures 2-1, 2-6**).

In addition to Notch signaling, whose endosomal activation requires V-ATPase function, the role of endocytic trafficking has been established downstream of a number of morphogenic receptor-based signaling pathways in *Drosophila* development (reviewed in **González-Gaitán 2003, Fischer et al 2006**), including long-range acting morphogens such as decapentaplegic (Dpp), short-range diffusible morphogens like wingless (Wg) and hedgehog (Hh); as well as membrane-bound non-diffusible morphogens such as Boss (bride of sevenless), the ligand of the sevenless (Sev) RTK (**Krämer et al 1991, Cagan et al 1992, Krämer and Phistry 1996**). Interestingly, Sev signaling from the endosomal

compartment (early endosomes and MVBs) following receptor internalization is required for the attainment of the threshold of MAPK signaling that is necessary for the differentiation of the R7 photoreceptor (**Halfar et al 2001**), whose precursors are the only cells in the imaginal eye disc to internalize the Boss ligand during photoreceptor fate determination (**Krämer et al 1991**). In the following chapter, following a brief review of the role of the endomembrane compartment (featuring early endosomes, multivesicular bodies, and late endosomes/lysosomes) in signaling downstream of growth factors such as insulin/IGF1 and EGF in mammalian cells, the results of our experiments examining bafilomycin-dependent changes in the subcellular distribution and activation of ISP signaling components (as well as the subcellular localization of various components of endomembrane traffic) are presented and discussed.

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF BAFILOMYCIN AS AN INHIBITOR OF PKB/AKT SIGNALING IN NIH-3T3 FIBROBLASTS

3-1 – Introduction

Although the plasma membrane (PM) is the initial site of receptor activation in the transfer of information from circulating extracellular factors to the cellular interior, it is not the sole cellular locale of receptor-mediated signal transduction. The traditional conception of RTK signaling throughout the 1980s and early 1990s had assumed that receptor signaling occurred solely at the PM, but this inaccurate preconception was challenged by various studies in the mid-1990s, including subcellular fractionation and co-immunoprecipitation studies that demonstrated the EGF-dependent accumulation of EGFR signaling complexes in early endosomes of liver parenchymal cells (**Di Guglielmo et al 1994**). Similar examinations of insulin-treated adipocytes would reveal that internalized IR complexes were more heavily phosphorylated than those at the PM (**Kublaoui et al 1995**), consistent with a previous study that had demonstrated the preferential activation of PI3K in internal (rather PM-localized) receptor complexes in response to insulin treatment (**Kelly and Ruderman 1993**). Moreover, the signaling potential of endosomes was recapitulated in hepatic tissues downstream of insulin activation (**Bevan et al 1995**), and has since been extended to include the PDGF (**Wang et al 2004**) and VEGF receptors (**Lampugnani et al 2006**) in that tissue. Accordingly, the endocytic network, which directs vesicular traffic between the cellular surface and various intracellular compartments (**Diagram 3-1**), and whose maintenance requires V-ATPase function (see **Section 1-5.2**), has since been established as a *bona fide* contributor in determining signal duration and intensity downstream of diverse classes of cell-surface receptors in various tissues (reviewed in **Seto et al 2002**, **Murphy et al 2009**, **Sadowski et al 2009**, **Platta and Stenmark 2011**).

Following the initial response to extracellular signals, ligand-stimulated RTKs are internalized into endosomes through endocytosis, where they can continue to actively propagate the ligand-dependent signal through their cytoplasmic kinase domains (reviewed in **Miaczynska et al 2004a**, **Hoeller et al 2005**, **Polo and Di Fiore 2006**, **Sorkin and von Zastrow 2009**, **Sadowsky et al 2009**). As shown in **Diagram 3-2**, following ligand binding and receptor activation, RTKs (as well as GPCRs) are localized from the PM to early endosomes (endocytic vesicles), and can enter the endocytic

network through two main mechanisms – clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE), after which they are directed to either late endosomes/multivesicular bodies (MVBs), and subsequently to lysosomes for degradation; or alternatively, rerouted back to the PM through recycling endosomes and the exocytic pathway (reviewed in **Le Roy and Wrana 2005**). As such, endocytosis and progression through the increasingly acidic compartments of the endocytic network, during which the RTK may become dephosphorylated, ubiquitinated, and dissociated from its ligand, can on the one hand lead to receptor complex degradation and RTK signal attenuation; while alternatively, its recycling to the PM can increase the overall availability of RTKs at the cellular surface, thereby prolonging or intensifying the RTK-dependent signal. Accordingly, the balance of the signal terminating and prolonging influences of receptor endocytosis significantly contribute to both the strength and the duration of receptor-borne signals, and provide an important layer of tissue- and context-specific differential regulation of signaling downstream of particular receptors.

For a number of RTKs, the mode of internalization can impact the signal transduction process with respect to both the outcome and the extent of the subsequent cellular response. Independently of CME, some receptors can be internalized by PM invaginations called caveolae (reviewed in **Parton and Simons 2007**), which occur at PM lipid rafts ([Diagram 3-2](#)), and in the case of the EGF and PDGF receptors, have been proposed to sequester the receptor complex, thereby preventing their overactivation (**Matveev and Smart 2002**). The transforming growth factor β (TGF β) receptors can be internalized via both CME and caveolae, with the former leading to activation of downstream effectors from early endosomes, while the latter leads to the rapid degradation of the receptor complex and signal termination (**Di Guglielmo et al 2003**). Furthermore, the preferred pathway of internalization can be influenced by ligand abundance as in the case of EGF, whereby low levels of the ligand result in CME-mediated internalization, leading to increased recycling of the receptor complex and prolonged signaling; while high doses of EGF result in the additional promotion of CIE-mediated internalization, which enhances receptor degradation rates (**Sigismund et al 2005, Sigismund et al 2008**). Similarly, low PDGF concentrations result in CME-

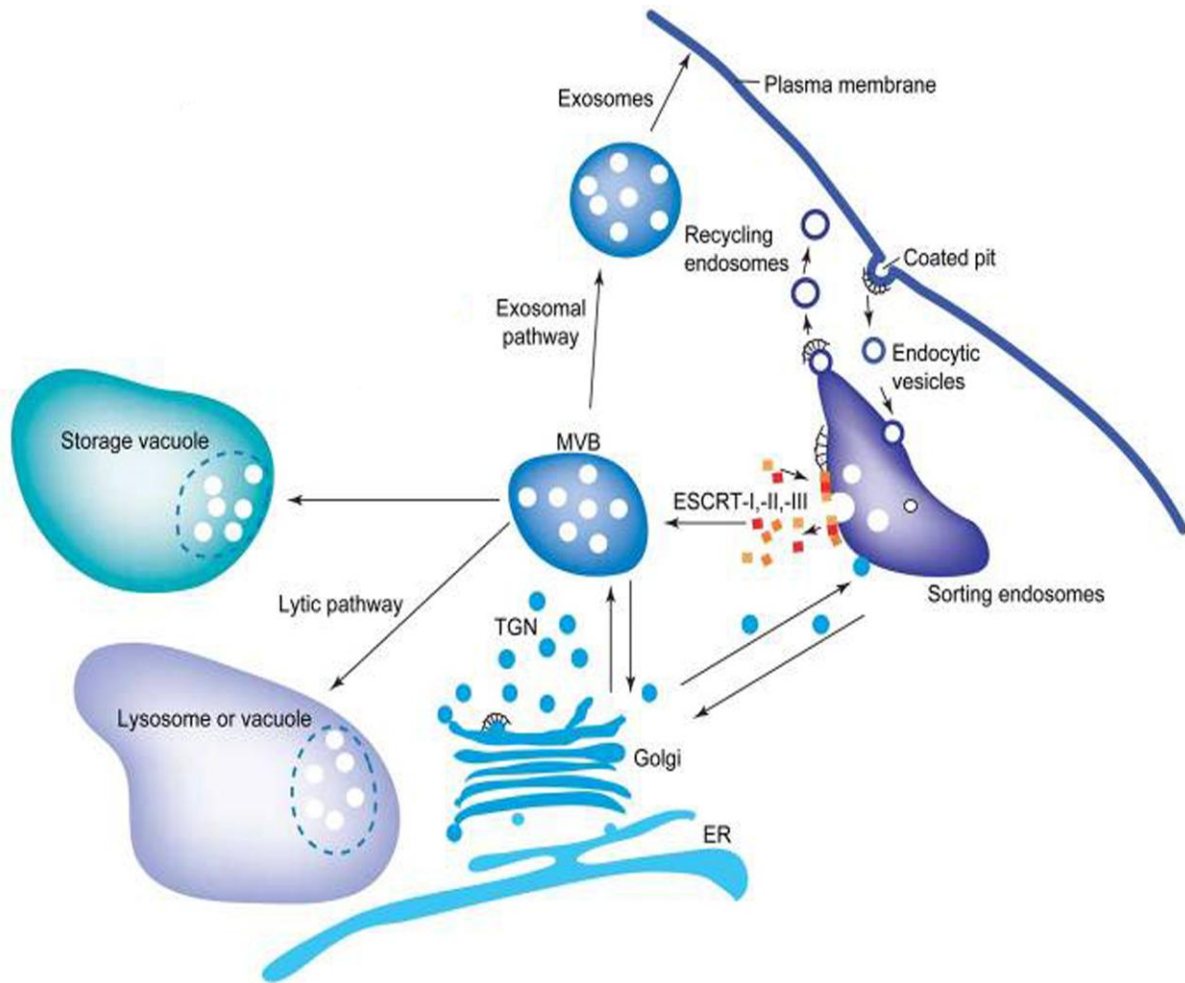


Diagram 3-1. The endocytic network. Adapted from **Winter and Hauser (2006)**. MVB (multivesicular body), TGN (*trans*-golgi network), ER (endoplasmic reticulum). See text for details.

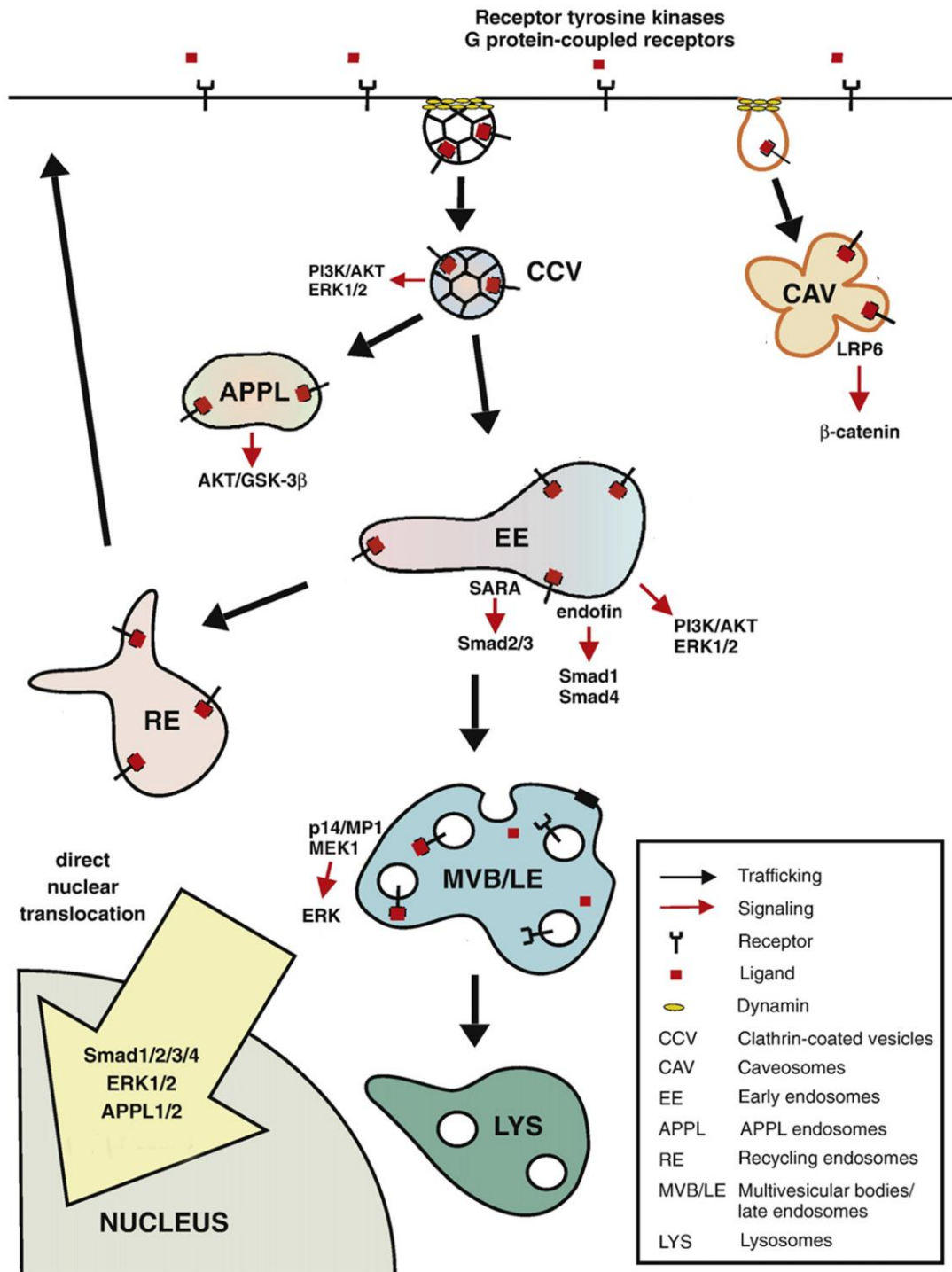


Diagram 3-2. Endomembranes as platforms of intracellular signaling. Adapted from Sadowski et al (2009). Schematic representation and selected examples of signaling molecules and transduction pathways acting locally on the surface of specific endomembranes. See text for details.

mediated cytoskeletal rearrangement, while increased PDGF levels induce mitogenesis and proliferation through lipid raft/caveolin-mediated endocytosis (**De Donatis et al 2008**). Interestingly, in the cases of the neurotrophin receptors TrkA and TrkB, activation by their respective ligands (NGF and BDNF) can result in CME, but whereas TrkA is mainly targeted to recycling endosomes, TrkB is predominantly degraded in the lysosome, and their divergent endocytic paths correlate with their particular signaling responsibilities and cellular responses (**Chen et al 2005**). Just as the endocytosis of cell-surface receptors can occur through two distinct mechanisms, their recycling can also be carried out in two ways – rapidly through early endosomes marked by the small GTPase Rab4, which are directly transported to the plasma membrane (**Diagram 3-3**); or through the slower route, whereby the early endosomes fuse with perinuclear recycling endosomes enriched with the small GTPase Rab11, which then directs Rab11-marked vesicles to the plasma membrane (reviewed in **Sheff et al 1999, Stenmark 2009**).

One of the first cytosolic proteins demonstrated to be essential for early endosome fusion was the small GTPase Rab5 (**Gorvel et al 1991**), which when localized to endomembranes, is preferentially targeted to early endosomes (**Chavrier et al 1990, Bucci et al 1992**). Numerous effectors have been identified for Rab5, including EEA1 (early endosome antigen 1), which is required for endosome fusion, and tethers endosomes together through homodimeric complexes (**Christoforidis et al 1999a**); and APPL1/2, which compete with EEA1 for Rab5 binding (**Zoncu et al 2009**), and are recruited to Rab5 in early endosomes, where they can regulate Akt activation and substrate specificity (**Schenck et al 2008**). Consistent with the large number of molecules with which it interacts, various functions have been proposed for Rab5 in the early steps of endocytosis, including internalization, early endosome fusion, and the shuttling of endocytic vesicles along microtubule tracks (**Horiuchi et al 1997, McLauchlan et al 1998, Christoforidis et al 1999b, Nielsen et al 1999**). Furthermore, in addition to endosomal traffic and receptor internalization, Rab5 function has been implicated in RTK-dependent signal transduction processes throughout the endocytic matrix, including its demonstrated requirement for the IRS1-p85 α interaction and Akt activation at signaling membranes (**Su et al 2006**); the appropriate localization and activation of

mTORC1 at lysosomes (**Bridges et al 2012**); nuclear localization of APPL-associated early endosomal complexes (**Miaczynska et al 2004b**); and through its interaction with p110 β , an important role in clathrin-mediated endocytosis and early endosome formation (**Christoforidis et al 1999b**). Early endosomes, then, which fuse with sorting endosomes, serve as the first transit station following internalization, from which they are either directed to recycling endosomes for relocalization to the cellular surface, or sent to lysosomes for degradation (**Diagram 3-1**). Canonical early endosomes are distinguished by the presence of the aforementioned EEA1 and Rab5 molecules (**Simonsen et al 1998**), and though distinct from the microenvironment of the PM, their only slightly acidic lumen (see **Section 1-5.2**) permits RTK-mediated signal transduction, while their cytoplasmic surface can host interactions with endosome-specific signaling molecules that may serve crucial roles in the biological response of the activated receptor (reviewed in **Sorkin and von Zastrow 2009**). The concept of early endosomes as a mobile signaling hub was largely uncovered in studies of neurons, in which the long distances between axonal termini and the main cellular body raised questions as to the range and speed of signal transduction, and its incompatibility with simple diffusion as an executive mechanism (reviewed in **Howe and Mobley 2005, Ibáñez 2007**). By tracking internalized NGF, it was shown that clathrin-coated vesicles (CCVs) and early endosomes are carriers of TrkA receptors and significant sources of TrkA-dependent ERK and PI3K/Akt activation (**Howe et al 2001, Delcroix et al 2003**), and furthermore, the microtubule- and dynein-mediated transport of endosomes was demonstrated to be required for NGF-dependent neuronal survival (**Ye et al 2003**).

Similarly, numerous other receptors were subsequently demonstrated to signal from early endosomes, including EGFR (**Pennock and Wang 2003**) and PDGFR (**Wang et al 2004**), as well as receptors for the TGF β family of ligands including activins, bone morphogenic proteins (BMPs) and TGF β itself. The endosomal activation of TGF β receptors induces the phosphorylation of Smad transcription factors and their translocation to the nucleus (**Diagram 3-2**), with the latter process shown to specifically require an interaction with SARA (Smad anchor for receptor activation), an adaptor protein that predominantly resides on early endosomes, with which it associates through its FYVE domain (**Hayes et al 2002**), a zinc finger present on numerous endosomal

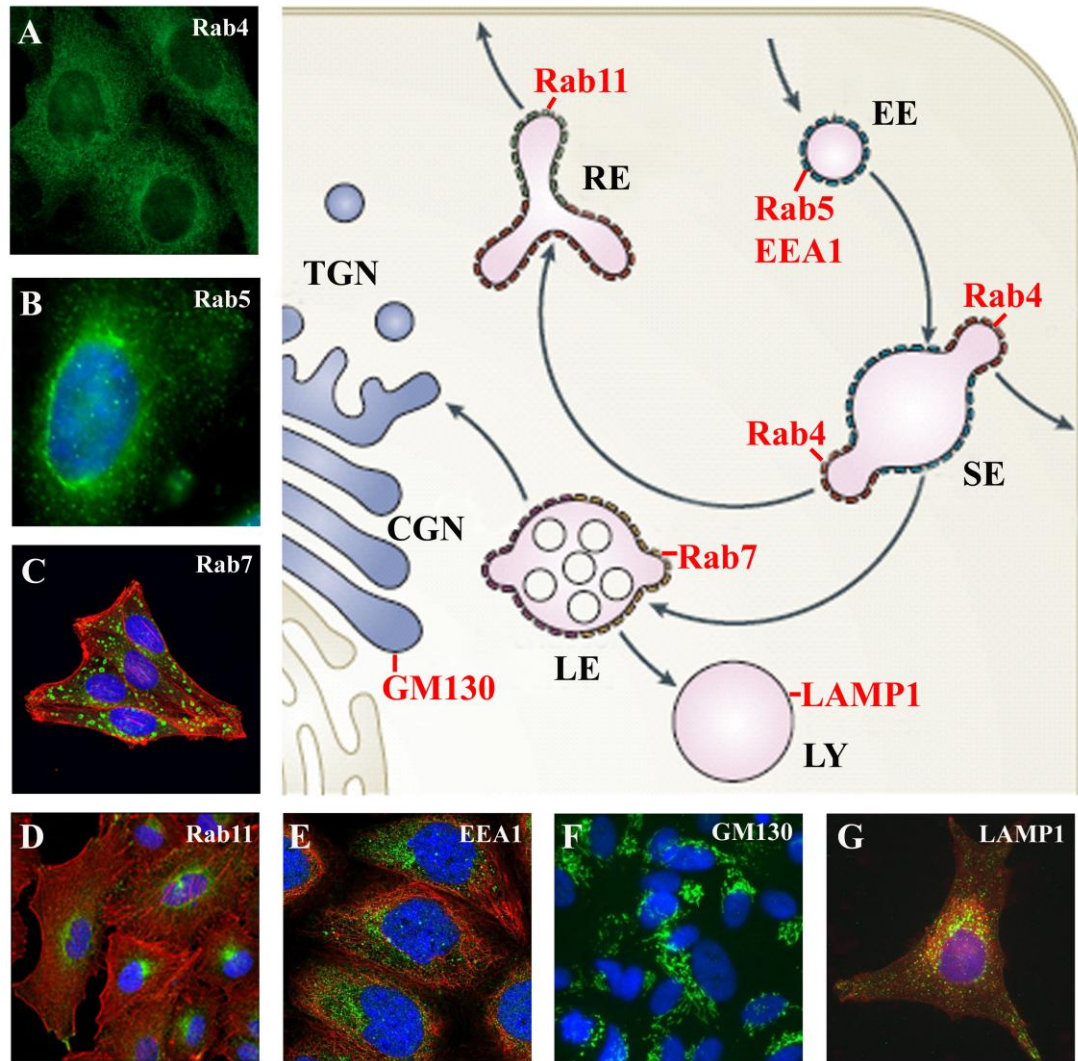


Diagram 3-3. Differential localization of endomembrane markers along the endocytic pathway. Selected molecules involved in endocytic processes are schematically represented with respect to their site-of-action: EE (early endosome), SE (sorting endosome), RE (recycling endosome), LE (late endosome), LY (lysosome), CGN (*cis*-golgi network), TGN (*trans*-golgi network). (A-G) Confocal immunofluorescence microscopy images of commercially available antibodies (vendor, cat. number) depicting the subcellular localization of the highlighted endomembrane markers (in green throughout). Nuclei are stained blue, and the membrane is stained red where applicable. Panels have been reproduced from images made publicly available by the respective vendors of the reagent in question. (A) Rab4 (Sigma Aldrich, R5780) in 3T3 fibroblasts. (B) Rab5 (Abcam, ab109534) in terminally differentiated HepaRG hepatic cells. (C) Rab7 (Cell Signaling Technologies, #9267) SK-MEL-28 melanoma cells. (D) Rab11 (CST, #5589) in A549 human lung adenocarcinoma epithelial cells. (E) EEA1 (Novus Biologicals, NBP1-91859) in human epidermal carcinoma cells. (F) GM130 (abcam, ab52649) in ARPE-19 human retinal pigment epithelial cells. (G) LAMP1 (abcam, ab24170) in HeLa human cervical carcinoma cells. See text and **Section 3-2.2** for details.

traffic-regulating molecules (reviewed in **Hayakawa et al 2007**). The FYVE domain selectively binds PI(3)P (**Diagram 1-5A**), a phospholipid enriched in endomembranes (**Gaullier et al 1998**), and is the major catalytic product of Vps34 (**Herman and Emr 1990**), the sole mammalian representative (*PIK3C3*) of class III PI3Ks in mammals (reviewed in **Vanhaesebroeck et al 2010**). Interestingly, the interaction of the TGF β receptor with endofin, another early endosome-specific FYVE domain-containing adaptor, promotes phosphorylation and nuclear translocation of Smad4 (**Chen et al 2007**); while similarly, Smad1-dependent signaling requires endofin downstream of the BMP receptor (**Shi et al 2007**). The subpopulation of early endosomes containing the Rab5 effectors APPL1 and APPL2 (adaptor containing phosphotyrosine-interaction domain, PH domain, and leucine zipper motif 1 and 2) have been reported to be distinct from EEA1-positive early endosomes, and shown to be crucial for the efficient transduction of mitogenic signals, providing another direct link between endosome-borne signaling events and nuclear signaling processes activated in their response (**Miaczynska et al 2004b**). Moreover, subsequent studies in zebrafish demonstrated that the localization of APPL1 to endosomes promoted Akt activation and contributed to the determination of substrate specificity (**Schenck et al 2008**), further entrenching endosome-specific interactions in the execution of Akt-dependent cellular processes.

One of the most relevant aspects of these studies is their support of the notion of endosome-assisted nuclear translocation of RTK effectors as the biologically relevant means of delivering signaling molecules to their target (reviewed in **Scita and Di Fiore 2010**). Depending on cell type and morphology, signals originating from the plasma membrane must often travel considerable distances to reach the nucleus or other membrane-distal structures (as mentioned above in the context of neurons). However, the precise mechanism by which endocytosed intact receptors and their associated signaling complexes travel from the surface of early endosomes through the pores of the nuclear envelope to the inner membrane of the nucleus remains poorly understood. As predicted by mathematical models (**Kholodenko 2003, Howe 2005**), and recapitulated in cell-culture studies using fluorescence reporters (**Kunkel et al 2005**), deactivating mechanisms such as dephosphorylation and ubiquitination during cytoplasmic diffusion can cause precipitous drops in the abundance of activated effectors, resulting in

potentially sub-threshold signaling magnitudes as they approach their intracellular target. Significantly, one such mathematical model went so far as to suggest that the majority of the relevant information transmitted from receptors at the PM to the nucleus can be associated with organelles of endocytic origin (**Howe 2005**), a finding supported by studies of subcellular compartment-specific Akt “pseudo-substrate” inhibitors, which revealed that nuclear and membrane-associated (but not cytoplasmic) pools of Akt were required for terminal adipocyte differentiation in cultured 3T3-L1 pre-adipocytes (**Maiuri et al 2010**). As such, the propulsion of endosomes along microtubule tracks provides a rapid means for the nuclear translocation of RTK effectors, and may also protect the activated effectors from molecular deactivating mechanisms. With endosomes as the major means of transport over large intracellular distances, the role of diffusion, which is comparatively inefficient, can be effectively limited to the final travel path once the transitory endosome’s destination is reached. The Akt molecule does not require phosphorylation for nuclear translocation (**Zhu et al 2007**), and various lines of evidence in diverse cell types demonstrating the presence of a nuclear PI-dependent signaling apparatus support this paradigm of receptor-dependent Akt activation networks in the nucleus, including the demonstrated presence of nuclear pools of PIP₃ (see **Figure 2-13**, reviewed in **Ye and Ahn 2008**), PI3K (**Neri et al 1994**, **Lu et al 1998**, **Kim et al 1998**, **Martelli et al 2000**), and the PI3K-enhancing GTPase PIKE (**Ye et al 2000**). Furthermore, in addition to the positive regulators of Akt function, the PIP₃ phosphatase PTEN is also localized to the nucleus (**Dél  ris et al 2003**), and PTEN nuclear translocation has most recently been demonstrated to occur through its SUMOylation (the addition of SUMO, or small ubiquitin-like modifier), and to control DNA repair and sensitivity to genotoxic stress (**Bassi et al 2013**). Moreover, downstream of the PI3K/PTEN junction, and mirroring the PI3K-dependent activation of Akt at the PM, PDK1 has also been demonstrated to be localized to the nucleus in a phosphorylation-dependent manner (**Lim et al 2003**, **Scheid et al 2005**).

Following their internalization into early endosomes and transition to sorting endosomes, endocytosed receptors are directed towards MVB/late endosomes, which constitute morphologically distinct large intracellular structures that serve as “distribution

centers” for internalized molecules, and are often marked by the enriched presence of LAMP1 (also present on lysosomes) and the small GTPase Rab7 (reviewed in **Gruenberg and Stenmark 2004**). MVBs are late endosomal structures with up to several hundred intraluminal vesicles that form by invagination and budding from the limiting endosomal membrane (**Diagram 3-1**). They are transitory pre-lysosomal stations, which either fuse with lysosomes, releasing their intraluminal vesicles, whose contents are then degraded by lysosomal enzymes; or alternatively, recycle the endocytosed contents back to the cell surface through the exosomal pathway (reviewed in **Winter and Hauser 2006**). Accordingly, in eukaryotes, MVBs are involved in the sorting and/or degradation of numerous membrane-associated molecules including transporters (for example, GAP1, Ste6, and PDR5) and the cell-surface receptors mentioned above. Protein sorting into MVBs is highly regulated, and genetic analyses in yeast have identified a number (>15) of soluble class E vacuolar protein sorting (VPS) genes in the recycling, degradation, or secretory pathways initiated at MVBs (**Raymond et al 1992**). Biochemical studies in both yeast and mammals suggest that these molecules are assembled into three distinct heteromeric complexes referred to as ESCRT-I, -II, and -III (Endosomal Sorting Complex Required for Transport), which direct cargo recognition, sorting, and concentration, as well as complex assembly, vesicle formation, and dissociation (reviewed in **Raiborg et al 2003, Babst 2005, Winter and Hauser 2006**).

Although MVBs/late endosomes are generally associated with cargo degradation and the termination of RTK-dependent signal transduction, they nonetheless play crucial roles in signaling processes that occur downstream of the receptor-borne signal and its immediate effectors (**Diagram 3-2**). The importance of endosomal transit from early endosomes to late endosomes/MVBs was illustrated by the disruption of c-Met (hepatocyte growth factor receptor) trafficking from peripheral to perinuclear endosomes, which prevented the nuclear accumulation of the transcription factor STAT3 (**Kermorgant and Parker 2008**); with similar blockages in EGFR peripheral/perinuclear traffic demonstrated to impinge on MAPK activation (**Taub et al 2007**). In fact, in the MAPK signaling pathway, the late endosomal adaptor p14, complexed to MP1 (MEK partner 1), serves as a scaffold for the recruitment of MEK1 to the late endosomal membrane (**Teis et al 2002, Teis et al 2006**). The importance of this interaction was

demonstrated by the mislocalization of the p14-MP1 complex to the plasma membrane, which rendered it incapable of potentiating MAPK signaling. Furthermore, during Wnt signal transduction, the sequestration of GSK3 inside MVBs has been suggested to result in the stabilization of downstream targets such as β -catenin (**Taelman et al 2010**), which in the absence of Wnt stimulation is normally phosphorylated by GSK3 and targeted for degradation by the “destruction complex” (see **Section 1-4.2**). The internalization of the Wnt co-receptors Frizzled (Frz) and low-density lipoprotein receptor-related 6 (LRP6) was shown to require the activity of V-ATPase (**Blitzer and Nusse 2006, Cruciat et al 2010**); and the subsequent endosomal sequestration of GSK3 has been proposed to insulate β -catenin from GSK3-dependent phosphorylation, allowing its accumulation and translocation to the nucleus where it acts as a transcriptional activator of Wnt target genes (reviewed in **Dobrowolski and De Robertis 2012**). As described in detail in the introductory chapter (**Sections 1-4.3, and 1-4.5**), and as discussed in Chapter 2, another example of lysosomal signal transduction that is directly relevant to our studies is the importance of Rheb farnesylation and endomembrane localization in mTORC1 activation (**Buerger et al 2006**), and the convergence of the GF- and nutrient-dependent branches of mTORC1 activation at the lysosomal surface, which was shown to require early/late endosomal conversion, and whose blockage was found to prevent the interaction of mTORC1 with Rheb in late endosomes and lysosomes (**Flinn et al 2010**).

Study rationale and objectives:

The mechanistic links described above between endocytosis and receptor-dependent signaling strongly suggest a strong interdependence between the two processes, and the importance of their interplay has been established in signaling downstream of various classes of receptors, diverse cell types, and distinct physiological processes. By providing a hierarchical network for the dynamic intracellular compartmentalization of signaling molecules, the endocytic matrix spatially and temporally controls the output of receptor-borne signals, and acts as a crucial determinant of both signal propagating and attenuating processes. Depending on the receptor system (and cell-type) in question, endosomes (whose subpopulations are specialized and allow for differential signaling outputs) generally seem to fulfill three major functions in signal

transduction: (1) they allow the prolongation of ligand binding-dependent signal transduction after internalization; (2) they promote the assembly of specific endomembrane-specific signaling complexes whose composition is predicated on the enriched endosomal presence of complex components; and (3) their role in cytoskeleton-mediated intracellular transport allows the delivery of their active signaling components to various targeted locales within the cell such as the nucleus. Our results in *Drosophila* demonstrated an important role for V-ATPase in the activation of Dakt, and suggested that endomembrane-associated and nuclear pools of Akt are crucial for the regulation of growth, which is consistent with the roles attributed to endomembranes in ISP signal transduction throughout the endocytic network, including its role in the translocation of Akt to the nucleus, where many of its important targets reside. Due to the availability of antibodies against a broader repertoire of ISP- and endomembrane-signaling components of mammalian origin, we examined the effects of prolonged V-ATPase inhibition on Akt phosphorylation and subcellular localization in bafilomycin treated NIH-3T3 fibroblasts *in vitro*, which was followed by cellular and subcellular fractionation studies examining the effects of bafilomycin treatment on the activity and/or subcellular localization of various endomembrane- and ISP-signaling components.

In turning to cultured mammalian fibroblasts, our first objective was the establishment of a temporal window over the course of which bafilomycin-induced changes in Akt localization and signaling could be investigated. The incubation of NIH-3T3 cells with 50nM bafilomycin over a 24 hour period was found to result in a near-maximal decrease in Akt phosphorylation within the first 12 hours of treatment (**Section 3-3.1**). This 12 hour treatment window was thereby adopted for our subsequent experiments addressing our second objective – the examination of subcellular compartment-specific changes in Akt abundance and phosphorylation, whereby a cellular fractionation protocol was designed and applied to the intracellular compartment-specific enrichment of signaling molecules (**Section 3-3.2**). This approach allowed us to generate subcellular fractions enriched for cytoplasmic, membrane, endosomal, and nuclear contents respectively. Accordingly, changes in Akt abundance and phosphorylation were tracked in NIH-3T3 cells over a 12 hour bafilomycin treatment period, with samples harvested after 2, 4, and 12 hours of treatment. Immunoblot analysis of the resulting

subcellular fractions indicated a significant reduction in the abundance and phosphorylation of Akt in the endosomal and nuclear compartments (**Section 3-3.3**), which was consistent with our previously discussed observations in *Drosophila* larval tissues. Considering the aforementioned importance of early endosomal populations in RTK-mediated signal transduction, and their role in the subcellular targeting of signaling complexes, our third objective consisted of determining whether the observed decrease of Akt abundance and phosphorylation in the endosomal fraction of bafilomycin-treated NIH-3T3 cells could in fact be traced to early endosomal subpopulations. The endosome-enriched cellular fraction, which is separated from the cytoplasmic, membrane, and nuclear compartments in our fractionation protocol, is a crude conglomeration of various vesicular subtypes, including early endosomes, late endosomes/MVBs, lysosomes, ER/Golgi-associated vesicles, secretory granules, and other endomembranes. The subfractionation of this crude endosomal fraction through an Optiprep density gradient allowed us to further separate the endosomal content of larval tissues or NIH-3T3 cells into 20 fractions of increasing Optiprep concentration. In both larval tissues and NIH-3T3 cells, immunoblot analysis of the endosomal subfractions demonstrated a distinct accumulation of Akt in early endosomal fractions in response to growth factor stimulation, whereas treatment with bafilomycin was shown to consistently and time-dependently deplete early-endosomal populations of Akt (**Section 3-3.6**).

3-2 – Materials and Methods

Cell lines, antibodies, and reagents.

Murine NIH-3T3 cells were purchased from ATCC (CRL-1658) and maintained in DMEM/5% FBS, and 5% CO₂. Bafilomycin A1 was purchased from Sigma Aldrich (B1793), and dissolved in DMSO. The antibodies used in immunoblot analyses include: Caspase 3 (both cleaved and uncleaved, CST #9665), cleaved Lamin A (CST #2031), Akt (CST #9272), phospho-Akt S473 (CST, #4060), phospho-Akt T308 (CST #9275), GAPDH (Abcam ab9484), Histone H3 (Abcam ab1791), CREB (Abcam ab325515), non-phosphorylated β -catenin (CST #4270), Calnexin (generously provided by Dr. David Williams), ATP6V1A (Abcam ab137574), PDK1 (CST #3062), phospho-PDK1 S241 (CST #3061), phospho-PDK1 Y373/Y376 (Abcam ab52893), GSK3 α/β (Upstate 05-412), phospho-GSK3 α/β S9/S21 (CST #9331), phospho-GSK3 β S9 (Abcam ab107166), phospho-GSK3 α S21 (Abcam ab28808), mTOR (CST #2972), phospho-mTOR S2448 (CST #2971), S6K1 (CST #9202), phospho-S6K1 T389 (CST #9205), phospho-S6K1 S411 (Santa Cruz sc7983), Rictor (CST #2140), Rab4 (CST #2167), Rab5 (CST #2143), *Drosophila* Rab5 (Abcam ab31261), Rab7 (CST #9367), Rab11 (CST #3539), EEA1 (CST #2411), GM130 (BD Bioscience 610822), Lamp1 (ab24170), dHook (generously provided by Dr. Helmut Krämer), HA (CST #2367), HRP-linked secondary antibodies (CST #7074 and #7076).

Cell viability assays.

Six-well tissue culture plates (Invitrogen) were seeded with $\sim 1 \times 10^4$ NIH-3T3 cells, and grown at 37°C (5% CO₂) in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen 11965-092) supplemented with 5% FBS (Gibco). When nearly confluent (3-4 days), the cultured medium was exchanged for DMEM/5%FBS containing bafilomycin at the indicated final concentrations (5-100nM), or an equal volume of the drug vehicle DMSO. At the indicated time intervals, the medium-only blank, vehicle-treated control, and bafilomycin-treated samples were subjected to the CellTiter 96® AQueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega G5421) as per the manufacturer's recommendations. The colorimetric reagent consists of a mixture of a novel tetrazolium compound (MTS) and an electron coupling agent (PMS). MTS is reduced by cellular bioactivity (catalyzed by dehydrogenase enzymes found in metabolically active cells) into a formazan product that is soluble in tissue culture medium. Following an incubation of ~ 15 -30 minutes with the colorimetric reagent (a mixture of MTS and phenazine methosulfate, or PMS), during which the culture media of viable samples (such as the control) take on a deep-brown color, 200 μ l aliquots of each sample's culture medium were transferred into a 96 well plate in quintuplicate, and the abundance of the formazan byproduct (which is directly proportional to the number of metabolically active cells) was measured directly based on its photic absorbance at 490nm on a microplate reader (VersaMax, Molecular Devices). The experiment itself was conducted in triplicate. **These experiments were carried out by the author of the thesis (SK).**

Fluorescence-activated cell sorter (FACS) analysis.

Six-well tissue culture plates were seeded with $\sim 1 \times 10^4$ adherent NIH-3T3 cells, and grown at 37°C (5% CO₂) in 2mL of DMEM supplemented with 5% FBS. Following 2-3

days of growth, all samples (in duplicate) were refreshed with DMEM/5%FBS, while experimental samples were treated with bafilomycin in a staggered fashion 12, 24, and 36 hours prior to the assessment of drug treatment on viability. Following the end of the incubation period, cells were briefly trypsinized (Gibco 25300-054), neutralized in 2mL of culture medium, and pelleted by centrifugation at 300g (1200 rpm Eppendorf 5810R). The neutralizing medium was aspirated and discarded, and the pelleted cells were twice resuspended in ice cold PBS (phosphate buffered saline), and recentrifuged. The washed pellet was resuspended in 400µl of 1x binding buffer (Biovision 1035-100), to which 250ng of AnnexinV-FITC (1001-1000) and propidium iodide (Biovision 1056-1) were added, and analyzed immediately after staining. Flow cytometry analyses were carried out on a FACScalibur instrument using the ModFit program (Becton Dickinson, USA), for which Rakesh (Casey) Nayyar's expertise and assistance is greatly appreciated. Data was collected for 50,000 cells from each sample, and the experiment was done in triplicate. AnnexinV is a Ca^{2+} -dependent phospholipid-binding protein that exhibits a high affinity for phosphatidylserine (PS), a phospholipid usually confined to the inner leaflet of the plasma membrane, and that is translocated to the outer surface of apoptotic cells. Propidium iodide is a non-specific DNA dye that is excluded from live cells with intact plasma membranes, but incorporated into non-viable cells. As such, viable cells are doubly negative for both PI and AnnexinV-FITC. Single positive populations are considered either early apoptotic (AnnexinV-positive/PI-negative) or necrotic cells (AnnexinV-negative/PI-positive), whereas double positive (AnnexinV-positive/PI-positive) cells are considered to be in late stages of apoptosis. **These experiments were carried out by the author of the thesis (SK).**

Generation of NIH-3T3 whole cell lysates for immunoblotting.

After 3-4 days of growth in DMEM/5% FBS, simultaneously seeded ($\sim 5 \times 10^5$ cells/100mm tissue culture plates, Corning 430167) near-confluent samples of NIH-3T3 cells were incubated with bafilomycin (final concentration of 50nM) or an equal volume of the DMSO vehicle, and following a wash with ice-cold PBS, harvested at the indicated times with a cell lifter (Corning 83-3008) in $\sim 1\text{mL}$ of ice-cold RIPA buffer (see **Section 2-2** for composition), flash frozen in a dry ice/ethanol bath, and stored at -70°C until all samples were harvested. Whole cell lysates for Western blot analysis were generated by first homogenizing the RIPA samples with a 23G needle (10-15 passages) on ice, followed by centrifugation at 10,000g (10min, 4°C) to generate a supernatant clear of cellular debris. While avoiding the upper lipid layer, approximately $\sim 350\mu\text{l}$ of the supernatant was collected, from which $300\mu\text{l}$ was mixed with $100\mu\text{l}$ of 4x Sample Buffer/ β -mercaptoethanol and used for SDS-PAGE analysis; while $\sim 50\mu\text{l}$ was set aside for protein quantitation (Bradford assay). Based on quantitation values, equal amounts of the protein samples were separated by SDS-PAGE electrophoresis, transferred to a PVDF membrane, and immunoblotted as previously described. **These experiments were carried out by the author of the thesis (SK).**

Generation of subcellular fractions for immunoblotting.

A total of eight 150mm tissue culture plates (Corning 430599) were each seeded with $\sim 1 \times 10^6$ NIH-3T3 cells and grown in DMEM/5% FBS. When nearly confluent after 4-5 days of growth (DMEM/5% FBS culture medium was refreshed after 3 days), two plates

were identically mock-treated with the vehicle (DMEM/5% FBS, DMSO) 12 hours prior to harvest (referred to as TP0), while the experimental samples (two plates for each time point) were treated with bafilomycin (DMEM/5% FBS, 50nM bafilomycin) 12, 4, and 2 hours prior to harvest (referred to as TP12, TP4, and TP2). At the conclusion of the time course, cells were harvested starting with the first TP2 plate, which was washed twice in ice-cold 1x hypotonic buffer [1x HB – 20mM Hepes, 50mM NaF, 1.5mM MgCl₂, pH 7.1, prior to use add 1mM benzamidine, 0.1mM sodium orthovanadate, 5.4mg/mL β-glycerophosphate, and protease inhibitor cocktail tablet (Roche 11873580001)], and harvested with a cell scraper in 3mL of HB. The process was repeated with the duplicate plate, whose cells were harvested in the ~3mL HB solution containing the harvested cells from the first plate, essentially pooling the two samples. The resulting ~3mL HB solution was contained in a 15mL tube (Falcon), and incubated on ice for ~15 minutes (occasionally shaking the tubes gently) for hypotonic lysis of the harvested cells. The process was repeated with the TP4, TP12, and lastly, the TP0 samples. Following the harvest of all samples, the 3mL HB lysates were transferred to a Dounce homogenizer and gently sheared (10-15 strokes) on ice, and further equally gently homogenized (~10 passages) with a 23G needle (Becton Dickinson 305194Z). The resulting lysates were then individually filtered through a 64μm Nitex nylon mesh (Genesee scientific) in order to exclude intact cells from the lysate. The generation of enriched subcellular compartments from these resulting lysates is described as a flowchart in **Figure 3-4A**.

Generation of the nuclear (N) fraction: The homogenized hypotonic lysates were first centrifuged at 300g (10 min, 4°C) to generate a post-nuclear supernatant (**S1**) and a crude nuclear pellet (**P1**) for each of the four samples (TP0, 2, 4, and 12). The **S1** supernatants were set aside on ice for further fractionation (see below), while the **P1** crude nuclear pellets were gently resuspended in 3mL of ice-cold B1 buffer [250mM sucrose, 10mM MgCl₂, 1mM benzamidine, 0.1mM sodium orthovanadate, 5.4mg/mL β-glycerophosphate, protease inhibitor cocktail], gently layered over 3mL of ice-cold B2 buffer [880mM sucrose, 0.5mM MgCl₂, 1mM benzamidine, 0.1mM sodium orthovanadate, 5.4mg/mL β-glycerophosphate, protease inhibitor cocktail], and centrifuged at 2,600g (10 min, 4°C). Following the aspiration and discarding of the supernatants, the purified nuclear pellets were washed by resuspension in 3mL of ice-cold 1x HB, and recentrifuged at 300g (10 min, 4°C). The supernatants were once again aspirated and discarded, while the purified and washed nuclear pellets were gently resuspended in 1mL of ice-cold 1x RIPA buffer (which contains detergents, see **Section 2-2**), and the nuclear suspensions were sonicated on ice for 10 x 1 second bursts (with a 1 second intervals between each burst) using a Misonix XL 2020 sonicator fitted with a microtip probe and set at power setting 5, repeating the sonication cycles 5 times for each sample. Following sonication, the nuclear lysates were centrifuged at 10,000g (10 min, 4°C) to pellet nuclear debris, and ~500μl of the supernatants containing solubilized nuclear contents (the “N” fraction) were recovered and set aside for quantitation (Bradford assay) and SDS-PAGE electrophoresis as previously described.

Generation of the membrane (M) fraction: The post-nuclear supernatants **S1** (~2.5mL) of all four samples were centrifuged at 2,600g (10 min, 4°C), resulting in a supernatant (**S2**) consisting of cytoplasmic contents and endomembranes, which was collected and set aside for further fractionation (see below); and a pellet (**P2**) consisting of membrane structures. As a washing step, the **P2** membrane pellets were gently resuspended in 3mL

of ice-cold 1x HB, recentrifuged at 2,600g (10 min, 4°C), resuspended in 1 mL of ice-cold 1x RIPA buffer, homogenized with a 23G needle (10-15 strokes) to solubilize membrane-bound proteins, and prepared for quantitation and SDS-PAGE electrophoresis as previously described.

Generation of the cytoplasmic (CY) and endomembrane (EN) fractions: The S2 supernatants (~2mL) consisting of cytoplasmic and endomembrane contents were transferred to high-speed polycarbonate centrifuge tubes (Beckman Coulter 355647) and centrifuged at 200,000g (60min, 4°C) with an MLA-80 fixed-angle rotor in an Ultima TLX-120 (Beckman Coulter) ultracentrifuge. From the resulting supernatant (S3), ~500µl was collected as the cytoplasmic fraction, and prepared for quantitation and SDS-PAGE electrophoresis as previously described. The endosomal pellet (P3) was gently washed (without disrupting the pellet) with ice-cold 1x HB, and thereafter resuspended in 1mL of ice-cold 1x RIPA, homogenized with a 23G needle (10-15 strokes), and prepared for quantitation and SDS-PAGE electrophoresis as previously described. **These experiments were carried out by the author of the thesis (SK).**

Endosomal subfractionation for immunoblotting.

For the generation of endomembrane fractions from *Drosophila* larval tissues, ~50 Akt-HA-expressing heads were incubated in insect culture media supplemented with 5% FBS and either mock-treated for 12 hours with the DMSO vehicle, treated for 12 hours with 0.2U/mL humulin, or treated for 12 hours with both bafilomycin (500nM final concentration) and 0.2U/mL humulin. Following the incubation period, the incubation media of the control and treated samples were aspirated and discarded, and treated heads were Dounce homogenized (10-15 strokes) in 1.5mL of ice-cold 1x HB on ice, and further homogenized by passage through a 23G needle. The resulting lysates were centrifuged at 3,000g (10 min, 4°C) in order to pellet unlysed cells, nuclei and membranes. The supernatants (~1.2mL) containing the cytoplasmic and endomembrane fractions were then centrifuged as described above at 200,000g (60min, 4°C) in order to generate endomembrane pellets. The supernatant (cytoplasmic content) was discarded, and following gentle washes with ice-cold 1x HB, the endosomal pellets were resuspended in 1mL of ice-cold 1x HB, and layered atop 10mL solutions of ice-cold 10-30% OptiPrep (Sigma Aldrich D1556) gradients of 1x HB (prepared with an Isco Model 160 gradient former) in thin-wall polyallomer tubes (Beckman Coulter 331372). The OptiPrep gradients and layered endomembrane samples were then centrifuged at 100,000g (16 hours, 4°C) using an SW41 Ti swinging bucket rotor in a Beckman Optima XL-100R ultracentrifuge. At the conclusion of the centrifugation run, a total of twenty 0.5mL fractions of the ~10mL OptiPrep gradients were collected beginning from the top of the gradients using a Brandel Density Gradient Fractionation System (BR-188). In the case of NIH-3T3 cells, the EN fraction (as well as the N, M, and CY fractions) were generated as described above for control (TP0) and bafilomycin-treated (TP2, TP4, and TP12) samples. Approximately 1/10th of the resuspended EN pellet was set aside for diagnostic Western blotting (**Figure 3-14C**), while the remaining 9/10th (~1mL) was layered atop a 10-30% OptiPrep gradient as described above for *Drosophila* samples. **These experiments were carried out by the author of the thesis (SK), with the exception of the preparation of the Optiprep gradients, and the collection of the Optiprep fractions, which were done with Dr. Rick Bagshaw's assistance.**

3-3 – Results

3-3.1 – Bafilomycin downregulates Akt phosphorylation and induces apoptosis in NIH-3T3 cells

In the extensive literature documenting its use, the *in vitro* effective concentrations of macrolides like bafilomycin A1 and concanamycin A can vary greatly depending on experimental design, cell-type, and the particular physiological responses under investigation. As discussed in the previous chapter's discussion, studies examining the bafilomycin-dependent inhibition of lysosomal acidification as well as lysosome/autophagosome fusion, and the induction of apoptosis in cultured cells, have been carried out with concentrations ranging from 10nM to 1μM, while concentrations of bafilomycin below the nanomolar range (<1nM) are ineffective for the inhibition of endosomal acidification, and induce neither the accumulation of autophagosomes nor apoptotic cell death (**Shacka et al 2006**). Some studies examining the acute effects of V-ATPase inhibition have used bafilomycin A1 (or the structurally related macrolide concanamycin A) at concentrations at or above the upper effective range (>1μM), and usually over a short period of treatment (<1 hour). As examples, 2μM concanamycin was shown to block endocytic transport in *Arabidopsis* cultures (**Dettmer et al 2006**); treatment with 10μM bafilomycin for 30 minutes was demonstrated to inhibit insulin-dependent glucose uptake as a result of the defective V-ATPase-dependent osmotic swelling and exocytic membrane-localization of GLUT4-containing vesicles in adipocytes (**Choi et al 2007**); and more recently, the use of concanamycin concentrations ranging from 2μM to 10μM by Sabatini's group in their aforementioned investigation of the V-ATPase/Ragulator interaction in nutrient starved HEK-293T cells, which was conducted within a 1 hour treatment window (**Zoncu et al 2011a**). Other studies, however, including our own, have examined the cumulative effects of bafilomycin-dependent V-ATPase inhibition in the lower range of concentrations (<200nM). Some of these studies, like Barry Posner's investigation of the effects of inhibiting endomembrane acidification on insulin signaling in hepatocytes (**Balbis et al 2004**), have been conducted using a short (30 minute) treatment window. Other studies examined the effects of similar

concentrations over an extended period (24 hours), such as Stephen Pennington's demonstration of the bafilomycin-dependent inhibition of mitogen-induced DNA synthesis in Swiss 3T3 fibroblasts treated for 24 hours at a concentration of 125nM (**Saurin et al 1996**); or Guido Kroemer's study in which bafilomycin at a concentration of 100nM over a period of 24 hours was shown to induce autophagosome accumulation and apoptotic death in cultured HeLa cells (**Boya et al 2005**), and which was published just prior to my transition in 2006 into the PhD program as a student in Dr. Manoukian's laboratory.

To further investigate the intracellular consequences of the pharmacological inhibition of V-ATPase activity with respect to Akt activation, growth, and survival, we established a time and concentration window within which treatment with bafilomycin could be analyzed in cultured NIH-3T3 murine fibroblasts. Accordingly, with the concentrations and treatment period used by the two studies mentioned above (**Saurin et al 1996**, **Boya et al 2005**), we incubated semi-confluent cultures of growing NIH-3T3 cells (DMEM medium supplemented with 5% FBS) with various concentrations of bafilomycin (DMSO vehicle control, 5nM, 20nM, 50nM, and 100nM) within the lower range (<200nM) of effective concentrations, and examined the time- and concentration-dependent effects on viability over a span of 3 days at 24 hour intervals using the MTS cell viability assay (**Figure 3-1A**). Within the first 24 hours of treatment, all four concentrations of bafilomycin caused a comparable nearly twofold decrease in cell viability (<40% viability compared to vehicle-treated); and by 48 hours of treatment, cell viability was negligible (<5% compared to vehicle-treated) at all concentrations examined. Our observation that cell viability was significantly reduced within the first 24 hours of bafilomycin treatment irrespective of the concentration used (5-100nM) prompted us to examine the nature of the observed bafilomycin-induced cytotoxicity within the first 24 hours of treatment. We treated cultures of NIH-3T3 cells with the mean concentration of bafilomycin in the range we tested (50nM) for up to 24 hours, and harvested cells at 4 hour intervals. Caspase 3 is an executioner caspase (see **Section 1-4.7**), and its proteolytic activation is prevented by insulin/IGF, thereby promoting cell survival, and this anti-apoptotic mechanism has been shown to be compromised in IRS1-

deficient cells, suggesting its dependence on PI3K/Akt signaling (**Tseng et al 2002**). Western-blotting for cleaved (and therefore pro-apoptotic) caspase-3 and LaminA suggested that latent bafilomycin-induced mortality in treated cells is initiated approximately 16 hours following treatment in these cells, and maximal at the last interval (24 hours) examined, at which point cleaved caspase-3 cannot be detected in vehicle-treated (non-apoptotic) samples (**Figure 3-1B**). These results suggested that the decrease in viability was associated with apoptotic death, and were consistent with the aforementioned temporal course of bafilomycin-induced apoptosis in HeLa cells (**Boya et al 2005**), and our own MTS viability data with bafilomycin-treated NIH-3T3 cells (**Figure 3-1A**). To further establish the onset of apoptosis in response to bafilomycin treatment, NIH-3T3 cultures were incubated with 50nM bafilomycin (or the vehicle control DMSO) for up to 36 hours, labeled with FITC-conjugated AnnexinV and propidium iodide (PI), and subjected to FACS analysis (**Figure 3-2A**). Whereas the proportion of apoptotic cells gated was negligible in vehicle-treated controls throughout the 36 hour time course, the fraction of apoptotic/dead cells in bafilomycin-treated samples was found to time-dependently increase, consisting of ~10% of the gated population of cells after 12 hours of bafilomycin treatment, ~60% of the gated population by 24 hours of treatment (a value comparable to the decrease in viability observed in the MTS assay at the corresponding time point), and >90% after 36 hours of exposure to bafilomycin (**Figure 3-2B**).

Having established a 24 hour window within which 50nM bafilomycin could time-dependently induce apoptotic cell death, including the initiation of “executioner” caspase-3 cleavage at 16 hours post-treatment (**Figure 3-1B**), we investigated the status of Akt phosphorylation at S473 and T308 over the same time span in identically treated NIH-3T3 cells (50nM bafilomycin, DMEM/5% FBS). Whole-cell lysates extracted from bafilomycin-treated samples were harvested at 4 hour intervals over a 24 hour incubation period, and protein samples from whole cells lysates of each time point were analyzed by Western blotting. A nearly twofold decrease in the phosphorylation of both residues (~40% for S473 in the HM, ~50% for T308 in the T-loop) was observed in the first examined interval (4 hours), and this decrease was found to peak by the 12th hour of

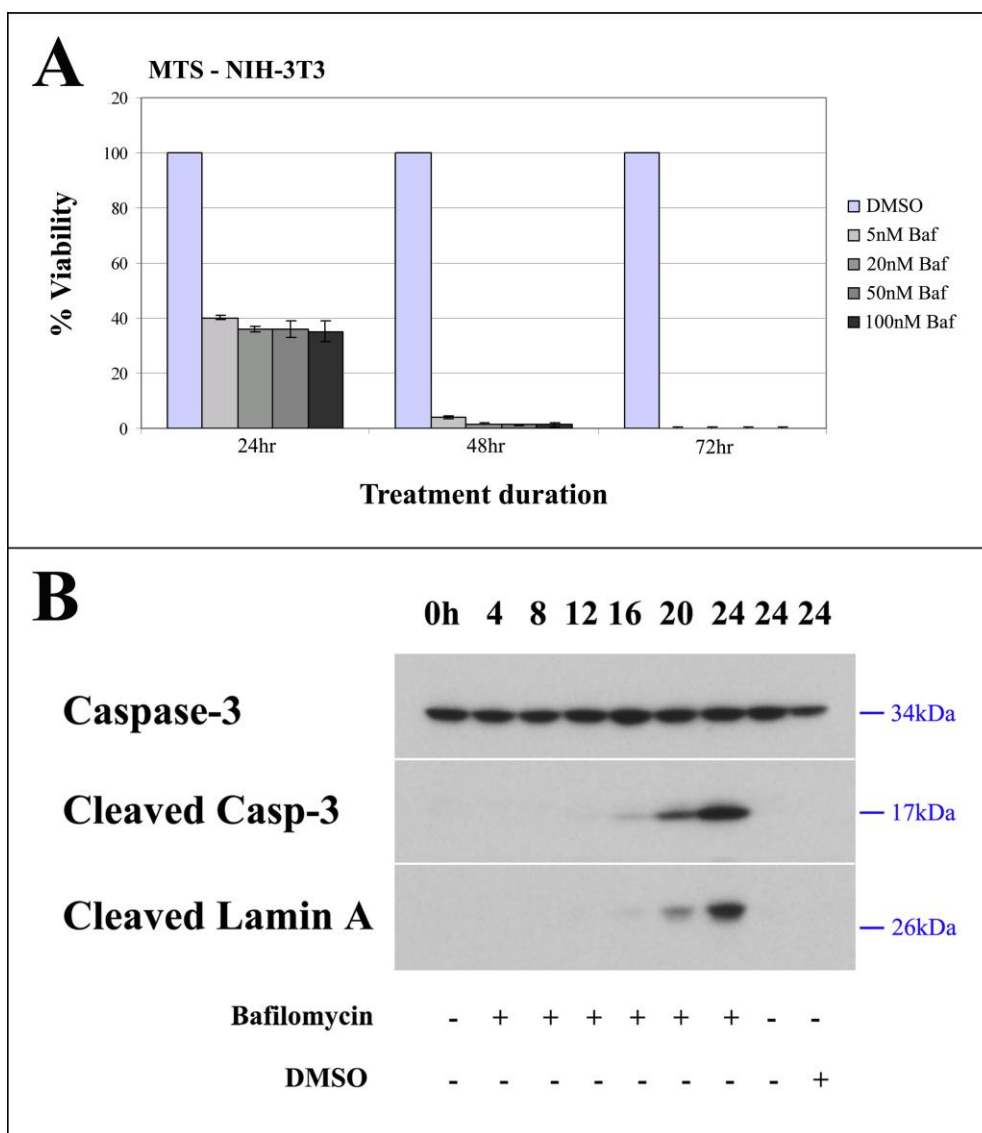


Figure 3-1. Bafilomycin treatment significantly reduces the viability of NIH-3T3 cells. (A) Cultured NIH-3T3 cells were grown in DMEM supplemented with 5% FBS, and either vehicle- (DMSO) or bafilomycin-treated (at indicated concentrations) in triplicate for 24, 48, and 72 hours. The viability of vehicle- and bafilomycin-treated cells was assessed at each indicated time interval with the MTS cell viability assay (Promega Corp). Absorbance was measured at 490nm using a PowerWave XS microplate reader (BioTek), and graphically depicted as a percent value normalized to the vehicle-treated control at each time point, which was arbitrarily assigned a value of 100%. (B) NIH-3T3 cells (DMEM/5% FBS) were treated with 50nM bafilomycin for up to 24 hours and harvested at 4 hour intervals, with concomitant untreated and vehicle-treated (DMSO) samples collected at the end of the time course. Whole-cell lysates from each time point were analyzed by Western blotting for the accumulation of cleaved caspase-3 and Lamin A peptides with uncleaved pro-caspase-3 as a loading control. Immunoblots are representative of three repetitions of the experiment. The molecular weight values in the right-hand margin are based on the PageRuler™ (Fermentas) prestained protein ladder.

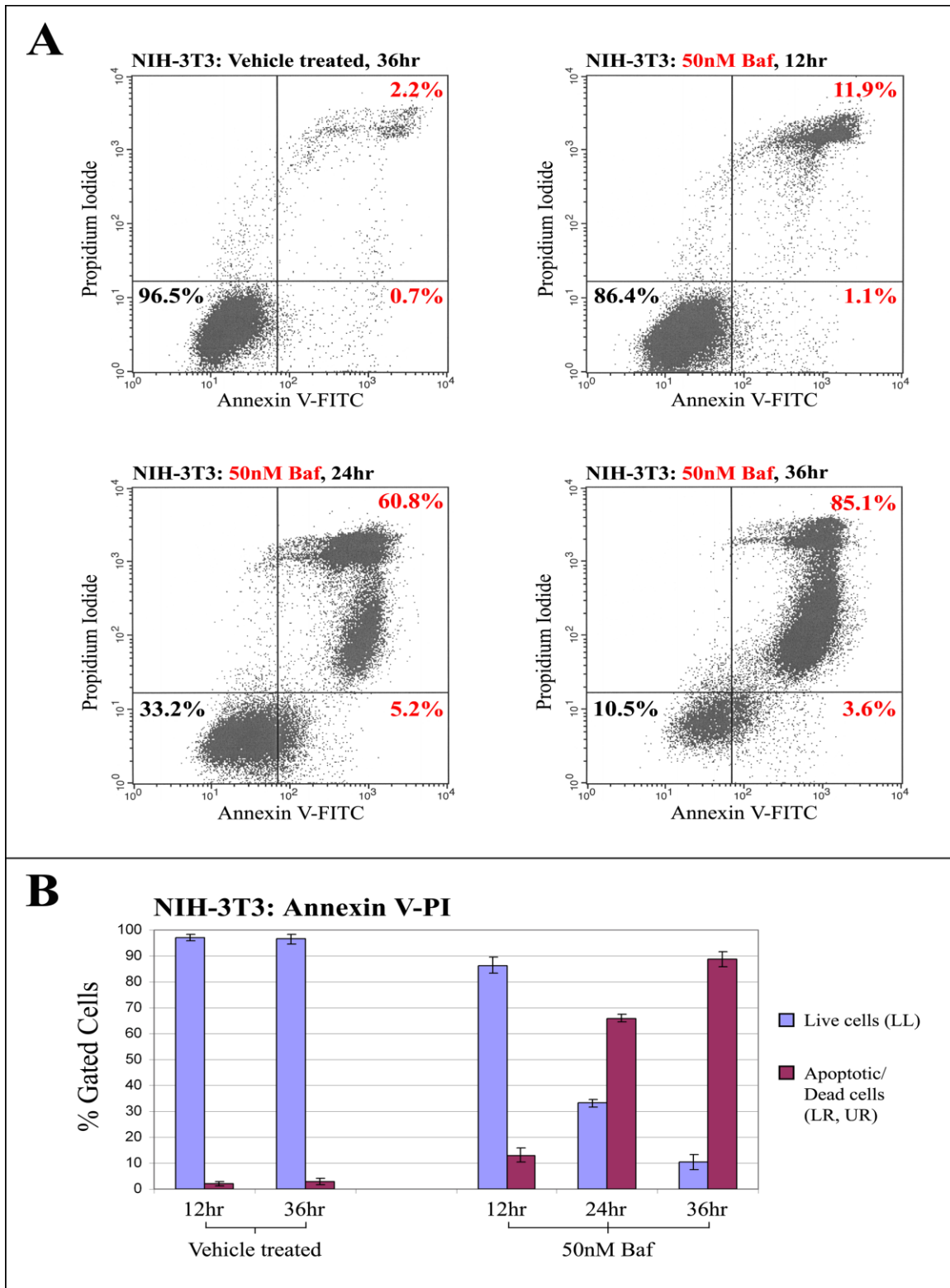


Figure 3-2. Bafilomycin treatment induces apoptosis in NIH-3T3 cells. Cultured NIH-3T3 fibroblasts (DMEM/5% FBS) were either vehicle- (DMSO) or bafilomycin-treated (50nM) for up to 36 hours, and harvested at the indicated time intervals. Harvested cells

were then labeled with AnnexinV- fluorescein isothiocyanate (FITC) and propidium iodide (PI), and subjected to FACS analysis. The results of these experiments, done in triplicate, are presented as: **(A)** representative dual parameter scatter plots with indicated percentage values of viable cells (AnnexinV- and PI-negative, Lower Left quadrant, in black), apoptotic cells (AnnexinV-positive and PI-negative, Lower Right quadrant, in red), and dead cells (AnnexinV- and PI-positive, Upper Right quadrant, also in red) over the course of the bafilomycin treatment period as compared to vehicle-treated control cells over the same time span; and **(B)** a graphical depiction of the percentage distribution of viable and apoptotic/dead gated cells in response to vehicle and bafilomycin treatment over the same time span. LL-Lower Left, LR-lower right, UR- Upper Right.

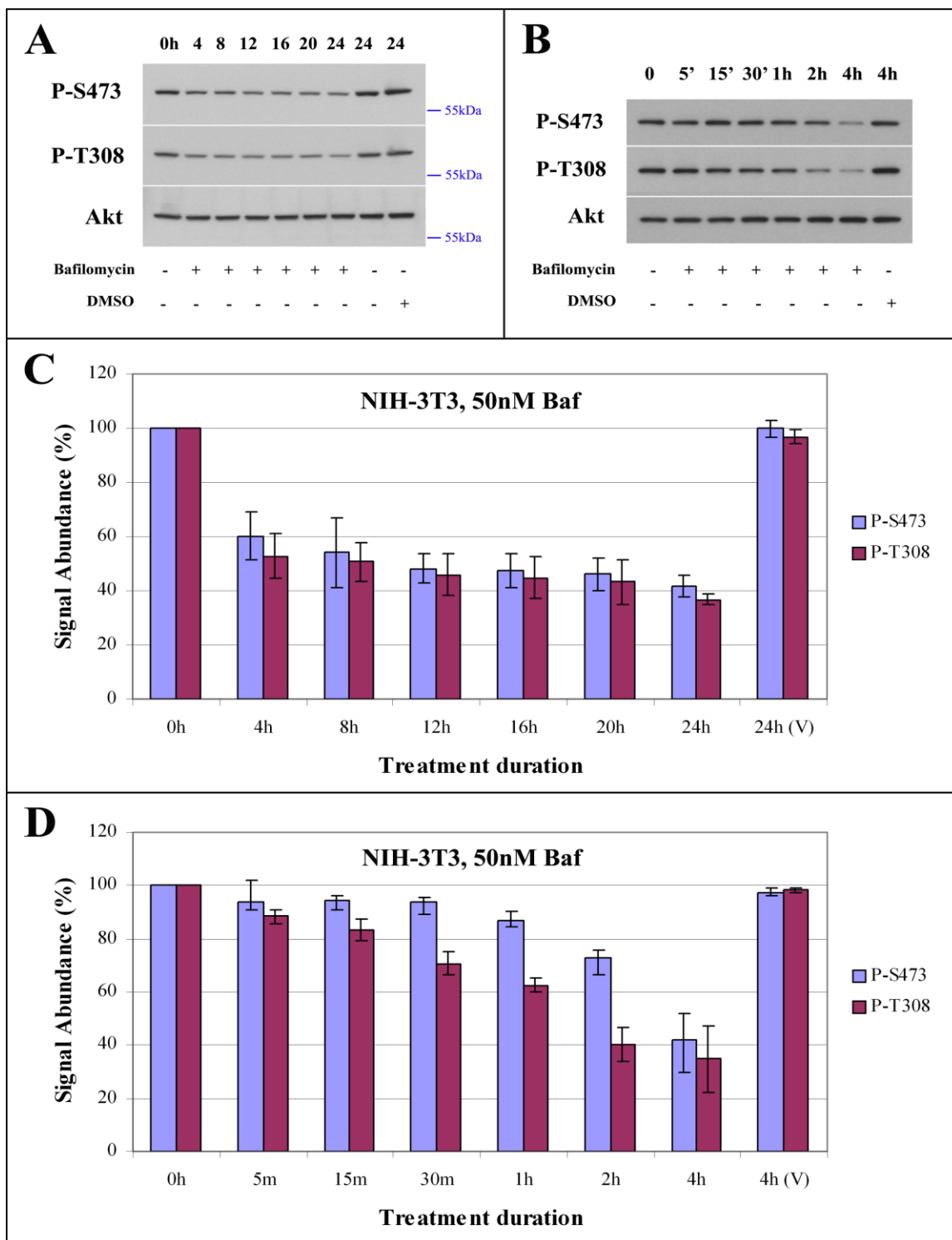


Figure 3-3. Bafilomycin time-dependently downregulates Akt phosphorylation in NIH-3T3 cells. Cultured NIH-3T3 fibroblasts (DMEM/5% FBS) were treated with 50nM bafilomycin for the indicated time spans and analyzed by Western-blotting. **(A)** Cultured NIH-3T3 cells were treated with 50nM bafilomycin for up to 24 hours and harvested at 4 hour intervals, with concomitant untreated and vehicle- (DMSO) treated samples

collected at the end of the time course. Whole-cell lysates from each time point were analyzed by Western-blotting for changes in the levels of Akt phosphorylation at S473 and T308 over the 24 hour incubation period, with total Akt protein as the loading control. The molecular weight values in the right-hand margin are based on the PageRuler™ (Fermentas) prestained protein ladder. **(B)** Cultured NIH-3T3 cells were treated with 50nM bafilomycin for up to 4 hours, and harvested at the indicated time intervals (in minutes and hours), with the concomitant vehicle- (DMSO) treated sample collected at the end of the 4 hour time course. Akt phosphorylation at S473 and T308 was analyzed by Western blotting, with total Akt protein as the loading control. The experiments described in **(A)** and **(B)** were carried out in triplicate, and representative Western blots were quantitated densitometrically for inverted luminosity in order to generate the graphical representations shown in **(C)** and **(D)**, respectively. The derived densitometric values for the S473 and T308 immunoblot signals at each examined time point were normalized to their corresponding total Akt values, and expressed as a percentage of the initial (0 hour) S473 and T308 signals, which were arbitrarily assigned a value of 100%.

treatment, thereafter reaching a plateau (~60%) maintained until the end of the 24 hour time course (**Figure 3-3A,C**).

Since much of the decrease in Akt phosphorylation in NIH-3T3 cells was observed in the first time interval examined, the first 4 hours of treatment with 50nM bafilomycin were further investigated at six intervals – 5, 15, 30, 60, 120, and 240 minutes of treatment). Examination of Akt phosphorylation over this shortened time span of bafilomycin treatment revealed only a *slight* decrease (~15%) in T308 phosphorylation 5-15 minutes after bafilomycin treatment, with a comparable decrease in S473 phosphorylation noticeable after ~1 hour of bafilomycin exposure (**Figure 3-3B,D**). Within the first 4 hours of bafilomycin treatment *as a whole*, however, the largest drop in the levels of T308 phosphorylation occurred between 1 and 2 hours of treatment (decrease from 60% to 40%), while a similar decrease was observed for S473 phosphorylation between 2 and 4 hours of treatment (decrease from 70-50%). These results suggested that the effects of bafilomycin on Akt phosphorylation, which are (1) noticeable within the first 4 hours of treatment, and (2) nearly-maximal (in relation to the level achieved at 24 hours) by the 12th hour of bafilomycin treatment. Further more, these effects were found to temporally precede the latent induction of caspase cleavage in the apoptotic response by at least 4 hours with respect to the near-maximal decrease in Akt phosphorylation, and by approximately 12 hours with respect to the onset of diminished Akt activation. However, although the cell death precipitated by bafilomycin treatment is of an apoptotic nature in NIH-3T3 cells, the precise mechanism of apoptotic induction is not addressed by these experiments, and cannot be directly correlated to any protective effects Akt activation may have in this “baseline” *in vitro* setting. For example, whereas the induction of caspase cleavage we observe in response to the treatment of NIH-3T3 cells with 50nM bafilomycin is latent with respect to our observed reduction in Akt phosphorylation, the apoptosis reported to occur in HeLa cells treated with 100nM bafilomycin was shown to occur over 24 hours concomitantly with autophagosome accumulation (**Boya et al 2005**). As such, in order to further assess the *subcellular* effects of bafilomycin-dependent V-ATPase inhibition, we restricted our temporal window of bafilomycin (50nM) treatment to 12 hours in the cellular fractionation experiments

described in the forthcoming sections of this chapter. Lastly, the incubation of larval tissues with bafilomycin, which was first referred to in **Section 2-3.3**, is carried out routinely in our lab with various pharmacological agents of diverse origin and structure, and has generally required concentrations that are approximately tenfold higher than those typically used in cultured cells, a distinction that is likely attributable to differences in the local exposure of the drug to cells in an intact *tissue* as opposed to individual *cells* grown in suspension or *monolayers* of adherent cultured cells. As such, the experiments we carried out in incubated larval tissues were also conducted out over a period of 12 hours, but were treated with 500nM bafilomycin, a concentration ten times higher than that used in cultured NIH-3T3 cells. As previously described in **Section 2-3.3**, the treatment of larval tissues with 500nM bafilomycin over a time course of 12 hours had effects on Akt-HA phosphorylation that were comparable to those we observed in NIH-3T3 cells treated with 50nM bafilomycin over the same time span (**Figure 3-3A**).

3-3.2 – Subcellular fractionation and compartmental enrichment of bafilomycin-treated NIH-3T3 cells

As described in the Materials and Methods and **Figure 3-4A**, near-confluent NIH-3T3 cells maintained under standard culture conditions (DMEM/5%FBS) were treated with 50nM bafilomycin (2, 4, and 12 hours prior to harvest), harvested simultaneously in hypotonic lysis buffer (as opposed to the detergent-containing RIPA buffer utilized for the harvest of whole cell lysates in the 24 hour time course described in the previous section), and fractionated by sequential centrifugation steps to produce distinct subcellular lysates enriched for cytoplasmic (CY), membrane (M), endomembrane (EN) and nuclear/perinuclear (N) proteins for each time point (TP). The resulting subcellular fractions from vehicle-treated control cells (TP0, which represents the baseline of signaling activity in the presence of 5% FBS in the culture medium), or cells treated in parallel with 50nM bafilomycin (TP2, TP4, and TP12) were first analyzed by Western blotting for the assessment of fractionation efficiency with respect to the fraction-specific enrichment within each purified compartment of established molecular markers with known subcellular distribution patterns. As such, the examination of *uncleaved* pro-

caspase-3 (**Figures 3-4C, 3-5B**), whose nuclear translocation requires its proteolytic activation (**Kamada et al 2005**), and GAPDH (**Figures 3-4B, 3-5A**), both predominantly cytoplasmic proteins, confirmed the enrichment of the cytoplasmic fraction itself, while the lack of detectable contaminating signals in the other subcellular fractions suggested the efficient separation of the cytoplasmic compartment from the remainder of the cell in our fractionation protocol. Similarly, blotting for the nucleosomal protein Histone H3 and the transcription factor CREB (cAMP response element-binding protein), two peptides with established roles in the nucleus, where they are known to predominantly reside, confirmed the enrichment of the nuclear/perinuclear fraction, with a similar absence of contamination in non-nuclear fractions, with the exception of the trace presence of H3 in the M fraction (**Figures 3-4D,E and 3-5C,D**). Both the membrane fraction (M), which predominantly consists of stretches of the plasma membrane lipid bilayer, but can also include the cisternal membrane of the Golgi apparatus, and sloughed-off portions of the endoplasmic reticulum (likely the source of H3 in the M fraction); and the endosomal fraction (EN) which is a collection of heterotypic endomembrane vesicles of various size, density, subcellular origin and destination; tend to contain proteins whose subcellular localization is dynamic in nature, and therefore, unlike exclusively nuclear (H3, CREB) or cytoplasmic (GAPDH, pro-caspase 3) molecules, are likely to be found in multiple intracellular compartments. This is exemplified by the subcellular distribution of EEA1 (**Figures 3-9E, 3-10D**), which though enriched in the endosomal fraction, can be detected in appreciable abundance throughout all four compartments isolated in our assay, and is further discussed in **Section 3-3.5** in the context of changes in the localization of selected membrane traffic regulators.

Nonetheless, in order to address the enrichment of plasma membrane proteins in the membrane fraction (M), we examined the subcellular distribution of unphosphorylated β -catenin with an antibody that only detects β -catenin molecules lacking phosphorylation at S33/S37/T41. In the absence of Wnt signaling, cytoplasmic β -catenin is usually phosphorylated at these residues by GSK3, and thereby destabilized, ubiquitinated, and targeted for degradation (**Yost et al 1996**). Accordingly, the cytoplasmic fraction was found to contain very low, nearly undetectable levels of non-phosphorylated β -catenin (**Figures 3-4F, 3-5E**), whereas a prominent signal was

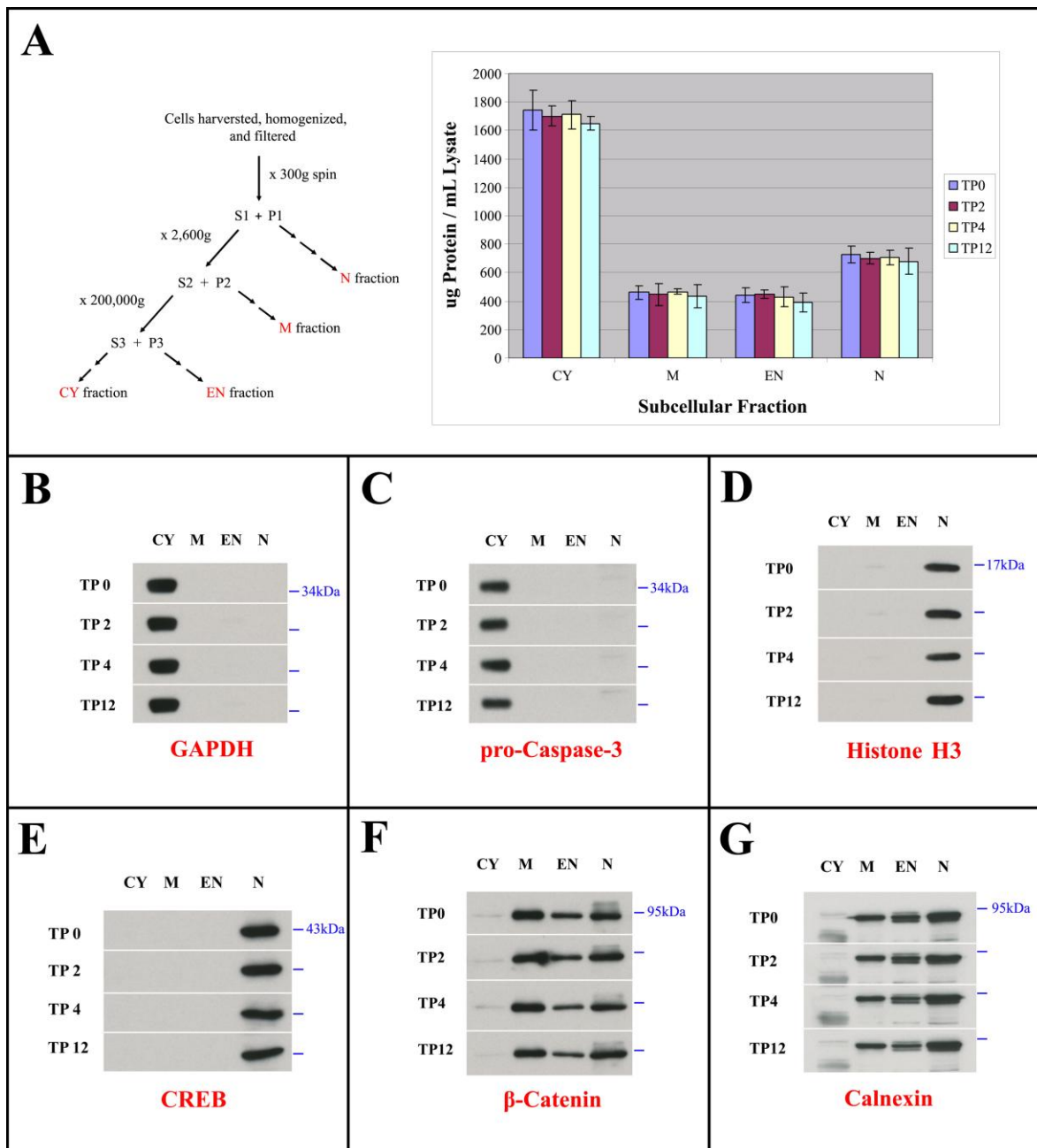


Figure 3-4. Subcellular fractionation of bafilomycin-treated NIH-3T3 cells. NIH-3T3 cells (DMEM/5%FBS) were incubated with 50nM bafilomycin for 2, 4, or 12 hours, and harvested simultaneously with the vehicle-treated control (TP0) in hypotonic buffer. **(A)** As described in the Materials and Methods, the fractionation protocol results in the generation of four distinct intracellular fractions enriched with cytoplasmic (CY), membrane (M), endomembrane (EN) and nuclear/perinuclear (N) proteins for all four time points (0, 2, 4 and 12 hours). The bar graph is a representation of the subcellular distribution of the total amount of protein harvested in each fraction, expressed in $\mu\text{g/mL}$. The values expressed in the graph are based on the spectrophotometric values obtained from two separate fractionation experiments using the Bradford method of protein

quantitation. **(B-G)** Representative Western-blot images of the intracellular distribution of GAPDH **(B)** and pro-Caspase-3 **(C)**, which are highly enriched in the cytoplasm; Histone H3 **(D)** and CREB **(E)**, which are highly enriched in the nucleus; unphosphorylated β -Catenin **(F)**, which is enriched in the membrane and nuclear fractions; and Calnexin **(G)**, which displays a largely non-cytoplasmic distribution. The molecular weight values in the right-hand margins of panels **B-G** are based on the PageRulerTM (Fermentas) prestained protein ladder.

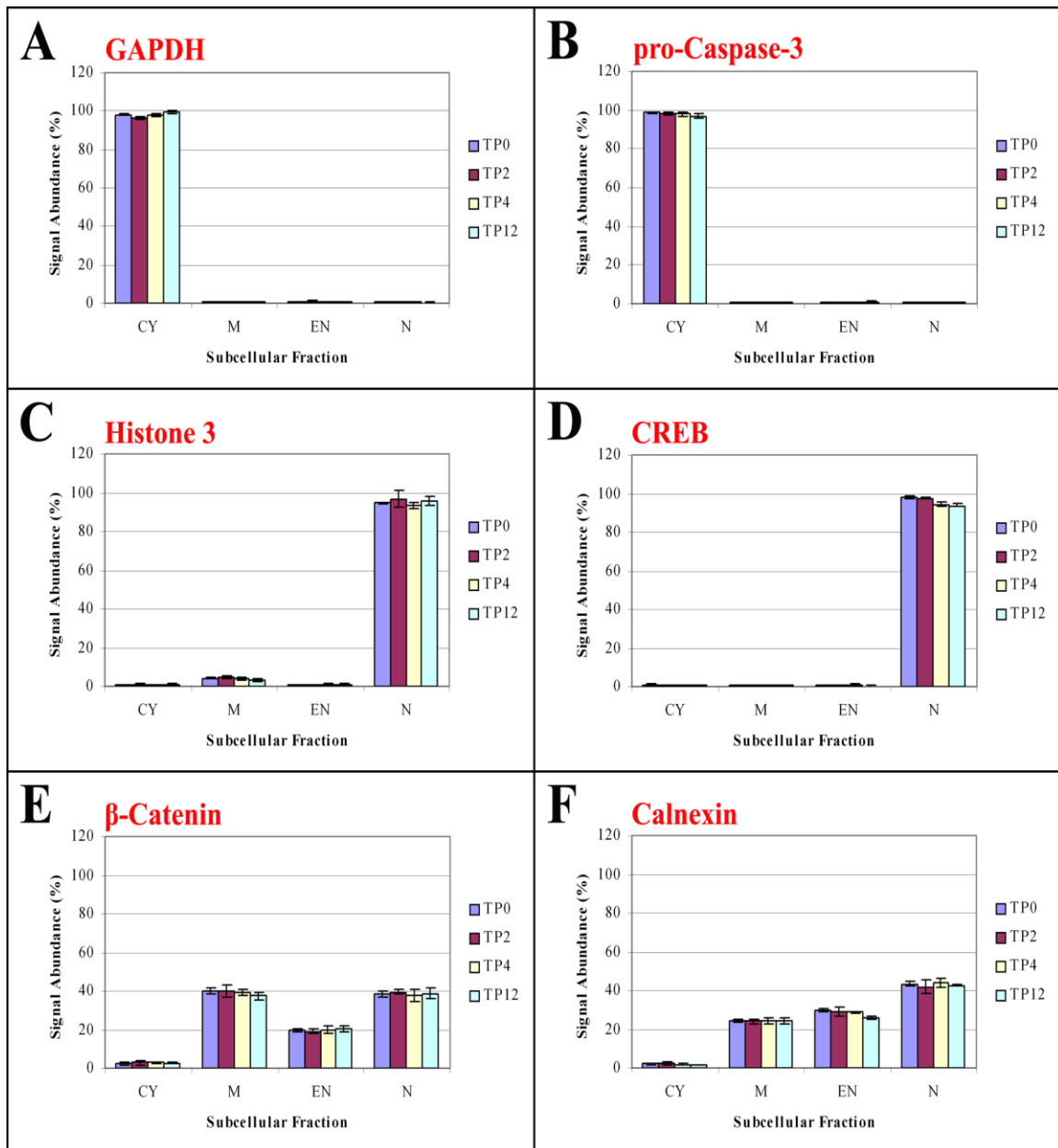


Figure 3-5. Fractional enrichment of subcellular markers in bafilomycin-treated NIH-3T3 cells. The representative Western blots described in **Figure 3-4**, and those from an identical duplicate experiment were quantitated densitometrically for inverted luminosity in order to generate the graphical representations shown for the intracellular distribution of: (A) GAPDH, (B) pro-caspase-3, (C) Histone 3, (D) CREB, (E) β -Catenin, and (F) Calnexin proteins. With all six data sets, the control densitometric values derived for each subcellular fraction (CY, M, EN and N) at TP0 were expressed as a percent of the total densitometric value calculated from the densitometric sum of all four fractions at TP0. The densitometric values subsequently derived for each subcellular fraction at TP2, TP4 and TP12 were then expressed as percent values normalized to the percent TP0 value (represented by the blue bars) of their respective subcellular fractions.

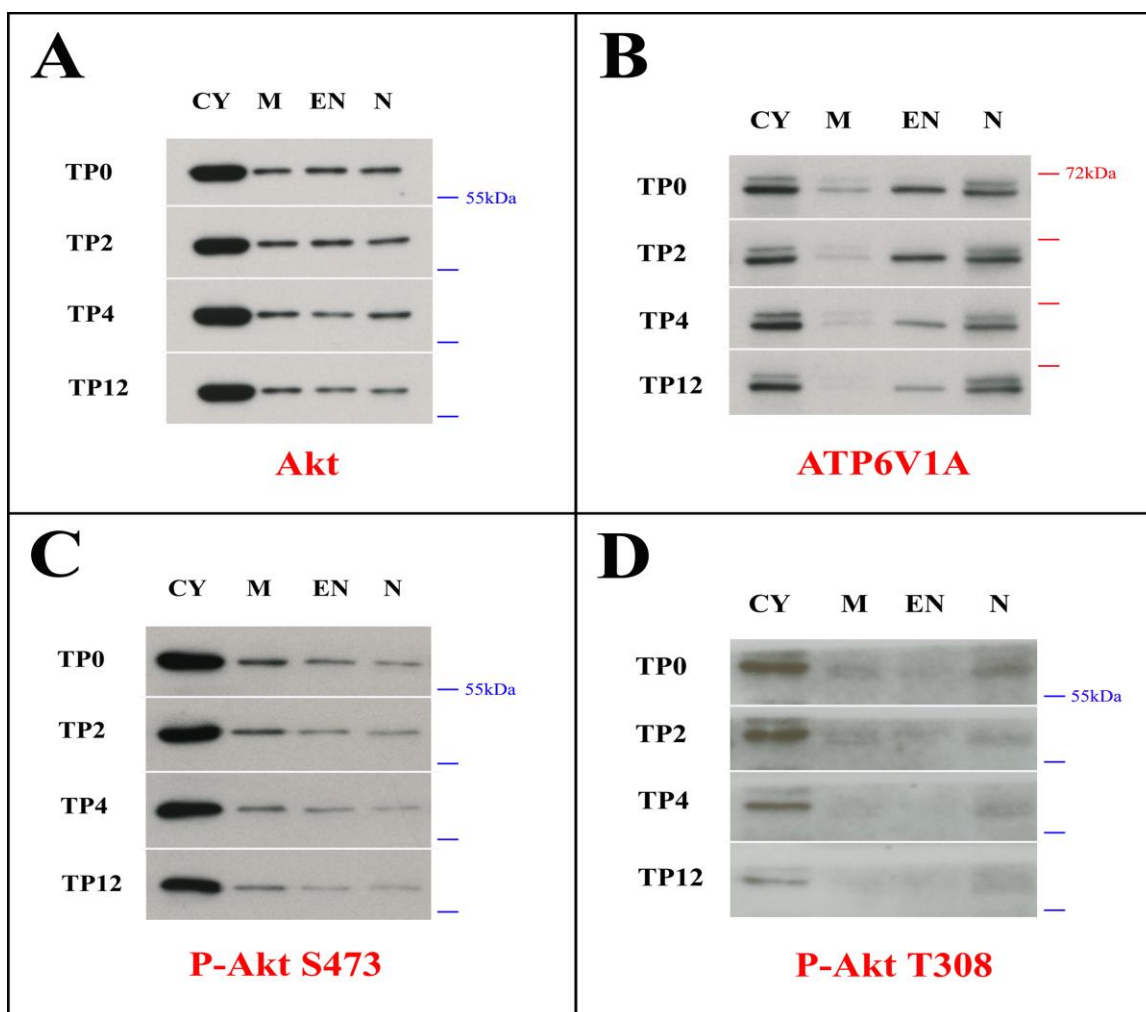


Figure 3-6. Intracellular distribution of Akt and V-ATPase in bafilomycin-treated NIH-3T3 cells. As in **Figure 3-4**, samples from all 4 time points of bafilomycin treatment (0, 2, 4, and 12 hours) were analyzed by Western blotting as a function of intracellular fraction – cytoplasmic (CY), membrane (M), endomembrane (EN) and (N) nuclear/perinuclear). A representative Western blot of Akt protein (**A**) demonstrates the presence of a predominant cytoplasmic (CY) reservoir, with discrete pools detectable in the M, EN and N fractions. (**B**) ATP6V1A (V-ATPase, subunit A) protein was detected abundantly in the CY, EN, and N fractions, with a very discrete pool present in the M fraction. (**C, D**) The phospho-Akt signals (S473 and T308), like total Akt, were predominantly cytoplasmic. With respect to the non-cytoplasmic pools of phospho-Akt, the S473 signal was readily detectable in all three subcellular fractions (M, EN, and N) whereas the main non-cytoplasmic presence of T308 was observed in the nuclear/perinuclear (N) fraction. The molecular weight values in the right-hand margins are based on the PageRulerTM (Fermentas) prestained protein ladder.

observed in the membrane fraction, presumably representing the plasma membrane localized pool of β -catenin involved in membrane adherens junctions (reviewed in **Hartsock and Nelson 2008**), and in the nucleus, where β -catenin (lacking phosphorylation at S33/S37/T41) can act as a transcription factor (see **Section 1-4.2**). Furthermore, the immunoblot signal intensity and subcellular distribution of these marker proteins was comparable throughout the examined time intervals of the experiment, recapitulating the consistency in the overall yield as well as in the protein content of each harvested fraction (**Figure 3-4A**), and exemplified by the unperturbed distribution of Calnexin, which can be detected in all but the cytoplasmic compartment (**Figures 3-4G, 3-5F**), consistent with its being a transmembrane molecule localized primarily in the perinuclear ER, but also present in various endomembrane compartments (**Myhill et al 2008**).

3-3.3 – Bafilomycin treatment induces changes in the intracellular localization and compartment-specific activation of Akt

Following our establishment of the efficiency of the subcellular fractionation protocol, the CY, M, EN and N fractions harvested from NIH-3T3 cells treated with 50nM bafilomycin (for 2, 4, and 12 hours) were analyzed by Western blotting to biochemically assess the subcellular distribution of Akt protein as a function of subcellular fraction at each examined time interval. The cytoplasm was found to harbor ~55% of total cellular Akt in untreated samples (**Figures 3-6A, 3-7A**), with the remaining ~45% being equally distributed in the M, EN, and N fractions. This relative pattern of Akt distribution was not overtly disturbed over the course of the 12 hour bafilomycin time course; although a distinct time-dependent reduction in Akt abundance was reproducibly observed in the EN and N fractions, with corresponding time-dependent increases in the cytoplasmic abundance of Akt. As shown in **Figure 3-7A**, in which the densitometric quantitation of the Akt signal in each compartment (displayed as a fraction of the total cellular abundance in all four compartments) is represented graphically, the time-dependent decreases in endosomal (EN) and nuclear (N) Akt abundance combine to represent a modest shift (combined loss of 10-15% in EN/N fractions, equivalent gain of

~10% in CY) in the localization of *total* cellular Akt. However, the *compartment-specific* loss of Akt localization in these fractions represents nearly *half* of the Akt pool present in these respective compartments in untreated (TP0) samples. In other words, though ~17% of total cellular Akt is localized to endosomes in untreated samples, the pool of endosomal Akt is reduced to ~10% after 4 hours of treatment with 50nM bafilomycin. This only represents a ~7% change with respect to total Akt distribution, but accounts for a significant portion of the endosomal population of Akt (~40%). Similarly, the nuclear pool of Akt accounts for ~15% of cellular Akt, and is reduced by nearly *half* after 12 hours of treatment, appearing more latently (but slightly more severely) than the effect observed in the endosomal fraction (40% decrease), which occurs 8 hours earlier at TP4 (**Figure 3-7A**), but preceding the induction of caspase-3 cleavage, which can be detected from TP16 onwards (**Figure 3-1B**), by ~4 hours.

As described in the introductory chapter (**Section 1-3**), phosphorylation of the Akt hydrophobic motif (HM, S473 in Akt1) is primarily carried out by mTORC2 in most signaling contexts, while phosphorylation of the Akt T-loop is PDK1-dependent. Like the general distribution pattern of total cellular Akt, the phospho-S473 signal prior to bafilomycin administration (TP0) is most abundant in the cytoplasmic fraction (**Figures 3-6C, 3-7C**), in which it accounts for ~60% of cellular Akt phosphorylated at this residue, with the remainder of phospho-S473 distributed in intracellular compartments (~18% in M, ~12% in EN, and ~10% in N). A decrease in levels of S473 phosphorylation over the duration of the treatment period was observed in all four subcellular fractions, with EN and N pools of Akt nearly devoid of S473 signal by the conclusion of the 12 hour treatment period. The CY fraction, which accounts for ~60% of the total cellular phospho-S473 signal, time-dependently diminishes to ~45% after 12 hours of bafilomycin treatment (or a ~25% compartment specific-decrease), but the largest relative compartment-specific decreases were found to occur (also time-dependently) in the M, EN, and N fractions. By TP12, the compartment-specific abundance of phospho-S473 was decreased by ~50% in the M fraction (from ~18% of the total cellular signal at TP0 to ~9% at TP12), ~80% in the EN fraction (from ~12% at TP0 to ~2% at TP12), and ~66% in the N fraction (from ~9% at TP0 to ~3% at TP12). The CY fraction also accounts for ~60% of the phospho-T308 signal at TP0 (**Figures 3-6D, 3-7D**), but in

contrast to the ~25% CY-specific decrease observed in phospho-S473 levels over 12 hours of treatment, the phospho-T308 signal was compartment-specifically diminished by ~66% in the CY over the same time span (from ~60% of the total cellular signal at TP0 to ~20% at TP12). The second-largest pool of phospho-T308 was in the N fraction (~25% of the phospho-T308 signal at TP0), in which its levels time-dependently decrease by ~60% in response to bafilomycin administration (from ~25% of the total cellular signal at TP0 to ~10% at TP12). The remaining ~15% of total phospho-T308 (CY and N combine to represent ~85% of the phospho-T308 signal) is roughly evenly distributed in the M and EN compartments (~8% each), and is negligible (levels below 2%, >75% loss) after 4 hours of bafilomycin treatment.

We also examined the subcellular localization of the V-ATPase A subunit, (encoded in *Drosophila* by the *Vha68* 1-3 genes), represented in mammals by *ATP6V1A*. As mentioned in the introduction (see **Section 1-5.1**), the A subunit is a component of the peripheral ATP-hydrolyzing V_1 domain, which is cytoplasmic, and whose recruitment to the integral V_0 domain is an important regulatory step in V-ATPase activation at the surface of endomembranes along the endocytic pathway (**Lafourcade et al 2008**, reviewed **Toei et al 2010**). In contrast to the integral transmembrane V_0 domain (which is highly hydrophobic and exclusively membrane-associated), the V_1 domain is abundantly represented in the cytoplasmic fraction of growing NIH-3T3 cells at TP0 (~35% of total cellular *ATP6V1A*, **Figures 3-6B, 3-7B**), and maintained at that level in the CY compartment throughout the 12 hour time course. Prior to the administration of bafilomycin (TP0), it can also be readily detected (~30% of total *ATP6V1A*) in the N fraction, presumably in association with the multitude of perinuclear vesicles whose acidification and/or fusion is V-ATPase-dependent, and this perinuclear/nuclear pool of *ATP6V1A* is also unperturbed over the course of 12 hours of bafilomycin treatment. Interestingly however, the prominent pool of endosome-associated *ATP6V1A* molecules in the EN fraction (~25% of total *ATP6V1A* at TP0) and the smaller membrane-associated pool in the M fraction (~10% of total *ATP6V1A* at TP0) are significantly depleted over the 12 hour time course of bafilomycin treatment, including a ~50% decrease observed in the EN fraction (from ~25% of the total cellular signal at TP0 to ~12% at TP12), with the largest drop occurring between 2 and 4 hours of treatment

(**Figure 3-7B**). The bafilomycin-dependent effect on ATP6V1A abundance in the EN fraction mirrors the extent and time course of the diminished localization of total Akt in the same compartment (**Figure 3-7A**), suggesting a potential correlation between the bafilomycin-dependent diminished localization/activation of Akt in the endosomal compartment and the bafilomycin-dependent inhibition of V-ATPase activity, which may itself be partly due to the diminished localization of ATP6V1A to the endosomal surface.

3-3.4 – Bafilomycin treatment downregulates mTORC1/S6K1 signaling concomitantly with Akt inhibition

Having observed whole-cell (**Figure 3-3**) and compartment-specific (**Figures 3-6, 3-7**) bafilomycin-induced downregulation of Akt phosphorylation at both S473 and T308, we examined the activity of the upstream-acting kinases largely responsible for their phosphorylation (mTORC2 and PDK1, respectively), both in whole cell lysates generated over 24 hours of bafilomycin treatment (as in **Figure 3-3**), and in the subcellular compartments of fractionated cells treated with bafilomycin for 12 hours (or vehicle-treated) as described in **Section 3-3.2**. As discussed in the previous chapter, our experiments in insulin-stimulated *Drosophila* larval tissues suggested that while treatment with 500nM bafilomycin or concanamycin diminished Akt phosphorylation (**Figures 2-3, 2-11**), it had no effect on either PIP₃ abundance (**Figure 2-12**) or the PH domain-dependent recruitment of tGPH to PIP₃-rich intracellular domains (**Figure 2-13**). In contrast to Akt, whose PDK1-dependent T-loop phosphorylation is PH domain-mediated and agonist-dependent, the activating phosphorylation of the PDK1 HM motif (S241) is thought to be mediated by autophosphorylation, which even occurs in bacterially expressed molecules independently of agonist stimulation or PI3K activation (**Casamayor et al 1999**). Accordingly, Western blotting of whole-cell lysates generated from NIH-3T3 cells treated with 50nM bafilomycin showed no significant changes in S241 phosphorylation over the course of 24 hours of treatment, suggesting that the autophosphorylation and activation of PDK1 was undisturbed (**Figure 3-8A**). In addition to the requirement of S241 autophosphorylation for PDK1 activity, its tyrosine-phosphorylation at Y373/Y376, which is thought to be mediated by Src (**Grillo et al**

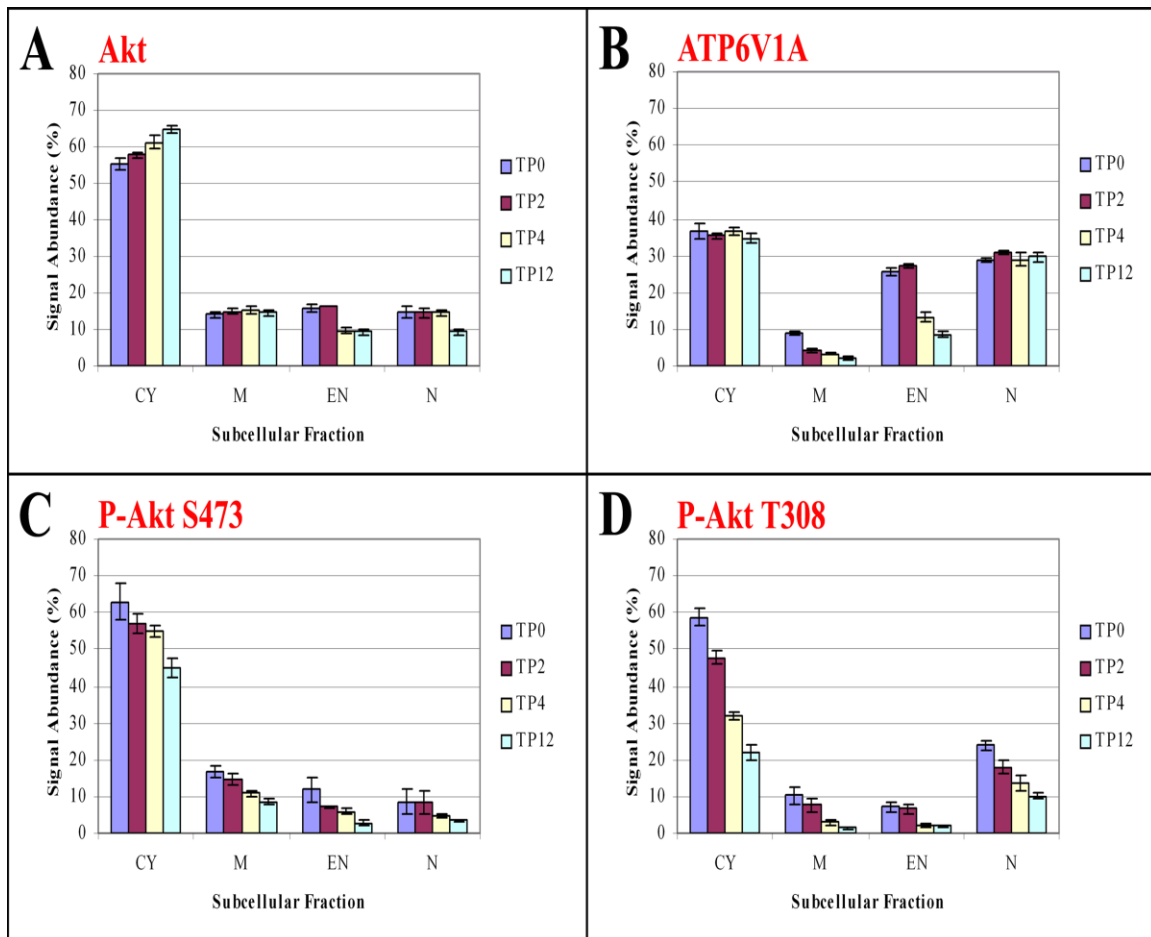


Figure 3-7. Differential downregulation of Akt phosphorylation in intracellular fractions of bafilomycin-treated NIH-3T3 cells. The representative Western blots described in **Figure 3-6**, and those from an identical duplicate experiment, were quantitated densitometrically for inverted luminosity in order to generate the graphical representations shown for the intracellular distribution of: **(A)** Akt protein, **(B)** ATP6V1A (V-ATPase, A subunit), **(C)** Akt protein phosphorylated at S473, and **(D)** Akt protein phosphorylated at T308. With all four data sets, the control densitometric values derived for each subcellular fraction (CY, M, EN and N) at TP0 were expressed as a percent of the total densitometric value calculated from the densitometric sum of all four fractions at TP0. The densitometric values subsequently derived for each subcellular fraction at TP2, TP4 and TP12 were then expressed as percent values normalized to the percent TP0 value (represented by the blue bars) of their respective subcellular fractions.

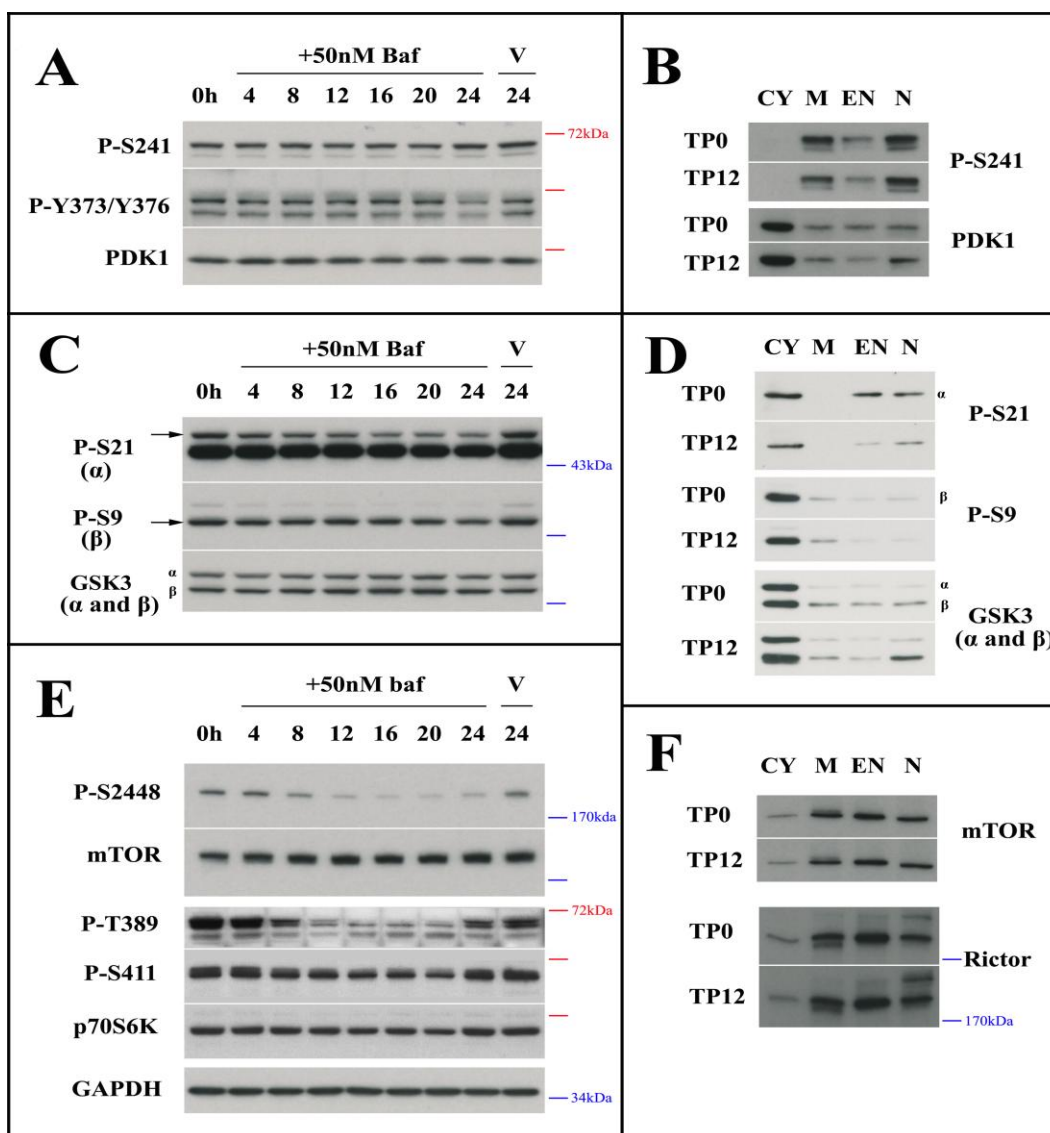


Figure 3-8. Intracellular distribution and activity of ISP signaling components in bafilomycin-treated NIH-3T3 cells. (A, C, E) As in **Figures 3-1B** and **3-3**, NIH-3T3 cells were treated with 50nM bafilomycin or the vehicle control (DMSO) for up to 24 hours, and harvested at 4 hour intervals. Whole cell lysates were Western blotted for: (A) total PDK1 protein and phospho-PDK1 (S241 and Y373/376); (C) total GSK3 (α/β) protein, phospho-GSK3 (S21 in GSK3α and S9 in GSK3β are both recognized by the CST #9331 antibody); and (E) total mTOR protein, phospho-mTOR (S2448), total p70S6K protein, and phospho-p70S6K (T389 and S411). GAPDH was used as a loading control throughout. (B, D, and F) As in **Figure 3-4**, fractionated samples of bafilomycin-treated NIH-3T3 cells were analyzed by Western-blotting as a function of intracellular fraction between 0 and 12 hours of bafilomycin treatment for differences in (B) Total PDK1 and phospho-PDK1 (S241); (D) total GSK3 (α/β), phospho-GSK3 S21 (ab28808), and S9 (ab107166); and (F) total mTOR, phospho-mTOR (S2448), and Rictor protein distribution. The molecular weight values in the right-hand margins are based on the PageRulerTM (Fermentas) prestained protein ladder.

2000, Park et al 2001, Yang et al 2008), has been shown to further activate PDK1 in response to insulin treatment in HEK-293T cells overexpressing IR (Park et al 2001), and to be required for insulin-mediated metabolic signaling in insulin-sensitive skeletal muscle cells (Fiory et al 2005). Similar to the status of S241 autophosphorylation, no changes were observed in the levels of Y373/Y376 Src-mediated phosphorylation, with the exception of a tangible drop in phosphorylation levels after 24 hours of bafilomycin treatment (Figure 3-8A), a latent effect that correlates with peaks of pro-caspase-3 cleavage (TP16-TP24, Figure 3-1B) rather than the inhibition of Akt phosphorylation at T308, which can be detected within the first 4 hours of bafilomycin treatment, Figure 3-3).

Despite the demonstrated *in vitro* capacity of PDK1 to agonist-independently promote the autophosphorylation of its HM, cytoplasmic pools of PDK1 (like those of Akt in *unstimulated* cells) are generally hypophosphorylated, whereas hyperphosphorylated pools of PDK1 (like those of Akt in *stimulated* cells) are associated with subcellular compartments such as the signaling membranes of stimulated cells (see Section 1-3.4). This suggests that though PDK1 is evidently capable of agonist-independent autophosphorylation *in vitro*, its cytoplasmic retention in intact cells correlates with the inhibition of its autoactivation, which is only facilitated, along with phosphorylation at other sites such as S396, upon its translocation to its designated (non-cytoplasmic) site(s) of activity. Accordingly, under standard tissue culture conditions (TP0, culture media supplemented with 5% FBS), Western blots of the fractionated NIH-3T3 cells revealed that although the predominant reservoir of PDK1 (like Akt) is situated in the cytoplasm (with subpopulations residing in the M, EN, and N fractions), the majority of PDK1 molecules with phosphorylated HM motifs resided in the subcellular compartments (Figure 3-8B), with highest levels found in the M fraction, its main site of agonist-induced Akt T-loop phosphorylation; and the nucleus (N fraction), to which it translocates in a phosphorylation-dependent manner (Lim et al 2003, Scheid et al 2005). Significantly, neither the subcellular PDK1 localization pattern, nor the levels of S241 phosphorylation varied significantly over the 12 hour bafilomycin time course (Figure 3-8B), which is consistent with the effect (or lack thereof) observed in whole-cell lysates (Figure 3-8A); and in addition to defective PIP₃ production and loss of PH domain-

dependent membrane recruitment, also eliminates defective PDK1 activation as a potential causal factor behind the downregulation of Akt T308 phosphorylation in response to bafilomycin treatment.

As described in the introductory chapter, Akt represents a dual nodal point of coordination between the ISP and mTOR-mediated signaling processes. Two distinct complexes feature mTOR catalytic activity – mTORC1 (minimally consisting of mTOR, Raptor, and mLST8), and mTORC2 (minimally consisting of mTOR, Rictor, mSin1, and mLST8), which both participate in Akt signaling but act at different levels in the signaling pathway and integrate distinct stimuli. The mTORC2 complex acts upstream of Akt, and in response to growth factor stimulation, phosphorylates Akt at S473 (see **Section 1-3.3**), while mTORC1 is active downstream of Akt, and jointly regulated by nutritional status through the Ragulator (see **Section 1-4.5**), and growth factor signaling through Akt-mediated Rheb activation (see **Section 1-4.3**). Importantly, though their respective mechanisms of membrane localization differ, the enzymatic activation of mTORC1 and mTORC2, like that of PDK1, is dependent on agonist stimulation, and is correlated with their membrane localization – the PM and the surface of endocytic signaling membranes in the case of mTORC2, and the lysosomal surface in the case of mTORC1. Although the agonist-dependent regulation of mTORC2 activity is not fully understood, Akt HM phosphorylation at S473 is a common read-out of mTORC2 activity, and as described above (**Figures 3-3, 3-6, 3-7**), is downregulated in response to bafilomycin treatment in NIH-3T3 cells. Our subsequent examination of mTOR cellular localization in Western blots of fractionated NIH-3T3 cells grown under standard culture conditions (DMEM/5% FBS) demonstrated that like active (autophosphorylated at S241) pools of PDK1, total mTOR protein in these growing cells was predominantly localized to intracellular compartments, with highest levels in the M and EN fractions (**Figure 3-8F**), consistent with the established importance of the PM and the surface of endomembranes as hubs of mTOR-dependent signaling (both mTORC1- and mTORC2-mediated). Both the basal distribution of mTOR and the highly similar distribution pattern of Rictor (as a marker of mTORC2 localization) observed in cultured NIH-3T3 cells (TP0, 5%FBS) was undisturbed following 12 hours of bafilomycin treatment

(**Figure 3-8F**), suggesting that the decreases in Akt S473 phosphorylation in the same time span of treatment are not due to gross mTORC2 mislocalization, but rather, may be caused by variables not addressed by our experiments, such as mTORC2 activation itself, and the physical interaction of mTORC2 with Akt, whose abundance in the intracellular M, EN, and N fractions, as mentioned above, is reduced over the course of 12 hours of bafilomycin treatment (**Figures 3-6, 3-7**).

Since mTORC1 activation, which occurs following its nutrient-dependent recruitment to the lysosomal membrane where it is activated by growth factor-mediated relief of Rheb inhibition, can be inhibited by either the prevention of its amino acid availability-dependent recruitment to the endomembrane surface (as demonstrated by Zoncu et al (**2011a**) in response to the acute treatment of cultured cells with high concentrations of concanamycin), or as we have demonstrated, through the (comparatively) latent downregulation of Akt in response to the chronic administration of lower levels of bafilomycin; the diminished activation of Akt that we observe in NIH-3T3 cells should also presumably downregulate mTORC1 activation through the diminished growth factor-dependent activation of Rheb. Accordingly, we investigated the phosphorylation status of the S6K1 HM site (T389), which is an established direct rapamycin-sensitive target of mTORC1 activity (**Pearson et al 1995**), and most closely correlates to S6K1 activity *in vivo*. In cultured NIH-3T3 cells treated with 50nM bafilomycin over a 24 hour treatment period, we observed a time-dependent decrease T389 phosphorylation that was noticeable by TP8, near-maximally downregulated by TP12, and maintained at these low levels for the remainder of the time course (**Figure 3-8E**).

Upon activation by mTORC1, S6K1 also participates in a poorly understood feedback loop involving mTORC1 itself as a substrate for S6K1 activity, which phosphorylates mTORC1 at S2448 (**Holz and Blenis 2005, Chiang and Abraham 2005**). However, since S2448A point mutants of mTOR exhibit normal signaling, phosphorylation of this site, currently of unclear significance, has nonetheless served as a reliable read-out of mTORC1-dependent S6K1 activity. Accordingly, Western blots of whole cell lysates derived from bafilomycin-treated NIH-3T3 cells revealed a significant time-dependent decrease in S2448 phosphorylation, whose time course matched that of

the bafilomycin-dependent decrease of S6K1 T389 phosphorylation (**Figure 3-8E**). In contrast to the mTORC1-mediated phosphorylation of S6K1 at T389, to which Akt activation contributes by promoting Rheb activation upstream of mTORC1 stimulation, and which is downregulated, like Akt phosphorylation, in bafilomycin-treated NIH 3T3 cells; the phosphorylation of S6K1 at S411, which is located in the S6K1 C-terminal autoinhibitory pseudo-substrate domain, and whose phosphorylation is required for subsequent mTORC1-dependent T389 phosphorylation, has been shown to be catalyzed independently of Akt signaling by numerous kinase activities, including ERKs, p38 SAPK, and various cyclin-dependent kinases (**Weng et al 1998, Papst et al 1998, Shah et al 2003, Hou et al 2007**). Accordingly, our examination of S6K1 phosphorylation at S411 in Western blots of whole cell lysates derived from NIH-3T3 cells treated with bafilomycin over the course of 24 hours revealed a subtle time-dependent decrease in S411 phosphorylation that, though evident over the course of 24 hours of treatment, was modest in magnitude in comparison to the significant decrease in T389 phosphorylation (or mTOR S2448 phosphorylation) observed over the same period of bafilomycin treatment (**Figure 3-8E**).

Lastly, we examined the phosphorylation state and subcellular distribution of the multifunctional kinase GSK3 (α and β isoforms), a canonical (but *not* exclusive) downstream target of Akt catalytic function, which is phosphorylated by Akt, among other kinases, at the N-terminal S21/S9 residues (α and β , respectively), creating a primed pseudo-substrate that inhibits GSK3 kinase activity towards the majority of its substrates, which include various growth and survival promoting factors negatively regulated by GSK3 in the absence of growth factor stimulation (see **Section 1-4.2**). In unstimulated cells, GSK3 is thought to play a constitutively inhibitory role in two hallmark biosynthetic cellular responses to insulin stimulation, namely GS-mediated glycogen synthesis and eIF-mediated protein synthesis (see **Section 1-4.2**); as well as direct and indirect pro-apoptotic roles in the cytoplasm and the nucleus (see **Section 1-4.7**). The growth-inhibitory and pro-apoptotic functions of GSK3 kinase activity are therefore relieved by growth factor stimulation, and accordingly, in growing NIH-3T3 cells cultured in the presence of 5% FBS, the growth factor-mediated activation of GSK3

regulators such as Akt maintains the inhibitory phosphorylation of GSK3 at S21/S9 as seen in Western blots of untreated and vehicle-treated NIH-3T3 whole-cell lysates (TP0, TP24, **Figure 3-8C**).

Over the course of 24 hours of bafilomycin treatment, the time-dependent decrease in the S21/S9 phosphorylation state of GSK3 α/β was most pronounced in the case of the α isoform, whose pattern closely resembled that seen for the bafilomycin-dependent decrease in Akt S473/T308 phosphorylation (**Figure 3-8C**); whereas the phosphorylation status of the β isoform was milder, and most similar to that observed for mTORC1-independent S6K1 phosphorylation at S411 (**Figure 3-8E**). Our subsequent examination of GSK3 phosphorylation and subcellular distribution in Western blots of fractionated vehicle- and bafilomycin-treated cells recapitulated the isoform-specific decrease in GSK3 phosphorylation. The GSK3 α isoform was predominantly detected in the CY fraction, with trace levels in the M, EN, and N fractions, and the GSK3 β isoform, which is expressed at comparable levels, is similarly cytoplasmic, but with a more pronounced representation in the M, EN, and N fractions (**Figure 8-3D**). GSK3 α phosphorylation at S21 is readily detectable in all but the M fraction of NIH-3T3 cells, and consistent with our observations in bafilomycin-treated whole-cell lysates (**Figure 3-8C**), was diminished in all three fractions (CY, EN, and N) following 12 hours of bafilomycin treatment, whereas the phosphorylation of GSK3 β at S9, which is primarily detected in the CY fraction, is relatively unaltered over the same period of bafilomycin treatment. Notably, the only overt change in GSK3 β abundance/phosphorylation was observed in the N fraction of bafilomycin-treated cells, in which a significant accumulation of *unphosphorylated* GSK3 β was detected following 12 hours of bafilomycin treatment, which though purely phenomenological and empirical in the context of our assay, is consistent with the nuclear upregulation of GSK3, whose pro-apoptotic consequences may contribute to the latent onset of caspase 3 cleavage (initiated after 16 hours of bafilomycin treatment, **Figure 3-1B**) and subsequent apoptotic cell death (**Figure 3-2**).

3-3.5 – Bafilomycin treatment causes discrete changes in the compartment-specific localization of selected endomembrane markers

As described in this chapter's introduction, most cellular functions depend on the appropriate compartmentalization of signaling molecules. This requirement mandates that transport between specialized organelles be tightly controlled in order to maintain the dynamic network of intracellular traffic within the required functional parameters of efficient cellular function. The various distinct compartments of the endocytic pathway, including the heterotypic population of early endosomes and exosomes, sorting stations like recycling endosomes and late endosome/MVBs; as well as destinations including terminal endomembranes such as lysosomes, the PM, the nucleus, and other organelles, are actively transported (rather than passively diffused) by microtubule- or actin-dependent molecular motors, and establish characteristic intracellular distributions depending on cell type and the nature/identity of cellular processes under endocytic regulation. For example, EEA1-containing early endosomes tend to be more peripherally distributed than LAMP1-marked late endosomes or lysosomes, which tend to cluster in the perinuclear region around the microtubule-organizing center (MTOC) (**Matteoni and Kreiss 1987, Mu et al 1995, Patki et al 1997**). The perinuclear steady-state distribution of lysosomes is thought to reflect a balance between long range vectorial movements towards the *plus* and *minus* ends of microtubules (analogous to highways), and short-range movements on actin filaments (analogous to streets).

With respect to “point-of-departure” and “destination” processes, the coordination of various vesicle budding, docking, and fusion steps is regulated in large measure by the Rab family of Ras-like small GTPases, which consists of ~40 members; four of which – Rab4, Rab5, Rab7, and Rab11 – were highlighted in the introduction . Although these four Rab molecules have well-characterized roles in the traffic of distinct endosomal subpopulations, the examination of their subcellular distribution by immunofluorescence microscopy, as shown in [Diagram 3-3](#), suggests that a large proportion of these molecules rests in the perinuclear region, where their physiological function is poorly understood. Nonetheless, though the physiological significance of the movement and steady-state localization of these traffic-regulating GTPase switches remains to be fully elucidated, their proper function is integral to the overall efficacy of endomembrane biogenesis, sorting, and transport. For example, Rab7 and its effector RILP (Rab7-

interacting lysosomal protein) regulate the attachment of late endocytic organelles to dynactin/dynein (**Jordens et al 2001**), which regulate movement towards the minus (MTOC-proximal) ends of microtubules, whereas MLN64 regulates the attachment of these organelles to actin filaments (**Hölttä-Vuori et al 2005**). Accordingly, interference with the function of these proteins (either by loss-of-function mutations or by overexpression) has been demonstrated by fluorescence microscopic analysis to modify the steady-state distribution of late endosomes and lysosomes, with the loss of their function generally resulting in late endosome dispersal and the loss of perinuclear clustering (**Harada et al 1998, Bucci et al 2000, Cantalupo et al 2001, Hölttä-Vuori et al 2005**).

Our confocal microscopy experiments in *Drosophila* larval salivary glands treated with bafilomycin (**Figures 2-13, 2-15**), suggested that changes in the composition and/or integrity of the endocytic network and the concomitant loss of intracellular acidification were likely contributing factors to the resulting downregulation of Akt phosphorylation; while our biochemical examination of compartment-specific fractions of cultured NIH-3T3 cells suggested that the bafilomycin-dependent downregulation of Akt activity in the endomembrane and nuclear/perinuclear compartments correlated with the diminished recruitment of Akt to these two crucial non-cytoplasmic signaling compartments, which have been demonstrated to mediate Akt-dependent cellular responses in various *in vitro* signaling contexts, including GLUT4-dependent glucose uptake in adipocytes (**Calera et al 1998, Hill et al 1999**), and the terminal differentiation of 3T3-L1 preadipocytes (**Maiuri et al 2010**). We therefore examined the subcellular compartment-specific distribution of the endocytic traffic regulators by biochemically analyzing the abundance of Rab4, Rab5, Rab7, and Rab11; as well as the early-endosomal Rab5 effector EEA1, the trans-Golgi network marker GM130, and the late endocytic marker LAMP1 in the CY, M, EN, and N fractions of cultured bafilomycin-treated NIH-3T3 cells. Although no *gross* changes in the general subcellular distribution patterns were observed for any of these markers, a number of subtle changes were observed that are consistent with both our own observations in bafilomycin-treated cells, as well as those of previously published reports of bafilomycin-dependent effects on endomembrane acidification, integrity, and endosome-based signaling processes.

The Rab4 molecule is translocated from its cytoplasmic (or perinuclear) reservoir to early endosomes and recycling endosomes, where it functions as a key regulator for the sorting, recycling, and regulated exocytosis of endosomal cargos (**Mohrmann et al 2002, Ohnishi et al 1999**). In our examination of Rab4 distribution in the cytoplasmic (CY), membrane (M), endosomal (EN) and nuclear/perinuclear (N) fractions of bafilomycin-treated NIH-3T3 cells, Western blots of untreated and bafilomycin-treated samples (50nM, 2, 4, and 12 hours) revealed a prominent (~50% of cellular Rab4) perinuclear/nuclear representation in the N fraction that remained relatively constant over the 12 hour time course of bafilomycin treatment (**Figures 3-9A, 3-10A**). The CY and EN fractions, which combined, account for ~40% of cellular Rab4 abundance, undergo reciprocal changes in compartment-specific Rab4 localization, whereby the ~25% decrease in endosomal Rab4 abundance (from 20% of the cellular pool at TP0 to ~15% at TP12) is accompanied by a comparable increase in the cytoplasm. This shift suggests a decrease in the translocation of Rab4 from the cytoplasm to its endosomal locale, and functionally correlates with the diminished recycling of endocytosed receptor complexes. This, in fact, is precisely the case in hepatocytes, in which Posner's group demonstrated the impaired IR recycling to the PM of cells pre-treated with bafilomycin (100nM, 30 minutes) independently of receptor internalization, which had remained intact (**Balbis et al 2004**). The Rab11 molecule functions as a key regulator in the "slow" route of perinuclear, plasma membrane and Golgi compartment endosomal recycling (**Ullrich et al 1996, Chen et al 1998**), and has been demonstrated to co-localize with Akt in GLUT4-containing vesicles (**Kessler et al 2000**), leading to the translocation GLUT4 transporters to the plasma membrane. Western blots for Rab11, like those for Rab4 and Rab5, revealed a prominent perinuclear (N) reservoir (~70% of cellular Rab11), but with the remaining Rab11 proteins evenly distributed in the CY, M, and EN compartments in growing, untreated NIH-3T3 cells (**Figures 3-9D, 3-10C**). Bafilomycin treatment was found to time-dependently deplete the membrane (M) and endosomal (EN) fractions (and by TP12, the CY compartment as well) of Rab11 protein, suggesting the downregulation of both ("fast") Rab4-mediated and ("slow") Rab11-mediated recycling of endosomal contents to the cellular surface.

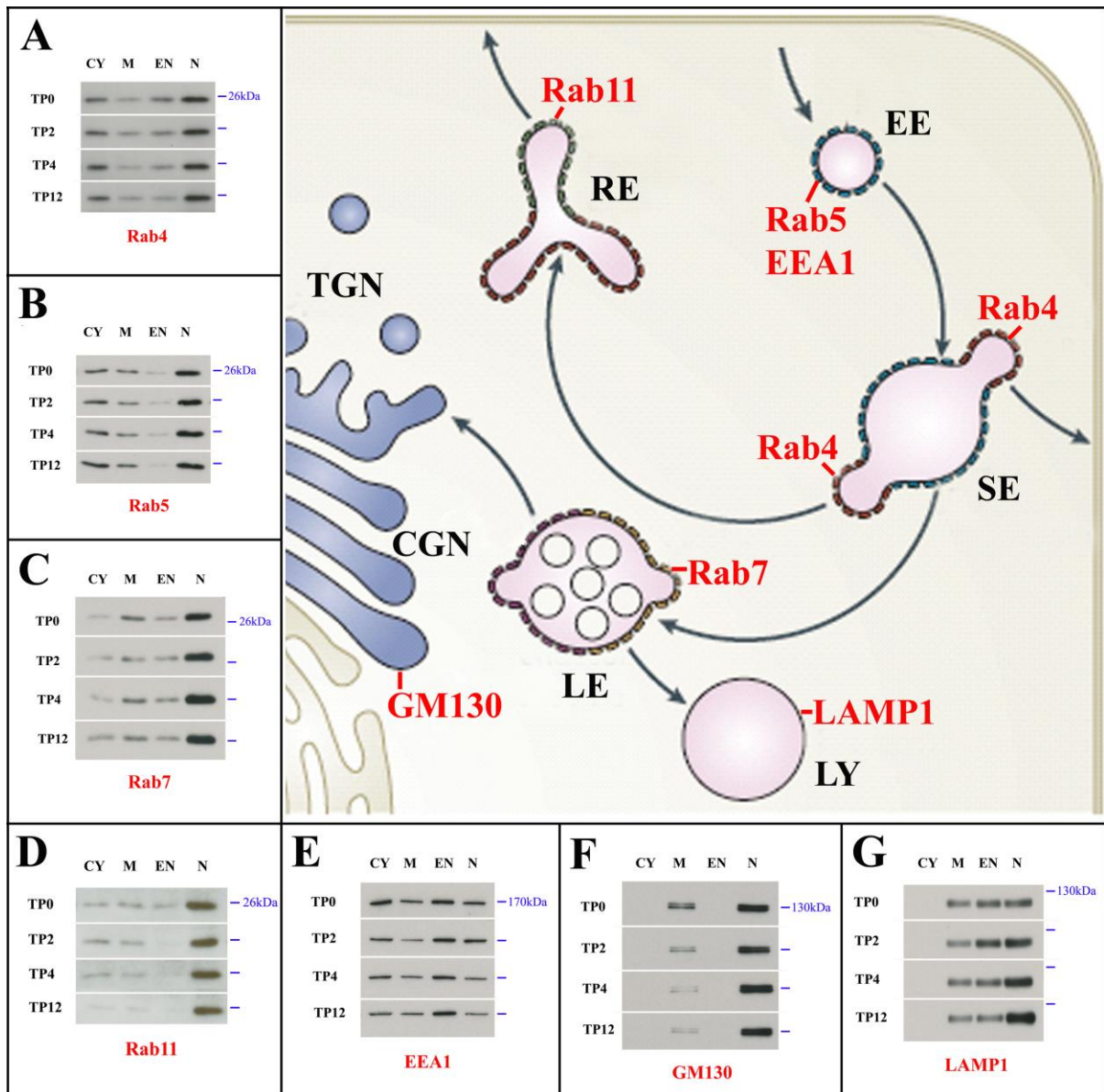


Figure 3-9. Intracellular distribution of endomembrane-associated signaling components in bafilomycin-treated NIH-3T3 cells. As in **Figure 3-4**, fractionated samples of bafilomycin-treated (50nM) NIH-3T3 cells were analyzed by Western-blotting as a function of intracellular fraction – cytoplasmic (CY); membrane (M); endomembrane (EN); and nuclear/perinuclear (N) over the course of 12 hours of bafilomycin treatment for differences in the subcellular distribution of selected endomembrane markers (**Diagram 3-3**). (**A-G**) Representative Western blots for the membrane traffic regulators Rab 4 (**A**), Rab 5 (**B**), Rab 7 (**C**), and Rab 11 (**D**); the early endosomal membrane fusion effector EEA1 (**E**); the Golgin protein GM130 (**F**); and LAMP1 (**G**), a transmembrane glycoprotein localized primarily in late endosomes and lysosomes. The molecular weight values in the right-hand margins are based on the PageRuler™ (Fermentas) prestained protein ladder.

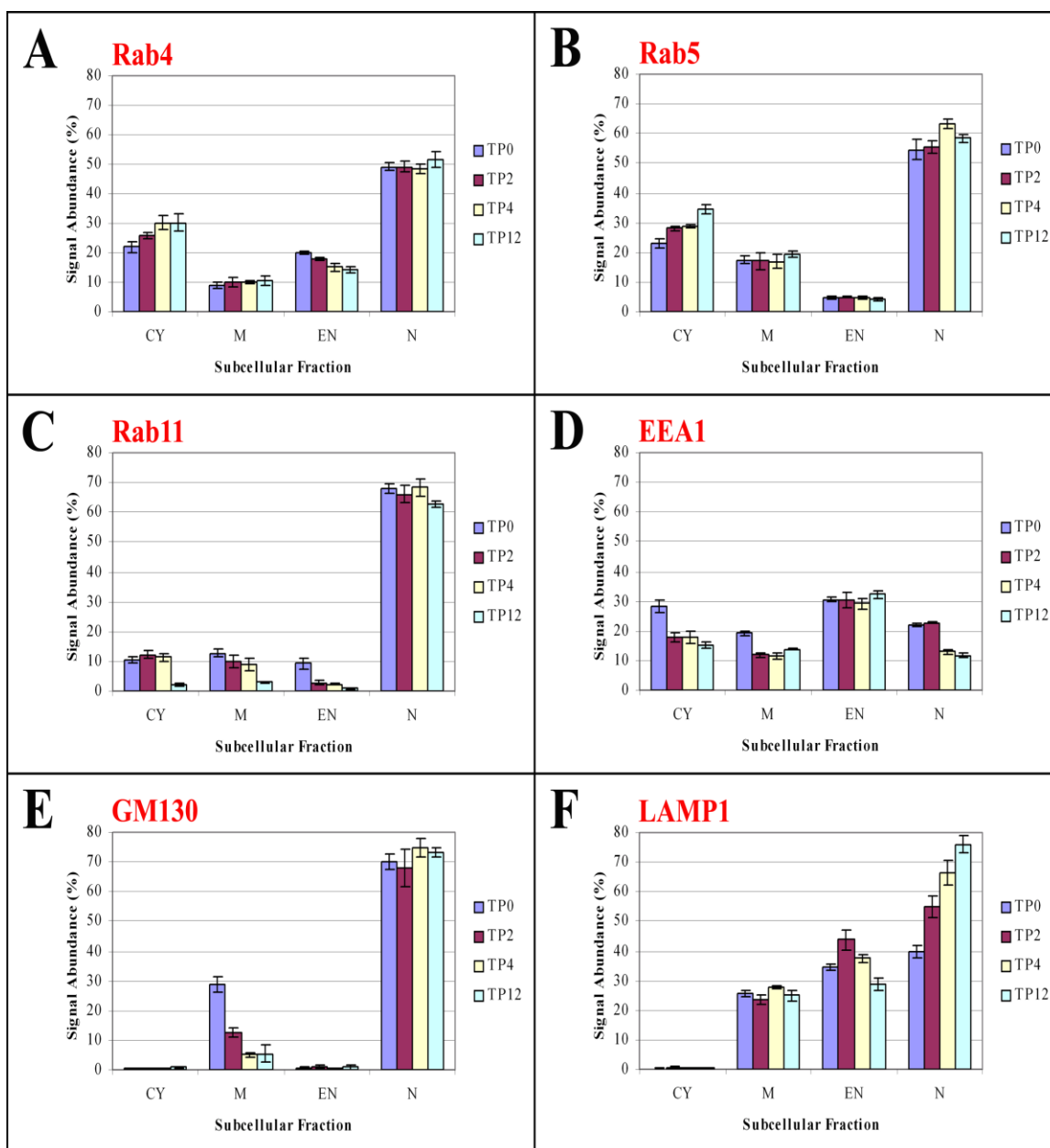


Figure 3-10. Bafilomycin-induced changes in the subcellular distribution of endomembrane-associated signaling components of NIH-3T3 cells. The representative Western blots described in **Figure 3-9**, carried out in duplicate, were quantitated densitometrically for inverted luminosity in order to generate the graphical representations shown for the intracellular distribution of Rab4 (**A**), Rab5 (**B**), Rab11 (**C**), EEA1 (**D**); GM130 (**E**); and LAMP1 (**F**). With all six data sets, the control densitometric values derived for each subcellular fraction (CY, M, EN and N) at TP0 were expressed as a percent of the total densitometric value calculated from the densitometric sum of all four fractions at TP0. The densitometric values subsequently derived for each subcellular fraction at TP2, TP4 and TP12 were then expressed as percent values normalized to the percent TP0 value (represented by the blue bars) of their respective subcellular fractions.

The Rab5 molecule, when localized to the plasma membrane and early endosomes, functions as a key regulator of vesicular trafficking during early endocytosis, in which it contributes to the biogenesis of membrane vesicles as an essential factor for (1) the assembly of clathrin-coated pits, (2) the fusion of homotypic early endosomes, and (3) for heterotypic endosomal vesicle to endosome fusion (reviewed in **Zerial and McBride 2001, Stenmark 2009**). Consistent with its association with PI3K activity in the process of clathrin-mediated endocytosis and early endosome formation, and the independence of our observed bafilomycin-dependent effects from upstream PI3K activation, our Western blots of Rab5 did not reveal any significant changes in non-cytoplasmic pools of Rab5 (**Figures 3-9B, 3-10B**). Interestingly, a significant time-dependent increase in the cytoplasmic (CY) and perinuclear (N) abundance of Rab5 protein was detected in the absence of reciprocal changes in the other compartments, and may represent an upregulation of Rab5 protein synthesis as a compensatory response to the bafilomycin-dependent decrease in Akt signaling despite normal levels of PI3K stimulation. Our other early endosomal marker examined by Western blotting was the Rab5 effector EEA1, which promotes the fusion of homotypic early endosomes, and tethers them in homodimeric complexes. The EEA1 C-terminus contains a FYVE domain which binds to PI3P, a phospholipid enriched in endomembranes (**Gaullier et al 1998**), and its stable association with the endosomal membrane is regulated by PI3K and Rab5 (**Simonsen et al 1998**), promoting early endosomal membrane docking and fusion events. Furthermore, with respect to Akt signaling, EEA1 has recently been demonstrated to be an obligate scaffold for PKC α -dependent Akt activation in endosomes (**Nazarewicz et al 2011**). Consistent with its predominant endosomal localization, EEA1 was most abundant (~35%) in the EN fraction of NIH-3T3 cells throughout the 12 hour bafilomycin time course (**Figures 3-9E, 3-10D**). Whereas the abundance of EEA1 in the endosomal fraction (its major site of function) was relatively unchanged by bafilomycin treatment, its representation in the other fractions, particularly the M fraction, which contains the PM (the source of endocytic vesicles); and the N fraction, to which numerous early endosomes are known to actively translocate as described above and in the introduction, was time-dependently diminished over 12 hours of bafilomycin treatment (**Figures 3-9E, 3-10D**), which considering its role in endosomal tethering,

suggests a downregulation of early endosomal traffic and biogenesis, with possible repercussions on the microtubule-mediated active transport of early endosomes to the nuclear periphery.

LAMP1 and LAMP2 are estimated to contribute ~50% of all proteins on the lysosomal surface (reviewed in **Eskelinen 2006**), and as such, their relative abundance is an effective biochemical readout for the presence and abundance of lysosomal structures, and correlates with lysosomal volume, as shown in [Diagram 2-14D](#). Although no significant changes were evident in bafilomycin-treated NIH-3T3 cells in the compartment-specific distribution of the late endosome-associated Rab7 molecule (**Figure 3-9C**), which mediates the maturation of late endosomes or phagosomes and their fusion with lysosomes; the subcellular distribution of LAMP1, which is non-cytoplasmic, and evenly distributed in the M, EN, and N fraction of untreated (TP0) NIH-3T3 cells, showed a distinct time-dependent pattern of perinuclear accumulation in response to bafilomycin treatment (**Figures 3-9G, 3-10F**), consistent with the lysosomal/autophagosomal swelling we observed in bafilomycin-treated *Drosophila* salivary glands (**Figure 2-13**).

Lastly, GM130 is a Golgin protein involved in the maintenance and integrity of *cis*-Golgi structures, including ER to Golgi transport (**Seemann et al 2000**), Golgi ribbon formation and cisternal maintenance (**Puthenveedu et al 2006, Marra et al 2007**), as well as mitotic Golgi fragmentation (**Nakamura et al 1997, Lowe et al 1998**), which also occurs (irreversibly) prior to apoptotic cell death in response to various death-inducing insults (**Walker et al 2004, Mukherjee et al 2007**). Like our inclusion of Calnexin as a marker of ER-associated structures, we had originally included GM130 in our panel of intracellular compartmental markers as a Golgi-specific epitope in order to assess the fractional location of Golgi derived membranes. Western-blot analysis of the intracellular abundance and distribution of GM130 in fractionated, bafilomycin-treated NIH-3T3 cells demonstrated its primary localization in the perinuclear compartment (**Figures 3-9F, 3-10E**), consistent with its abundant presence in the *cis*-Golgi network, which is the nucleus-proximal face of the Golgi apparatus that receives secretory granules from the ER. Unlike Calnexin however, whose abundance in the compartments in which it is present is unaltered over the course of bafilomycin treatment (**Figures 3-4, 3-5**), a

GM130 signal of lower abundance, whose intensity diminished over the 12 hour time course of bafilomycin treatment was also detected in the M fraction (**Figures 3-9F, 3-10E**). This signal likely corresponds to the minor *trans*-Golgi (nucleus-distal) localized pool of GM130, from which secretory vesicle traffic and recycling vesicle fusion is regulated, and which as mentioned in **Section 3-3.1**, may likely contaminates the M fraction in the process of cell homogenization, but may also indicate a downregulation of trans-Golgi network-modulated traffic.

3-3.6 – Bafilomycin diminishes the growth factor-stimulated recruitment of Akt to signaling endosomes

Considering the bafilomycin-induced decrease in the recruitment and activation of Akt in the endosomal (EN) fraction derived from fractionated NIH-3T3 cells (**Figures 3-6, 3-7**), and the presence of discrete changes in the subcellular distribution of endomembrane markers (**Figures 3-9, 3-10**), we further fractionated the heterotypic endosomal (EN) fractions of bafilomycin-treated NIH-3T3 cells and L3 *Drosophila* larval tissues in order to delineate the class(es) of endocytic structures to which Akt localizes in response to activating stimuli under growth-favoring conditions, and to further investigate the effects of bafilomycin treatment on the localization and activation of Akt with respect to its endosomal distribution. The total endomembrane fraction (EN) of bafilomycin-treated cells (*Drosophila* larval tissues or cultured NIH-3T3 cells) was harvested and further fractionated by OptiPrep (iodixanol) density gradient centrifugation (see Materials and Methods). The OptiPrep gradient (10-30%) was thereafter collected as 20 fractions of equal volume, and alternating fractions (1, 3, 5,...19) were analyzed by Western blotting for bafilomycin-dependent differences in the distribution of Akt and various endocytic markers (**Figures 3-11, 3-12, 3-13**). Endomembranes separated by density on OptiPrep gradients usually ranging from 5% to 30% (w/v) of the synthetic density gradient medium have been shown to migrate to generally reproducible domains, producing migration profiles characteristic of distinct endomembrane subpopulations, such as early endosomes, late endosomes/MVBs, and lysosomes; as well as Golgi-, and ER-associated intracellular structures. Tissues whose endomembrane contents have been

fractionated with OptiPrep gradients include cultured mammalian tissues such as HeLa cells (**Proikas-Cezanne et al 2006, Niu et al 2013**), kidney fibroblasts (**Fivaz et al 2002**), glial cells (**Weerth et al 2007**), various hematopoietic cells (**Schmidt et al 2009, Gibbings et al 2009**), and tumor cell lines (**Li and Donowitz 2008**); as well as non-mammalian tissues such as *Drosophila* larval tissues and embryos (**Khanna et al 2010**), and single-celled protozoans like *S. cerevisiae* (**Grunau et al 2011**). Consistent with our own panel of markers in *Drosophila* (Rab5 for early endosomes, dHook for MVBs, and Vha68 for lysosomes/vacuoles), the studies cited above, with some variability depending on cell type-specific differences, generally suggest that in a 10-30% OptiPrep gradient (which we collected as 20 fractions), sorting and endocytic vesicles, early endosomes and late endosomes/MVBs should settle in the first 5 fractions (10-15% OptiPrep), followed by lysosomal and vacuolar structures in the vicinity of fractions 6 to 11 (15-20% OptiPrep). The ER- and Golgi-associated endomembranes should migrate to fractions 12-16 (20-25% OptiPrep); while the highest density fractions (25-30% OptiPrep, fractions 17-20) commonly contain secretory granules and storage vesicles (**Figure 3-11A**).

Due to the logistical ease with which a high yield of endosomal structures can be extracted from a relatively small number (~20) of dissected larvae, the endosomal distribution of Akt-HA was first examined in preparations of OptiPrep endomembrane fractions generated from L3 larval tissues transgenically expressing HA-tagged bovine Akt. As described in detail in the Materials and Methods, the dissected tissues were either vehicle-treated (UT), insulin-treated (Ins) or treated with a combination of insulin and bafilomycin (Ins/Baf) for a period of 12 hours, and subsequently homogenized and cleared of debris and nuclei, generating a post-nuclear lysate, which was further centrifuged at 200,000g to generate an endomembrane pellet. This endomembrane pellet, which is analogous to the EN fraction generated in NIH-3T3 cells (see **Figure 3-4A**), was isolated, resuspended and layered onto a 10-30% OptiPrep gradient and subsequently centrifuged at 100,000g for 16 hours in order to separate the various species of endomembrane vesicles as a function of density.

In vehicle-treated samples of larval tissues, which are incubated in insect media with a volume of DMSO equal to that used in bafilomycin-treated samples, and henceforth simply referred to as untreated samples (UT), the early-endosomal marker

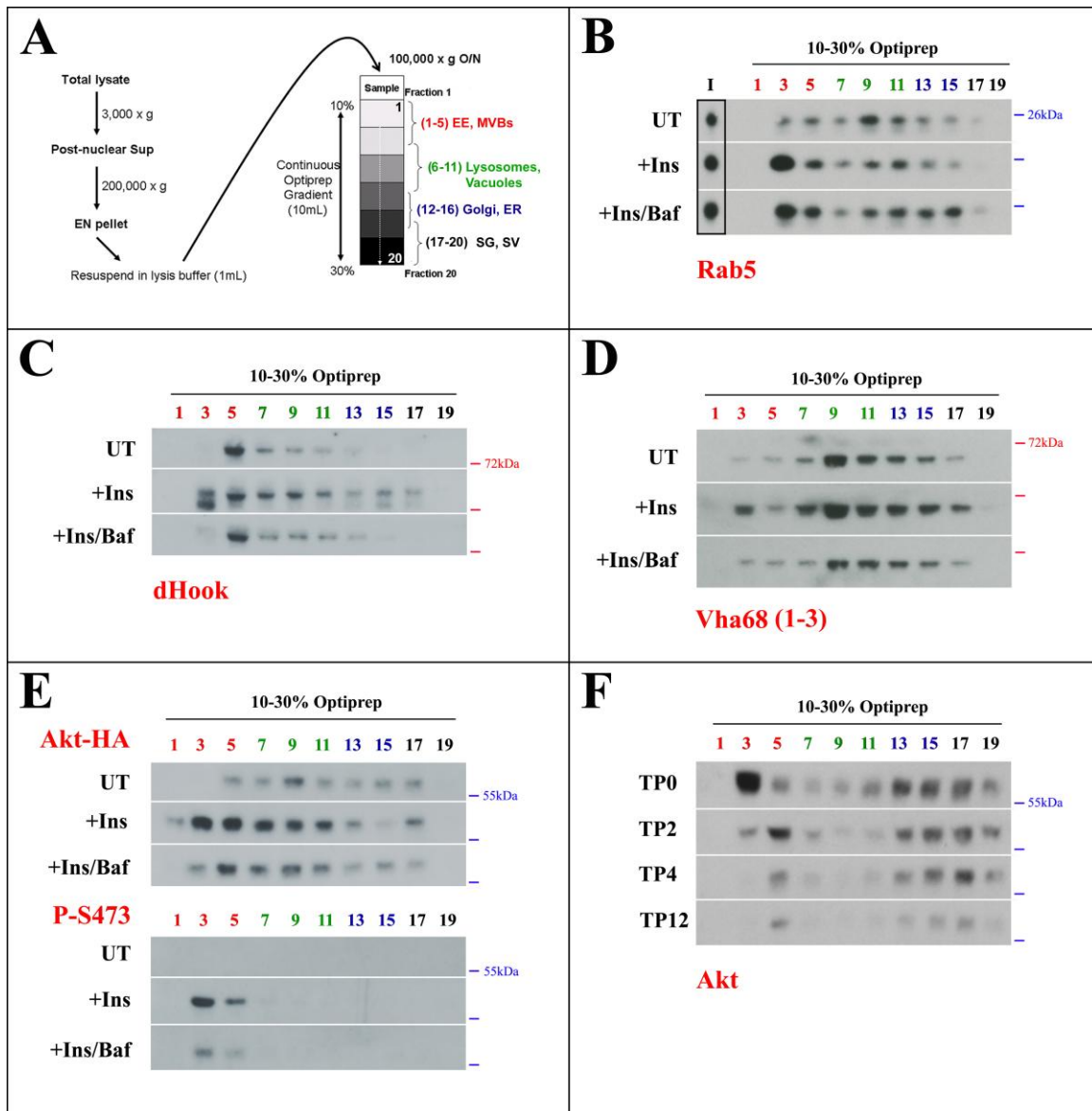


Figure 3-11. Bafilomycin treatment inhibits the insulin-induced recruitment of Akt to early endosomes and multivesicular bodies. As described in the Materials and Methods, the purified endomembrane (EN) fractions of dissected, incubated (untreated, 0.2U/mL insulin-treated, or 0.2U/mL insulin/500nM bafilomycin co-treated), and fractionated *arm-Akt-HA*-expressing 3rd instar larval tissues were layered on a 10-30% OptiPrep density gradient for further centrifugal subfractionation. **(A)** Early endosomes (EE) and late endosomes/multivesicular bodies (MVBs) are known to settle in low density fractions (1-5, in red); lysosomes and vacuoles are recovered in fractions 6-11 (in green); Golgi and ER vesicles accumulate in fractions 12-16 (in blue); and high-density fraction (17-20, in black) predominantly contain secretory granules (SG) and storage vesicles (SV). **(B-E)** Western-blots of: **(B)** the membrane traffic regulator Rab5, which is associated with EEs; **(C)** dHook, a protein required for endocytic trafficking predominantly localized in MVBs; **(D)** the V-ATPase A-subunit Vha68, which is particularly enriched in highly acidified lysosomes, but present throughout the endocytic

network; as well as (E) total transgenic HA-tagged bovine Akt protein as detected by the HA epitope, and mammalian phospho-Akt (S473). The input lane (I) in (B) is, by volume, 1/200th of the endomembrane fraction prior to OptiPrep layering; or in other words, the Rab5 input signal represents the mean Rab5 protein content of fractionated aliquots. (F) The resuspended endomembrane pellets of bafilomycin-treated and fractionated NIH-3T3 cells (see Materials and Methods) were similarly layered on a 10-30% OptiPrep gradient for centrifugal subfractionation. Western blot analysis of total (endogenous) Akt protein in vehicle-treated (UT), and 50nM Bafilomycin-treated (2, 4, and 12 hour) samples recapitulates the time-dependent loss of Akt enrichment in EE- and MVB-associated fractions (1-5) observed in identically-treated *Drosophila* larval tissues (E). The molecular weight values in the right-hand margins are based on the PageRulerTM (Fermentas) prestained protein ladder. OptiPrep gradient preparation and fraction collection following centrifugation were carried out by our collaborator Dr. Rick Bagshaw, a postdoctoral fellow in Dr. Tony Pawson's laboratory at the Samuel Lunenfeld Research institute, whose assistance and expertise were critical in the design and execution of the endosomal subfractionation protocol, and whose contribution of reagents and materials (including the OptiPrep solution) is *greatly* appreciated.

Rab5, which as described in the previous section has a sizeable perinuclear presence (**Diagram 3-3**, **Figures 3-9, 3-10**), was detectable throughout the endocytic network, but clearly most abundant in fraction 9 (~25% of total Rab5 signal in examined samples, **Figures 3-11B, 3-12A**), which is predicted to contain lysosomes, and likely corresponds to vesicles associated with this perinuclear reservoir. In insulin-stimulated samples, a distinct accumulation of Rab5 was evident in the lowest-density Rab5-positive fraction (fraction 3, **Figure 3-11B**), which generally corresponds to early endosomes. The combined treatment of larval tissues with insulin and bafilomycin did not significantly disrupt the early endosomal accumulation of Rab5, consistent with the aforementioned (see **Section 3-3.5**) independence of Rab5-dependent endocytic events from our observed bafilomycin-induced effects in NIH-3T3 cells. Consistent with the established role of Rab5 in growth factor-mediated endocytic events, the stimulation of dissected *Drosophila* tissues with insulin (irrespective of bafilomycin cotreatment) was found to result in a tangible increase in the endosomal content of Rab5 in comparison to *unsupplemented* (growth factor-starved) insect media (input lanes, **Figure 3-11B**). As described below (**Figures 3-11E, 3-13A,B**), Western blots of the endosomal contents of untreated tissues demonstrate that these compartments are essentially devoid of Akt-HA phosphorylated at S473 in the absence of growth factor stimulation, whereas in contrast, a significant presence of Akt-HA and the S473 signal in endosomal fractions (3 and 5) is clearly detected in insulin-treated samples. These results are consistent with the aforementioned synergistic growth factor-dependent activities of PI3K and Rab5 in the stimulation of downstream effectors and the promotion of receptor endocytosis, respectively.

The *Drosophila* Hook protein (dHook) promotes endocytosis of ligand/receptor complexes (**Krämer and Phistry 1996**), and is required for endocytic traffic and the maturation of MVBs (**Sunio et al 1999, Krämer and Phistry 1999**). In untreated larval tissues, dHook was largely located in fraction 5 (**Figure 3-11C, 3-12B**), consistent with its predominant association with MVBs in *Drosophila*. In insulin stimulated samples, a strong signal was maintained in fraction 5, and an insulin-specific accumulation was observed in fraction 3, suggesting an increase in its localization in early endosomes and endocytic vesicles. This insulin-induced accumulation in fraction 3, unlike the

accumulation of Rab5, was suppressed in Ins/Baf-treated samples, whose fractionation profile, despite the inclusion of insulin in the incubation medium, was nearly identical to that of untreated samples. This suggests that although the insulin-mediated biogenesis of Rab5-positive early endosomes is unhindered by bafilomycin treatment (**Figures 3-11B, 3-12A**), their fusion with MVBs is significantly inhibited by bafilomycin. Furthermore, as described below, the bafilomycin-induced loss of dHook localization in fraction 3 (early endosomes) is mirrored by the concomitant bafilomycin-induced loss of both Akt-HA localization and S473 phosphorylation in the same early endosome-enriched fraction (**Figures 3-11E, 3-13A,B**)

The *Drosophila* V-ATPase A subunit Vha68, which is a component of the peripheral V₁ domain that is recruited to the transmembrane V₀ domain, was detectable throughout the endosomal gradient in untreated samples (consistent with its functions throughout the endosomal network), but found in highest abundance in fractions 9-13 (**Figures 3-11D, 3-12C**), which predominantly contain lysosomes and lysosome-derived vesicular structures. Treatment with insulin enhanced Vha68 accumulation in fractions 9-13, and furthermore, induced an accumulation of Vha68 in other distinct domains, including fraction 3 (early endosomes), fraction 7 (late endosomes/lysosomes), fraction 13-15 (ER/Golgi), as well as in the storage or secretory vesicles (and possibly other high density endomembranes) of fraction 17 (**Figures 3-11D, 3-12C**). These effects were suppressed in samples treated with a combination of insulin and bafilomycin, resulting in a fractionation profile resembling that of untreated samples, including the bafilomycin-dependent loss of Vha68 recruitment to early endosomes (**Figures 3-11D, 3-12C**), an effect we also observed in the case of dHook (**Figure 3-11C**), and as described below, in the case of Akt-HA as well, itself mirrored by the loss of S473 phosphorylation in the same compartments (**Figures 3-11E, 3-13A,B**).

As alluded to above in the context of the insulin- and bafilomycin-induced effects on the endosomal localization of our selected endomembrane markers (Rab5, dHook, and Vha68), the examination of the endosomal distribution of transgenically-expressed HA-tagged Akt and its phosphorylation at S473 in untreated, insulin-treated, and Ins/Baf-treated *Drosophila* larval tissues demonstrated a distinct accumulation of Akt in endosomal compartments in response to insulin treatment, and conversely, a depletion of

its localization to these compartments as a result of co-treatment with bafilomycin (**Figures 3-11E, 3-13A,B**). Consistent with our hypothesis that Akt-mediated signaling downstream of agonist/receptor-dependent PI3K activation included a significant endomembrane-dependent dimension of Akt localization, and activation (or nuclear translocation), Western blots of untreated samples (growth factor-starved), in comparison to insulin-stimulated samples, contained low levels of Akt, which was most abundant in fraction 9 (lysosomes or colocalized ER/Golgi vesicles), with trace levels detectable from fractions 5 to 17. In insulin-treated samples, a significant accumulation of Akt could be observed in fractions 3 to 11, including highest levels of accumulation in fraction 3 (early endosomes), with progressively lower (but nonetheless elevated) levels in the MVB-associated (fraction 5), and lysosome-associated (fractions 7-11) fractions. This insulin-mediated effect was heavily muted in bafilomycin co-treated samples (Ins/Baf), including the loss of accumulation in fraction 3 (**Figures 3-11E, 3-13A**). Our examination of the S473 signal, consistent with our observations of the Akt-HA molecule, revealed (1) that the phosphorylation of Akt-HA at this residue was undetectable in all examined endosomal fractions of untreated (growth factor-starved) larval tissues; (2) that insulin treatment induced the accumulation of Akt-HA phosphorylated at S473 predominantly in fraction 3, and to a lesser degree in fraction 5, corresponding to early endosomes and MVBs, respectively; and (3) that co-treatment with bafilomycin severely diminished the abundance of the S473 signal in these two fractions (**Figures 3-11E, 3-13B**). These results suggest that early endosomes, to which Akt-HA is actively translocated in response to insulin, is the main site (within the endocytic network) of insulin-induced Akt phosphorylation at S473; and that both the enhanced localization of Akt-HA to early endosomes, and the upregulated phosphorylation of Akt-HA at S473 are diminished in response to bafilomycin treatment.

Lastly, in cultured NIH-3T3 cells, following a final repetition of the cellular fractionation protocol (as described in **Section 3-3.2**) in which all four subcellular compartments (CY, M, EN, and N) of untreated (TP0) and bafilomycin-treated (50nM, 2, 4, and 12 hours) samples were isolated; half of the resuspended endosomal (EN) fraction from each time point (TP0, TP2, TP4, and TP12) was further fractionated by centrifugation through a 10-30% OptiPrep gradient as described above for EN

compartments isolated from *Drosophila* larval tissues. The OptiPrep gradients of the centrifuged EN compartments were similarly collected in 20 fractions, and endosomal Akt distribution was examined at each time point by Western blotting. Consistent with our observations in *Drosophila* larval tissues expressing HA-tagged bovine Akt, the endomembrane compartments of NIH-3T3 cells growing in the presence of 5% FBS were found to primarily harbor *endogenous* Akt in fraction 3 (early endosomes), with less abundant but distinct subpopulations in fractions 13 to 17, spanning ER- and Golgi-rich domains (**Figures 3-11F, 3-13C**). Significantly, recapitulating our observations in *Drosophila* larval tissues, the accumulation of endogenous Akt in fraction 3 was lost within 4 hours of bafilomycin treatment, with a shift in this pool of Akt towards MVBs in fraction 5, which itself time-dependently diminishes over 12 hours of bafilomycin treatment. This overall and early-endosome/MVB-specific progressive depletion of Akt in the EN compartment is consistent with our observations both in *Drosophila* larval tissues, and in our experiments in fractionated NIH-3T3 cells, and the diminished abundance of these endomembranes themselves is consistent with the findings of recent experiments in *Arabidopsis* using concanamycin (**Scheuring et al 2011**). In order to ensure that the results obtained from the OptiPrep gradient were not due to any inconsistencies in our extraction protocol, the bafilomycin-dependent effects on relative Akt abundance and phosphorylation were examined in the CY, M, EN, and N fractions generated and set aside from the final repetition of the extraction procedure, and analyzed by Western blotting samples from each compartment as a function of *time* (TP0, 2, 4, and 12, **Figure 3-14**), as opposed to our previous method of analyzing all four time points as a function of subcellular compartment (ie **Figure 3-6**). As shown in **Figure 3-14C**, which examines all four time points in the EN compartment, the present repetition of the experiment in NIH-3T3 cells had reproduced our previously described effects with respect to time-dependent decreases in S473 phosphorylation, total Akt abundance, and Vha68 abundance in the EN compartment. The T308 signal was nearly imperceptible, consistent with our previous demonstration of its predominant localization in the cytoplasm and nucleus, while the EEA1 signal, which served as a control for EN protein content (and OptiPrep column input), was unchanged throughout the bafilomycin time-course. Similarly, the examination of relative changes in Akt abundance and

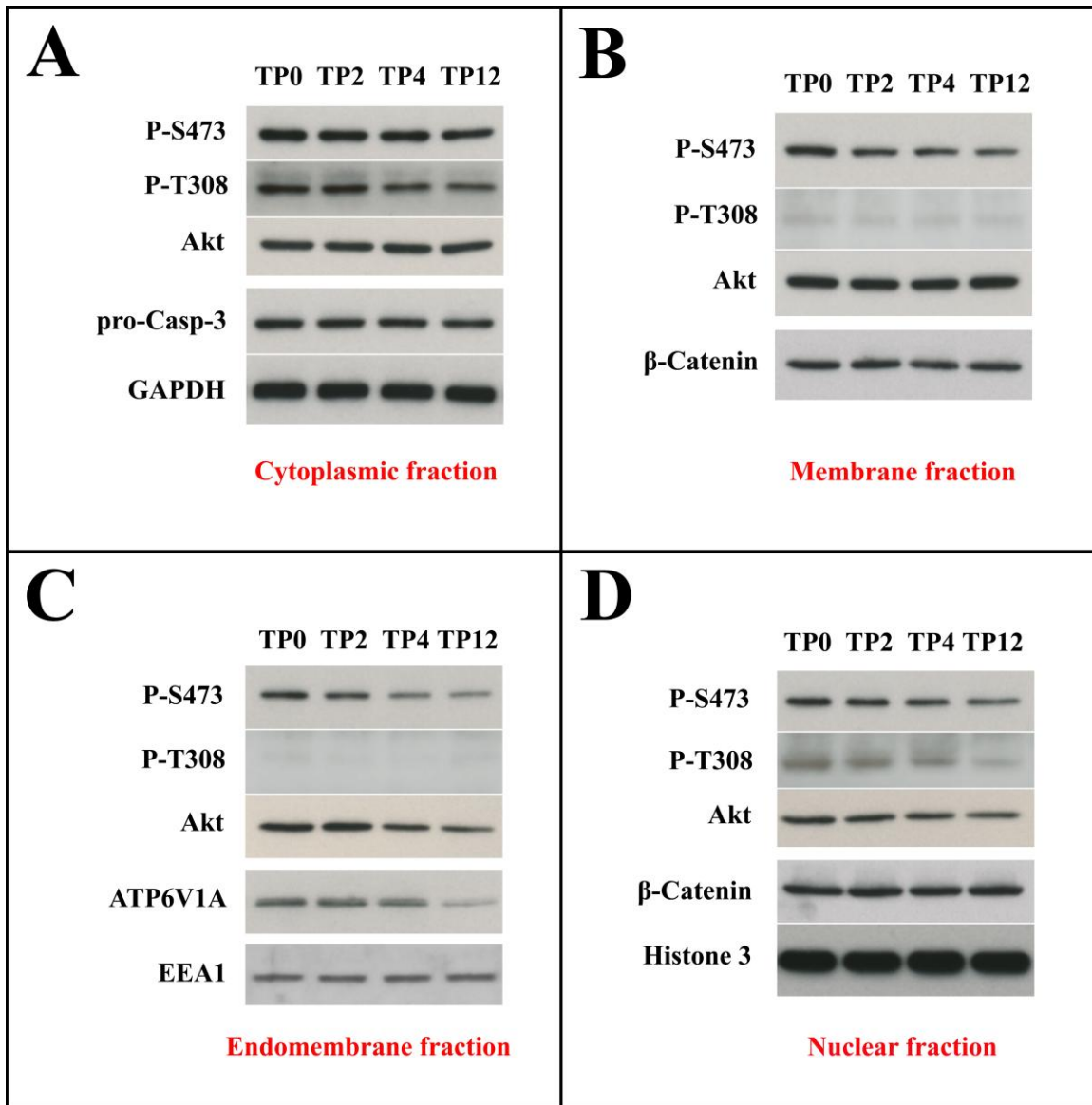


Figure 3-14. Fraction-specific effects on Akt phosphorylation in bafilomycin-treated NIH-3T3 cells. Samples from all 4 intracellular fractions of Bafilomycin-treated NIH-3T3 cells - cytoplasmic (**A**), membrane (**B**), endomembrane (**C**), and nuclear/perinuclear (**D**) were analyzed in duplicate as a function of treatment duration (0, 2, 4, and 12 hours) by Western-blotting. Levels of total Akt protein and phospho-Akt (S473 and T308) are shown for each fraction analyzed, with pro-Caspase-3 and GAPDH as loading controls for the cytoplasmic fraction (**A**), β-Catenin (unphosphorylated) as a loading control for the membrane fraction (**B**), EEA1 as a loading control for the endomembrane fraction (**C**), and both β-Catenin (unphosphorylated) and Histone H3 as loading controls for the nuclear/perinuclear fraction (**D**).

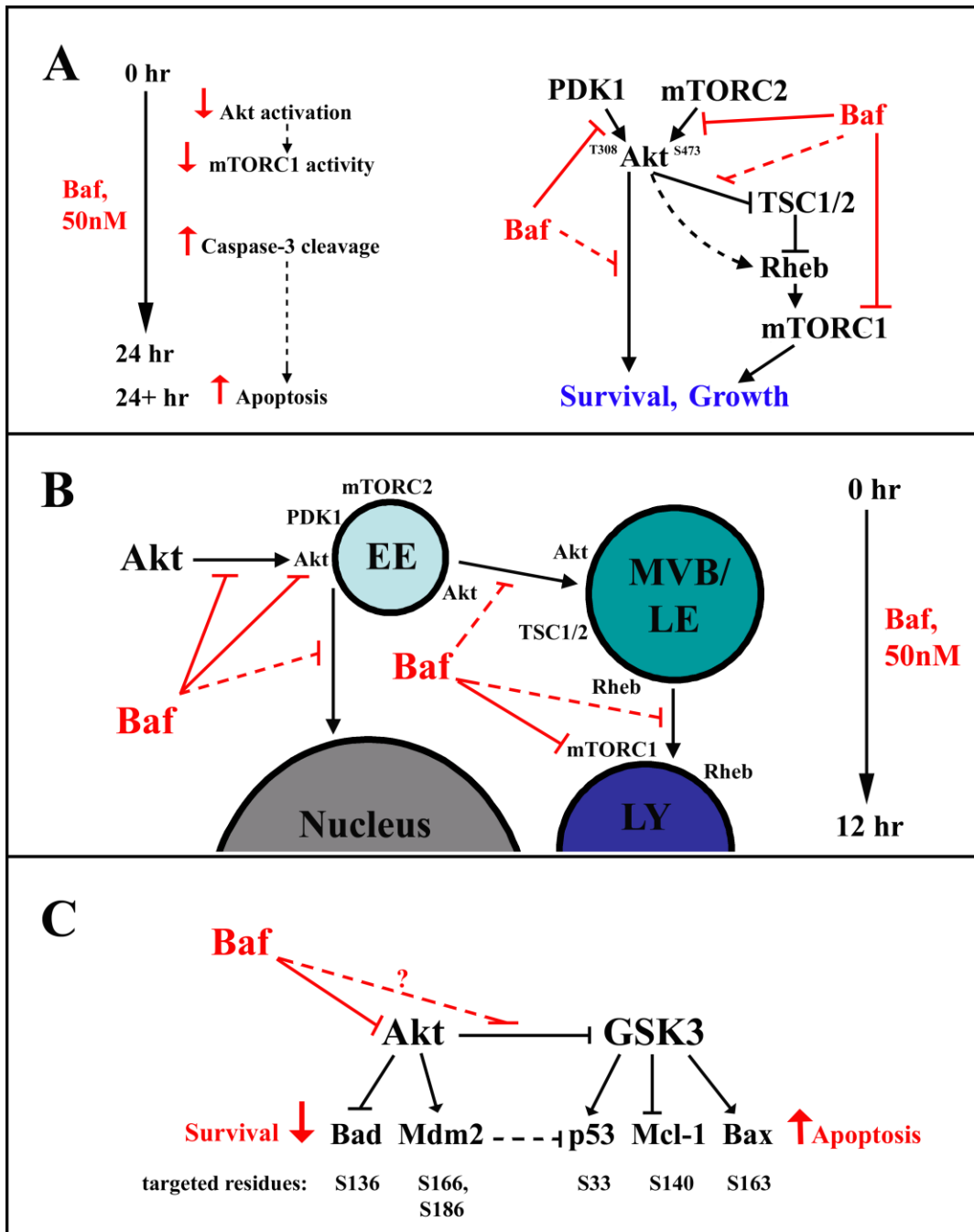


Diagram 3-4. Schematic representation of time-dependent bafilomycin-induced trends. (A) Time course of bafilomycin-induced effects detected in whole-cell lysates and FACS-analyzed NIH-3T3 cells. (B) Time course of bafilomycin-induced effects on endomembrane-associated Akt traffic and signaling in fractionated (and subfractionated) NIH-3T3 cells. (C) Candidate pro-apoptotic (Bad, p53, Bax) and anti-apoptotic (Mdm2, Mcl-1) substrates of Akt or GSK3 to be examined for phosphorylation (at indicated residues) and localization status in response to bafilomycin treatment. Bafilomycin induced effects are depicted in red, dashed lines indicate corollary or deduced effects. See text for details.

phosphorylation within the other three compartments (Figure **3-14A,B,D**) confirmed the proper execution of the extraction protocol and the reproducibility of the derived results.

3-4 – Discussion

While guarding against the over-interpretation of some of our more phenomenological observations (whose analysis is hampered by the subtlety of some of the observed bafilomycin-induced effects at our chosen concentrations of the drug, and over the time interval of our experiments), our *in vitro* analyses of cultured NIH-3T3 fibroblasts did reveal two *general* trends in the time line of bafilomycin-induced effects that allow us to identify a number of aspects of the Akt signaling paradigm whose mechanistic underpinnings warrant further investigation. The first trend (*Diagram 3-4A*) emerged from our biochemical analysis of the ISP in whole cell lysates (**Figures 3-1B, 3-3, 3-8**) and purified subcellular fractions (**Figures 3-6, 3-8**), which suggested that in bafilomycin-treated NIH-3T3 cells, the downregulation of Akt phosphorylation is an early event (onset at 2-4 hours, near-maximal by 12 hours); is soon thereafter followed by the downregulation of mTORC1 activity (onset at 4-8 hours of treatment, near-maximal at 12 hours); and concluded by the proteolytic activation of executioner caspases (onset at 16 hours of treatment, peak >24 hours), an effect that was recapitulated by the assessment of cell viability over the same period of bafilomycin treatment (**Figures 3-1A, 3-2**). The second trend to emerge (*Diagram 3-4B*) was detected by the subfractionation of the endosomal compartments of bafilomycin-treated *Drosophila* larval tissues and cultured NIH-3T3 cells (**Figure 3-11**), which demonstrated the robust accumulation of Akt in early-endosomal fractions in response to insulin stimulation in larval tissues (or serum treatment in NIH-3T3 cells), and an equally robust (and time-dependent) depletion of the early endosomal pool of Akt over 12 hours of bafilomycin treatment. The trend observed in this bafilomycin-induced depletion of Akt in the endomembrane compartments of growing NIH-3T3 cells consisted of its initial loss in early endosomal fractions, detectable after 2 hours of treatment, and accompanied by a shift in its predominant abundance (in low-density fractions) from the early endosomal population (fraction 3) to the late endosomal/MVB population (fraction 5), which is itself thereafter depleted by 4-12 hours of treatment (**Figure 3-11F**). Moreover, this bafilomycin-dependent endosomal depletion (first in EEs, then in MVBs) of Akt seems to temporally coincide with a decrease in Akt nuclear abundance and activation, which we detected time-dependently

between 4-12 hours of treatment (**Figure 3-7**), suggesting that V-ATPase inhibition results in the progressive loss of Akt-containing endomembrane structures, an effect first and most robustly manifested in early endosomes, and thereafter in more distal subcellular structures such as MVBs and nuclei. Beyond their suggestion as general trends (which is consistent with current conceptions of ISP signaling and endomembrane traffic), however, the formulation of a precise mechanistic explanation for these observed patterns requires a more detailed analysis of many of the paradigmatic concepts addressed in our experiments, whose limitations, along with potentially informative future experiments, are discussed herein.

First, our analysis of bafilomycin-induced cytotoxicity as measured by the viability of cultured NIH-3T3 cells incubated in its presence at a concentration of 50nM demonstrated near-complete loss of viability within 48 hours bafilomycin treatment (**Figure 3-1A**), and significant levels of apoptosis within 24 hours of bafilomycin treatment based on the FACS analysis of AnnexinV-positive cells and the levels of pro-caspase-3 cleavage (**Figures 3-1B, 3-2**); with the onset of pro-caspase-3 proteolytic activation narrowed down to TP16, or 16 hours following the administration of 50nM bafilomycin (**Figure 3-1B**). Although our biochemical examination of (1) whole cell lysates (**Figure 3-3**), (2) subcellular compartments (**Figures 3-6, 3-7, 3-8**), and (3) endosomal compartments (**Figures 3-11, 3-13, 3-14**) of bafilomycin-treated NIH-3T3 cells suggest that this latent apoptotic response is preceded by the downregulation of Akt activation, our experiments do not mechanistically examine this apparent correlation beyond (1) the diminished accumulation and activation of Akt in the nucleus (the site of many of its substrates involved in the decision between survival and apoptosis, such as BAD and MDM2); and (2) the subtle but tangible accumulation of (active, unphosphorylated) GSK3 β in the nuclei (or the perinuclear region) of NIH-3T3 cells, potentially resulting in the promotion of apoptosis through the intrinsic pathway (see **Section 1-4.7**). Our investigation of the subcellular distribution of PI3K/Akt signaling components was to include a panel of known Akt substrates (**Diagram 3-4C**) such as BAD phosphorylated at S136 and MDM2 phosphorylated S166 and S186 (in addition to GSK3 substrates such as Bax at S163 and Mcl-1 at S140; as well as p53 whose

phosphorylation at S33 is specific to the β isoform of GSK3). However, their phosphorylation status under our experimental conditions remains to be addressed.

The contribution of bafilomycin-induced Akt inhibition to the latent onset of apoptosis can also be assessed in cells transgenically expressing constitutively active forms of Akt (bearing serine/threonine to aspartic acid point mutations at the S473 and T308 sites, S473D/T308D, or AktDD), which cannot be further activated by insulin stimulation (**Alessi et al 1996a**), and renders the bafilomycin-dependent downregulation of Akt phosphorylation at these residues moot as direct causative agents of the apoptotic response. As such, the suppression of (or delay in) the onset of bafilomycin-induced apoptosis in AktDD-expressing cells in comparison to appropriate control cells (non-transgenic parental cell lines; mock-transfected and wildtype Akt-expressing transgenic controls) would confirm the suspected contribution of Akt inhibition to the apoptotic response. However, the opposite potential result of such an experiment, whereby the expression of AktDD does *not* suppress or even delay the onset of bafilomycin-dependent apoptosis, would not automatically eliminate the downregulation of Akt as a causative mechanism contributing to the apoptotic response, since an intact endocytic network may still be required for the nuclear translocation of Akt, even when constitutively activated by the DD mutation. As such, the examination of AktDD-expressing bafilomycin-treated cells would require further biochemical and/or confocal microscopic analysis to determine the efficiency of AktDD nuclear translocation in bafilomycin-treated cells. Moreover, the examination of the contribution of Akt-dependent signaling processes to the suppression of apoptotic cell death, and the relevance of their diminishment in the bafilomycin-induced apoptotic response would be best examined in cells that *intrinsically* require PI3K/Akt-mediated survival-promoting signals, such as PC-12 cells, which as discussed in **Section 1-4.7**, require stimulation by neurotrophins like NGF (which induce a significant PI3K/Akt signaling response downstream of receptor activation) for sustained survival (**Yao and Cooper 1995**), rather than fibroblasts grown under standard culture conditions, which are stimulated by the various growth factors and cytokines present in the culture medium supplemented with 5% serum.

Second, though our examination of whole-cell lysates (**Figure 3-3**) consistently demonstrated a time-dependent ~two-fold decrease in S473 and T308 phosphorylation that was near-maximal after 12 hours of bafilomycin treatment (and tangibly detectable after 2-4 hours), our examination of subcellular fractions enriched for cytosolic (CY), membrane-associated (M), endosomal (EN), and nuclear/perinuclear (N) constituents, though consistent with our observations in whole-cell lysates with respect to Akt phosphorylation, revealed distinct differences in the intracellular distribution of Akt itself, as well as its S473- and T308-phosphorylated forms, and furthermore, a differential effect in the extent of the depletion in their respective intracellular compartment-specific abundances in response to bafilomycin treatment (**Figures 3-6, 3-7**). With respect to the phosphorylation status of S473, which is mTORC2-dependent, both the mTOR protein, the catalytic centerpiece of mTORC2; and Rictor, the identity-defining component of mTORC2; were demonstrated to be predominantly localized in non-cytoplasmic fractions of growing NIH-3T3 cells (5% FBS), including the M, EN, and N compartments, with only nominal levels detectable in the CY fraction (**Figure 3-8F**). These observations suggest that in moderately (but continuously) stimulated NIH-3T3 cells (5% FBS), the S473-phosphorylating catalytic activity resides predominantly in intracellular components (consistent with the observed coordination of mTORC2 activity with that of PI3K-dependent PDK1 activity towards Akt, see **Section 1-3.3**), further implying that cytoplasmic pools of Akt phosphorylated at the HM accumulate following their release from intracellular signaling membranes (likely located in the M and EN fractions). A similar conclusion can be drawn in the case of T308 phosphorylation (whose detection was predominantly cytoplasmic, **Figures 3-6, 3-7**), as PDK1, whose cellular reservoir, like that of Akt, is cytoplasmic, could only be detected in its autophosphorylated form (phospho-S241) in non-cytoplasmic fractions (**Figure 3-8**), consistent with the largely localization-dependent regulation of PDK1 activity, the known requirement of membrane localization for PDK1-dependent Akt phosphorylation at T308, and the immediate release of T308-phosphorylated Akt from the PM surface (see **Section 1-3.2**). Curiously however, unlike the depletion of Akt from the EN and N fractions in response to bafilomycin treatment (which also correlates with their diminished phosphorylation in these respective compartments), the subcellular distribution of PDK1 and mTORC2

(mTOR/Rictor) were unchanged throughout the 12 hour incubation period. Accordingly, a number of inferences can be made that themselves require further investigation:

(1) The observed decreases in T308 and S473 abundance in the CY fraction likely represent a decrease in the membrane-associated phosphorylation of Akt, which is itself likely due to diminished Akt localization (in the case of the EN fraction) rather than alterations in PDK1 or mTORC2 localization. Furthermore, the aforementioned proposed primacy of endomembrane-associated pools of Akt in the propagation of downstream cellular responses, including Akt nuclear translocation (see **Section 3-1**), suggests that though the cytoplasm retains significant levels of S473 phosphorylation after 12 hours of bafilomycin treatment (**Figures 3-6, 3-7**), the levels of Akt and its phosphorylated forms in endomembranes may represent a more accurate gauge of Akt-dependent signaling, consistent with the severity of the local compartment-specific loss of Akt abundance and/or phosphorylation in the non-cytoplasmic fractions.

(2) In a somewhat related point, unlike the EN and N compartments, in which the loss of both Akt abundance and phosphorylation occurs concomitantly, the observed decrease in Akt phosphorylation in the M fraction (which, as previously noted, also likely contains ER/Golgi-associated structures as suggested by their GM130 and Calnexin content) is not due to the accompanying decrease in Akt abundance at membranes represented in the M compartment (**Figures 3-6, 3-7, 3-14**). The constancy of Akt localization to the plasma membrane in bafilomycin-treated samples was also observed in *Drosophila* salivary glands, whose examination by confocal immunofluorescence microscopy showed a fragmented *but maintained* accumulation of Akt at the plasma membrane in response to insulin treatment irrespective of V-ATPase inhibition (**Figure 2-12**); and consistent with the implication of V-ATPase function in the promotion of Akt activation *downstream* of PI3K-dependent PIP₃ production (**Figures 2-12, 2-13**). However, though the abundance of Akt at the plasma membrane was unaltered in these experiments, the levels of Akt phosphorylated at S473 *were* in fact diminished. Provided that the S473 signal in this compartment is in fact representative of the PM (from which phosphorylated Akt is known to be rapidly released from the membrane), the results suggest that although mTORC2 and Akt plasma membrane localization is unaffected, their physical interaction at this compartment, or alternatively, mTORC2 activity itself

may be (either directly, or indirectly through adaptor molecules) susceptible to bafilomycin treatment. Notably, although we examined the intracellular distribution of the S473 and T308 kinases, our analysis did not include the examination of the S473 and T308 phosphatases (PHLPP and PP2A, respectively), and their contributions to the observed downregulation of Akt in response to bafilomycin remains to be determined.

Third, our examination of ISP signaling components with known roles downstream of Akt activation (namely GSK3 α/β and mTORC1) demonstrated (1) a differential effect on the S9/S21 phosphorylation of the two GSK3 isoforms, and (2) a time-dependent downregulation of mTORC1 function in bafilomycin-treated NIH-3T3 cells (**Figure 3-8**). With respect to the bafilomycin-induced effects on GSK3 phosphorylation, our Western blot analysis of fractionated cells demonstrated that both isoforms are predominantly cytoplasmic, with discrete intracellular pools detectable in non-cytoplasmic membrane structures, a finding consistent with the experiments of Bijur and Jope (2003), and supported by the extensive literature on the subject (reviewed in **Beurel and Jope 2006**). Consistent with our analysis of whole-cell lysates (**Figure 3-8C**), our examination of subcellular fractions revealed that GSK3 β phosphorylation at S9 (**Figure 3-8D**), which (along with S21 of GSK3 α) can be targeted by a variety of kinases including RSK downstream of Ras/MAPK activation (which was not examined in our experiments), as well as Akt downstream of PI3K activation (see **Section 1-4.2**), was primarily detected in the CY fraction (and to a lesser degree, the M fraction), and unaffected by the bafilomycin-induced decrease in Akt activation. This suggests that either the latently diminished cytoplasmic pools of activated Akt are sufficient to maintain GSK3 β S9 phosphorylation, or that compensation by overlapping kinase activities maintains the S9 signal, in either case limiting its usefulness as a cytoplasmic read-out for Akt catalytic activity. In contrast, the phosphorylation of GSK3 α at S21 (unlike phospho-S9 GSK3 β) was readily detectable in the EN and N fractions, and its diminished abundance in these fractions, along with a modest decrease in the CY fraction, likely account for the time-dependent decrease in S21 phosphorylation observed in whole-cell lysates. Although the molecular basis for this differential effect on the N-terminal phosphorylation of GSK3 α and β in response to bafilomycin treatment is not

addressed in our experiments (and cannot be ascribed to the differential abundance of the two isoforms, which are equally represented), an examination of the phosphorylation status of established mediators of growth targeted by GSK3 for phosphorylation (including GS, eIF2B, and CREB), would more accurately demonstrate any bafilomycin-dependent effects on total GSK3 catalytic function in the context of the PI3K/Akt pathway. Similarly, our observed accumulation of GSK3 β in the N fraction, which based on the distribution of the phospho-S9 signal, is largely unphosphorylated and therefore catalytically uninhibited, needs to be further investigated in order to clarify whether this accumulation takes place in the nucleus itself, where its activity can impinge on survival-promoting or apoptosis-inducing processes (see **Section 1-4.7**); or alternatively, in perinuclear structures (such as the ER). The examination of bafilomycin-treated cells by confocal microscopy analysis for changes in the subcellular localization of GSK3 would discern between the nucleus and perinuclear regions, a distinction on which any speculation regarding its significance in the context of bafilomycin-induced effects in the nuclear compartment is largely predicated.

Our observed downregulation of mTORC1 activity in whole-cell lysates of bafilomycin-treated NIH-3T3 cells (**Figure 3-8E**), which we assessed by examining mTORC1-dependent S6K1 HM (T389) phosphorylation, and mTOR S2448 phosphorylation (which as mentioned in **Section 3-3.4**, is S6K1-dependent), revealed that unlike the acute inhibitory effect of V-ATPase inhibition (at concanamycin concentrations $>2\mu\text{M}$) on mTORC1 activity (**Diagram 2-14**), which was observed by Zoncu et al (**2011a**) to occur in the absence of any effects on Akt HM (S473) phosphorylation within the first hour of treatment; the time line of mTORC1 inhibition during the *prolonged* (24 hour) administration of lower concentrations of bafilomycin (whose effect on Akt phosphorylation is comparable to bafilomycin at similar concentrations, see **Figures 2-3, 2-12**) produced a comparable but *latent* reduction in mTORC1 activity that was detectable (following 4-8 hours of treatment) *after* the observed decline in Akt phosphorylation at both S473 and T308 (which is detectable within 2-4 hours of treatment with 50nM bafilomycin, **Figure 3-3**). These results suggest that in the context of NIH-3T3 cells, which we treated with 50nM bafilomycin, the comparatively latent downregulation of mTORC1, which occurs shortly after the onset of

Akt downregulation, may be a direct effect of the downregulation of Rheb activation within the growth factor-dependent branch of mTORC1 regulation; whereas the acute downregulation of mTORC1 in HEK-293T cells treated with $>2\mu\text{M}$ concanamycin has been attributed by Zoncu and colleagues to the diminished amino acid-dependent Ragulator-mediated recruitment of mTORC1 to the lysosomal membrane in the nutrient-regulated branch of mTORC1 regulation (**Zoncu et al 2011a**).

This possibility, which cannot be addressed by Western blots of whole-cell lysates, would be best examined by first, examining the efficiency of the localization of mTOR and mTORC1 components such as Raptor to the lysosomal membrane of 50nM bafilomycin-treated NIH-3T3 cells, while also examining the phosphorylation status of TSC2 and the localization of the TSC1/2 Rheb-inhibitory complex (either by confocal microscopy or subcellular fractionation and biochemical analysis). As discussed below, the growth factor-mediated recruitment of the V-ATPase A subunit (and by extension the V_1 domain) to the EN compartment (**Figures 3-6B, 3-7B**), which includes both early and late endosomes; and in particular, low-density endosomal fractions (**Figure 3-11D**), which are enriched in signaling endomembranes such as early and late endosomes (as well as some lysosomal structures); is time-dependently diminished in bafilomycin-treated samples, and temporally correlates with the decreases in EN-specific Akt phosphorylation. This suggests that (1) the growth factor-dependent branch of mTORC1 activation (leading to Rheb activation downstream of the Akt-TSC1/2 junction), and (2) the nutrient availability-dependent branch of mTORC1 activation (which V-ATPase-dependently recruits mTORC1 to the lysosomal endomembrane for Rheb-dependent activation), both of which employ the surface of endomembranes as locales for the execution of their signaling functions, may potentially contribute to the observed downregulation of mTORC1 signaling in response to the prolonged treatment of NIH-3T3 cells with chronic doses of bafilomycin. The experiments described above would delineate the contribution of the potential deregulation of these converging branches of the mTORC1 activating mechanism to the observed bafilomycin-induced downregulation of mTORC1 activity at our chosen concentration of the inhibitor, and over the treatment period we have investigated.

Lastly, whereas Akt activation at signaling membranes, which results in the release of Akt from the membrane-associated activation complex (see **Section 1-3.4**), provides (presumably) unhindered access to its cytoplasmic substrates, its maintenance at (1) signaling endomembranes such as early endosomes and MVBs, where its phosphorylation-dependent activation takes place; (2) in distal endomembranes such as late endosomes and lysosomes, where downstream effectors such as the TSC1/2 complex and Rheb are known to reside, and which serve as crucial hubs of mTORC1 regulation; as well as (3) the nuclear compartment, to which its translocation is thought to be endocytically regulated; likely requires various compartment-specific protein-protein interactions that delineate its subcellular endomembrane/nuclear distribution, and regulate its transition along the endocytic network to its appropriate destination. Accordingly, with the PM as a starting point, the activated receptor/ligand complex is endocytosed into early endosomes (EE). Early endosomes are not a homogeneous mass, and consist of subpopulations, some of which are associated with competing molecules, such as the Rab5 effector EEA1, which regulates the association of homotypic EEs and promotes their fusion; and APPL1/2, which compete with EEA1 for Rab5 binding, and promote the nuclear translocation of APPL-positive endosomes (see **Section 3-1**). As such post-EE traffic impinges on both (1) the nuclear translocation of distinct early endosomal structures, and (2) the subsequent fusion of endocytosed EEs into sorting endosomes, from which endocytosed molecules are either recycled to the membrane, or further transported to progressively more acidified endomembranes such as late endosomes/MVBs and lysosomes. The inhibition of V-ATPase activity seems to interfere with both of these post-EE trafficking routes, with each being capable of impinging on Akt activation and activity in distinct ways.

With respect to the nuclear post-EE path, our experiments examining the nuclear localization and phosphorylation of Akt consistently demonstrate its diminished activation in both cultured *Drosophila* salivary glands and in cultured NIH-3T3 cells (**Figures 2-11, 3-6, 3-7**). The second post-EE path, as alluded to above, is bifurcated, with one route leading to membrane recycling, and the other leading to lysosomal fusion. As described in **Section 3-3.5**, the recycling pathway was previously shown to be downregulated in response to bafilomycin treatment (**Balbis et al 2004**), and supported

(or at least not contradicted) by our own results examining the localization of endosomal markers, which revealed a decrease in Rab11 and (to a lesser degree) Rab4 abundance in the EN compartment (**Figures 3-9, 3-10**), both of which are implicated in endosomal recycling, and whose inhibition can result in the reduced availability of receptors at the cellular surface, in turn diminishing PI3K/Akt signaling. The inhibition of V-ATPase also results in the diminishment of transition along the endocytic pathway – from EEs to MVBs, late endosomes, and lysosomes – which requires V-ATPase activity for the progressively increased acidification on these endomembranes (reviewed in **Jefferies et al 2008**, see **Section 1-5.2**). This requirement is reflected by our findings in the subfractionated EN compartments of bafilomycin-treated NIH-3T3 cells (**Figures 3-11F, 3-13C**), in which a time-dependent loss of endocytic Akt localization is seen first in EEs (fraction 3, TP2), then in late endosomes/MVBs (fraction 5, TP4-12). These preliminary findings in our fractionation studies can be built upon in order to fill a number of conceptual gaps in the compartmental regulation of Akt activation and downstream signaling events.

With respect to our cellular fractionation experiment, through which cellular contents are roughly separated into cytoplasmic, membrane, endosomal, and nuclear/perinuclear compartments, a number of Akt-interacting molecules whose functions have been implicated in Akt subcellular localization could also be examined. Potential changes in the distribution of some of these molecules – such as (1) Tcl-1, a transcriptional regulator that promotes Akt nuclear translocation (**Laine et al 2000**, **Pekarsky et al 2000**, **Hiromura et al 2004**, **Pekarsky et al 2008**); (2) ClipR-59, which has been shown to regulate Akt membrane association and endomembrane localization in adipocytes (**Ding and Du 2009**); (3) the endosomal protein WDFY2, a FYVE domain-containing protein, which like APPLs, define a distinct subpopulation of PM-proximal early endosomes, and have been shown to serve as a scaffold for Akt downstream of insulin signaling (**Walz et al 2010**); and (4) the aforementioned APPL1/2 molecules, which themselves define a distinct subpopulation of EEs destined for nuclear translocation (**Miaczynska et al 2004b**) – would be highly informative in establishing a mechanistic correlation between our observed effects on intracellular (non-cytoplasmic) Akt localization and/or activation (**Diagram 3-5**). In order to recapitulate the

bafilomycin-induced occurrence of (and changes in) the colocalization of such intracellular translocation-promoting molecules with Akt, these additional analyses of fractionated samples could also be complemented with the confocal microscopic analysis of the interaction in question, or alternatively, through the use of Förster resonance energy transfer (FRET) reporters whose use in the investigation of the Akt-PDK1 interaction (**Calleja et al 2007, Calleja et al 2009**) was described in **Section 1-3.4**.

Our endosomal compartment subfractionation experiments (**Section 3-3.6**), which provided our most robust observations with respect to the bafilomycin-induced changes in endosomal Akt content and phosphorylation, should also be further exploited to examine the OptiPrep migration pattern of molecules such as Tcl-1, ClipR-59, WDFY2, and APPL1/2, and importantly, to investigate the endosomal distribution of upstream-acting endomembrane-associated regulators such as PDK1 and mTORC2, as well as downstream-acting endomembrane-associated effectors, such as TSC2, Rheb, Ragulator components, and mTORC1 itself. Considering the integral roles of the early-late endosomal transition and endomembrane localization in mTORC1 signaling (**Flinn et al 2010, Buerger et al 2006**), as well as our own demonstration of the bafilomycin-sensitivity of early endosomal Akt accumulation (**Figure 3-11E,F**), the delineation of the localization of the PDK1/mTORC2/Akt and TSC/Rheb/mTORC1/Ragulator signaling components (all of which generally require endomembrane localization for proper function) in growth factor-stimulated and/or bafilomycin-treated states would contribute to the mechanistic assessment of both the GF- and nutrient-mediated branches of mTORC1 regulation, whose activity, as previously mentioned, has been shown to be inhibited by V-ATPase inhibition – both in the nutrient-regulated branch (**Zoncu et al 2011a**), and as suggested by our studies, the growth factor-dependent PI3K/Akt-mediated branch.

Finally, as part of our collaboration with Dr. Rick Bagshaw and the Pawson lab, which was interrupted by the commencement of this dissertation's composition, we had intended to repeat the OptiPrep gradient endosomal subfractionation procedure for subsequent Mass Spectrometry analysis of the similarly collected fractions using a lower range of gradient density (2.5- 15%, as opposed to our standard use of the 10-30% gradient) in order to further separate the fractions represented in the 10-30% gradient as

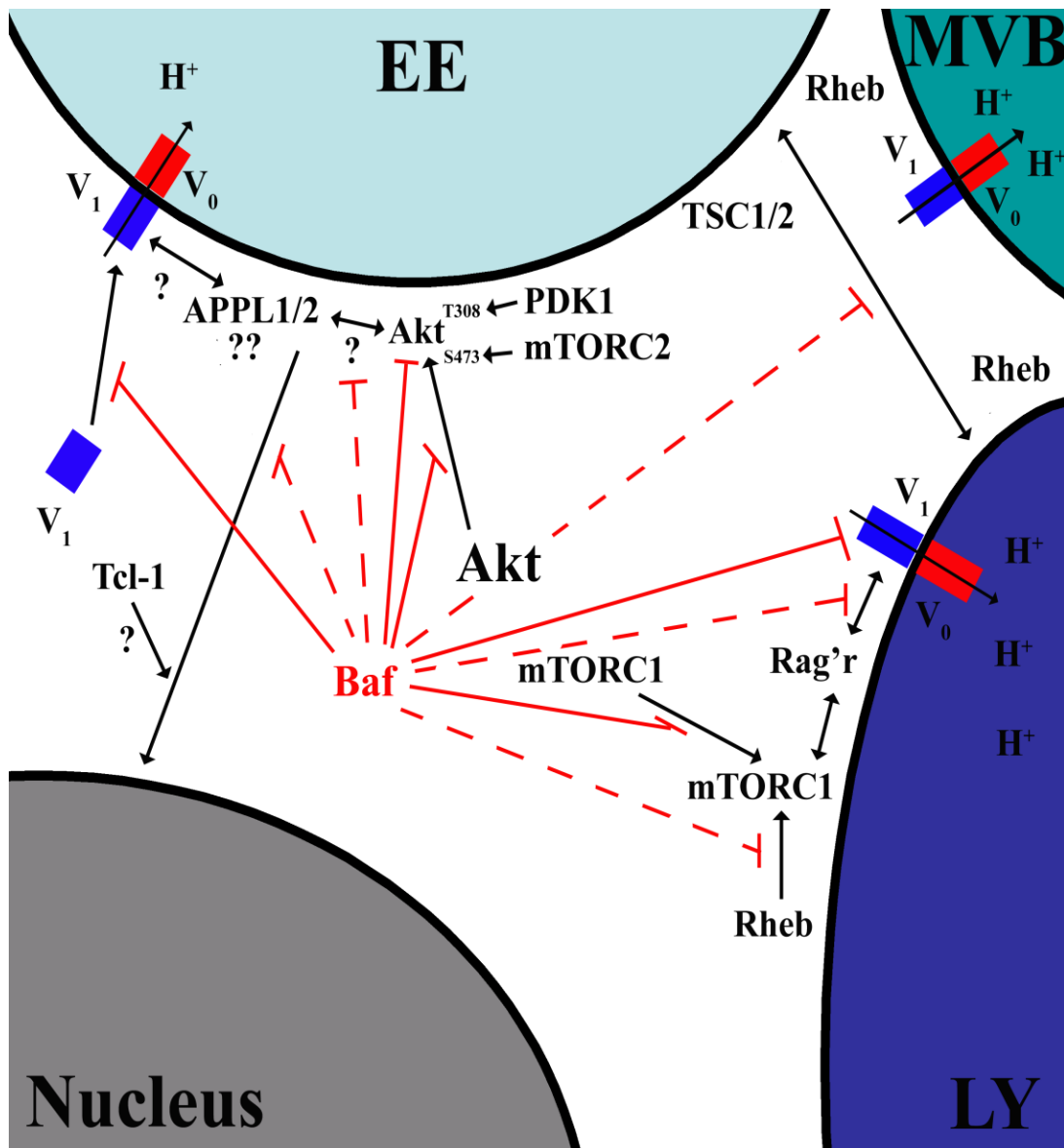


Diagram 3-5. Schematic map of demonstrated and suspected sites of bafilomycin-induced ISP inhibition. (EE) early endosome, (MVB) multivesicular body/late endosome, (LY) lysosome. Bafilomycin induced effects are depicted in red, dashed lines indicate corollary or deduced effects. With respect to Akt signaling in EEs, bafilomycin diminishes Akt recruitment to the endomembrane, downregulates its phosphorylation at the endomembrane, and decreases V_1 recruitment to the endosomal surface. The bafilomycin-induced diminished recruitment and activation of Akt at endomembranes and its translocation to the nucleus may require its interaction with endosomal markers such as APPL, and promoters of nuclear translocation such as Tc1-1. The bafilomycin-induced inhibition of mTORC1 activity can stem from a combination of the diminished V-ATPase-mediated Ragulator-dependent recruitment of mTORC1 to the lysosomal surface, and the diminished activation of Rheb as a result of decreased endomembrane-specific Akt activation. See text for further details.

fractions 1-10, which include EEs, MVBs, and lysosomes (**Figure 3-11**), the main endocytic compartments of interest with respect to growth factor-mediated Akt signaling. As mentioned above and shown in **Figures 3-6B** and **3-11D**, in addition to its well-characterized role in the dose- and time-dependent physical inhibition of V-ATPase catalytic activity (see **Sections 1-5.1, 3-3.1**), bafilomycin treatment was also found to diminish the abundance of the peripheral A subunit (Vha68/ATP6V1A) in the EN fraction, while its cytoplasmic (CY) and perinuclear (N) content was relatively unchanged. This suggests that in addition to the inhibited catalytic activity of V-ATPase in compartments requiring its function, the diminished recruitment of the A subunit to structures of the EN compartment also potentially results in the abolishment of V₁ domain-dependent protein-protein interactions at affected endomembrane surfaces (**Diagram 3-5**). These interactions may be crucial for the physical interaction of signal transducers such as mTORC2 and Akt through a mechanism analogous to that demonstrated to occur on the surface of lysosomes between V-ATPase and the Ragulator, which promotes mTORC1 lysosomal recruitment and allows its Rheb-dependent activation (**Zoncu et al 2011a**). Mass Spectrometry analysis of the collected fractions from 2.5%-15% OptiPrep gradients would allow a high-throughput assessment of molecules that co-migrate with Akt-positive endosomes in response to growth factor stimulation (and conversely, potentially depleted from endosomal populations in response to bafilomycin treatment), and could serve not only as a screening method for the identification of potentially novel Akt-interacting molecules in specific endosomal populations susceptible to bafilomycin, but would also allow the investigation of the distribution pattern of known or suspected interacting molecules with roles in Akt-dependent processes.

Taken together, our examination of the phosphorylation and subcellular distribution of Akt, ATP6V1A, and selected components of the PI3K/Akt signaling pathway, including PDK1, mTOR, S6K1, and GSK3, demonstrate that in NIH-3T3 fibroblasts cultured under standard conditions (5%FBS), the administration of 50nM bafilomycin for up to 24 hours (1) time-dependently downregulates Akt phosphorylation at both S473 and T308, including distinct compartment-specific losses of S473 and T308

phosphorylation in membrane (M), endosomal (EN) and nuclear (N) fractions within 12 hours of bafilomycin administration; (2) diminishes the endosomal, and nuclear pools of Akt concomitantly with the observed decreases in Akt phosphorylation; (3) diminishes the phosphorylation of downstream-acting signaling components of the PI3K/Akt pathway, including the downregulation of mTORC1-dependent S6K1 HM phosphorylation at T389, and the diminished phosphorylation of GSK3 α at S21; and (4) does not overtly affect the subcellular distribution (and in the case of PDK1, the activity) of upstream-acting Akt regulators such as PDK1. These results are consistent with our demonstration in *Drosophila* of the independence of the downregulation of Akt activation and nuclear translocation from PI3K-dependent PIP₃ production and PH domain-dependent membrane localization, and suggest that the bafilomycin-mediated decrease in Akt phosphorylation in endomembrane compartments results from the loss of Akt-specific endosomal compartments required for the endosomal concentration of Akt, and/or its diminished recruitment to intracellular signaling membranes for activation or nuclear translocation. As discussed in the fourth and final chapter, the prevalence in diverse cancers of Akt-activating mutations in upstream-acting signaling components such as various RTKs, PI3K, and the tumor suppressor PTEN; and the positive role of V-ATPase in Akt signaling downstream of these molecules, including its capacity to suppress the loss of PTEN function in *Drosophila*, makes V-ATPase a highly amenable and potentially pharmacologically exploitable molecular target, especially in tumors bearing inactivating mutations of PTEN, in which the pharmacological reconstitution of PTEN-mediated PI3K inhibition is inherently fraught with difficulty, in that whereas excessive activity of positive regulators such as PI3K can be (both theoretically and in practice) pharmacologically reduced to the desired level, the loss of tumor-suppressing negative regulatory function cannot be easily restored with synthetic molecular agents. As such, we investigated the cytotoxic capacity of V-ATPase inhibition and its correlation with levels of Akt phosphorylation in two tumor models – glioblastoma multiforme (GBM), and in collaboration with Dr. Keith Stewart, multiple myeloma (MM) – in which PI3K/PTEN/Akt axis signaling defects are frequently detected, and usually correlate with aggressive stages of the disease and poor prognosis.

CHAPTER 4

IN VITRO ASSESSMENT OF V-ATPASE INHIBITION AS A VIABLE AVENUE OF CANCER PHARMACOTHERAPY

4-1 – Introduction

Aberrant activation of the PI3K/Akt pathway has been widely implicated in many cancers, with estimates suggesting that mutations in PI3K/Akt signaling pathway components account for nearly 30% of all tumors (reviewed in **Luo et al 2003, Shaw et al 2006**). Accumulating evidence from genetic, biochemical and clinical studies ascribe a prominent role for the PI3K/Akt pathway in cancer cell growth and survival (**Diagram 4-1**), and have culminated in the aggressive development of PI3K/Akt pathway small molecule inhibitors alongside Ras/MAPK inhibitors for the *targeted* chemotherapy of various cancers (reviewed in **Engelman 2009, Bartholomeusz and Gonzallez-Angulo 2012**). Oncogenic mutations that result in the overactivation of PI3K/Akt signaling have been characterized and mapped to components acting at various steps between RTKs and Akt itself, and usually consist of activating mutations in oncogenes that act as positive regulators of the PI3K signal, and/or loss-of-function mutations in tumor suppressors that negatively regulate PI3K/Akt-dependent signaling.

As such, numerous RTKs with significant dimensions of downstream PI3K/Akt activation have been identified in various cancers as targets of oncogenic mutation, including the IGFR, which is overexpressed in numerous cancers including multiple myeloma (reviewed in **Baserga et al 2003, Hartog et al 2007, Pollak 2008**); the FGFR receptor family, which is activated by mutation, or (more rarely) overexpressed/amplified in cases of multiple myeloma (MM), as well as bladder, cervical, and prostate cancers (reviewed in **Grose and Dickson 2005, Turner and Grose 2010**); and the EGFR family of receptors, activating mutations of which have been identified in lung and colorectal cancers, as well as glioblastoma (GBM) tumors (reviewed in **Barber et al 2004, Normanno et al 2006, Sharma et al 2007**). Core signaling components acting downstream of these RTKs with known modulatory functions in PI3K/Akt signal transduction and susceptibility to oncogenic mutations include PI3K, PTEN, PDK1, and Akt itself (**Appendix I**). In addition to its ectopic activation in cells expressing oncogenic Ras mutants (reviewed in **Yuan and Cantley 2008, Zhao and Vogt 2008**), activating mutations (or amplification) of PI3K are often detected in cancer, and most commonly map to the p110 α isoform (*PIK3CA*). As shown in **Appendix I**, these genetic

modifications in PI3K function are found in several types of cancer including GBM, hepatocellular carcinoma, as well as breast, endometrial, and colorectal cancers (reviewed in **Courtney et al 2010**). Although rare in comparison to the diversity of tumors bearing *PIK3CA* mutations (and the relatively high frequency of detection in these cancers), activating mutations of the p85 α regulatory subunit of PI3K (*PIK3R1*) have also been detected in cases of GBM, ovarian, and colon cancer (**Philp et al 2001, Parsons et al 2008, Cancer Genome Atlas Research Network 2008**); and furthermore, though similar activating mutations of *PIK3CB* (p110 β) have not yet been found, and the amplification of p110 β is a rare occurrence, a significant proportion of colon and bladder cancers have been found to exhibit increases in p110 β activity and/or expression (reviewed in **Liu et al 2009b**), and its activity has been implicated in a murine tumor development model (**Ciraolo et al 2008**). As a negative regulator of PI3K/Akt signaling, the catalytically antagonistic activity of PTEN towards the PI3K product PIP₃ is also, as previously described in the introductory chapter (see **Section 1-2**), a hotspot of pathological deregulation in tumorigenesis (reviewed in **Song et al 2012**). In addition to germline mutations in the *PTEN* gene, which are thought to underlie the inherited cancer predisposition of PTEN hamartoma tumor syndromes, the somatic loss of PTEN due to genetic mutation, deletion, loss of heterozygosity, protein instability, or epigenetic modification also frequently occurs in many human tumors, including GBM and melanoma, as well as breast, prostate, gastric, endometrial, ovarian, and colon cancers (**Appendix I**).

Downstream of the PI3K/PTEN rheostatic junction, though less frequent in occurrence, mutations (or deregulated expression) have also been identified in PDK1, Akt, and various Akt-interacting proteins that modulate the latter's activity and function. Though its amplification or overexpression is detected in ~20% of breast cancers (**Brugge et al 2007**), activating mutations in the PDK1 (*PDPK1*) molecule itself are rarely found in most cancers – only two instances of its occurrence have been documented in colorectal cancer, and one in glioma (**Hunter et al 2006**). Downstream of PDK1, the amplification of *Akt* has been reported in a number of tumor types, including *Akt3* amplification in glioblastoma, and *Akt2* amplification in ovarian, breast and pancreatic cancers (**Appendix I**). More recently, an activating mutation in the PH domain

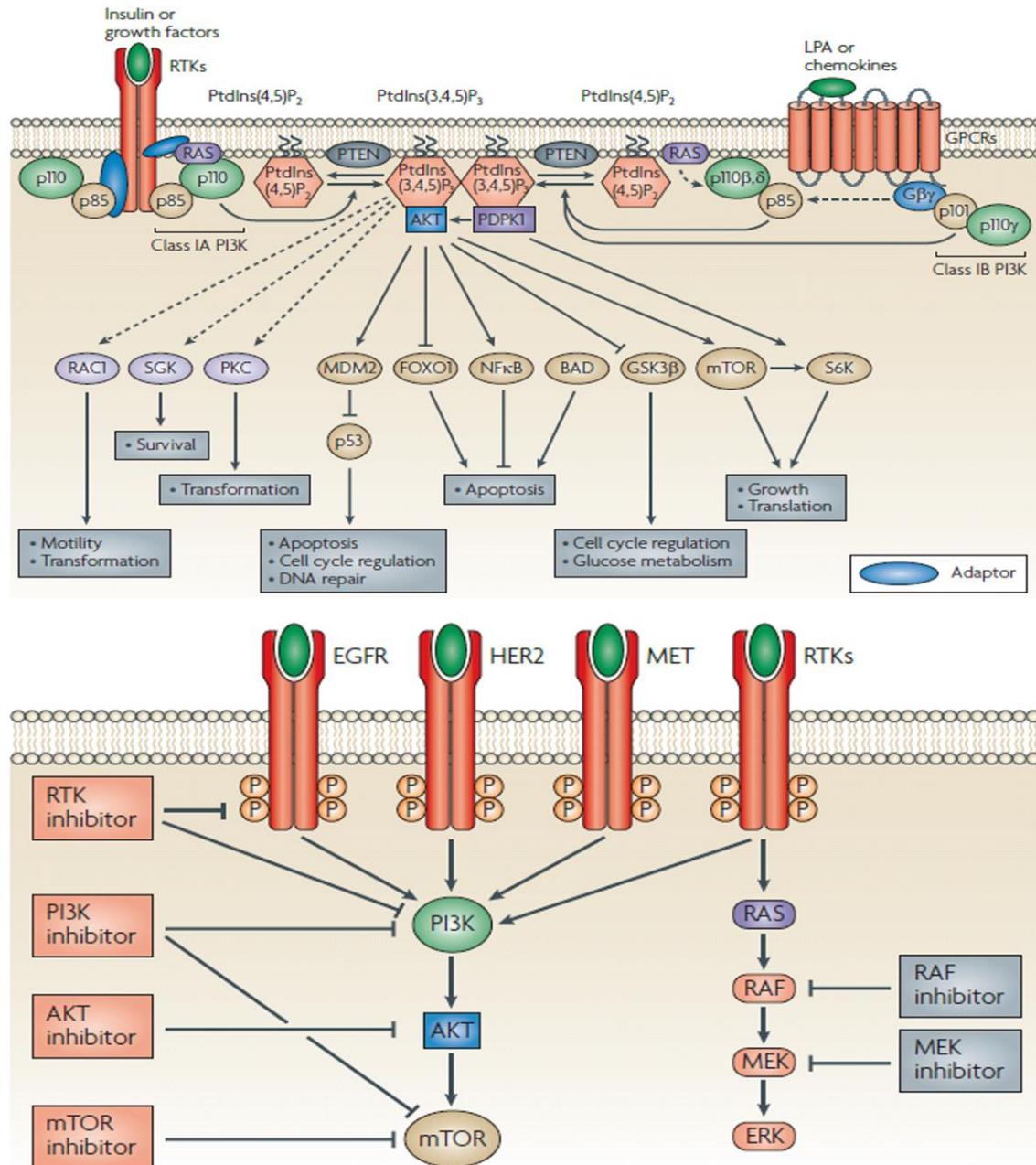


Diagram 4-1. Pharmacological targeting of the PI3K pathway in cancer 1.

Reproduced from Liu et al (2009b). In the upper panel, the contribution of PI3K/Akt-mediated signals to cellular processes relevant to tumorigenesis and therapy are graphically depicted. Akt and PDK1 substrates with crucial roles in tumor cell growth/survival are highlighted. The lower panel is a schematic representation of the current target-directed approach for the pharmacological treatment of tumors with elevated levels of PI3K and Ras-dependent signaling pathways.

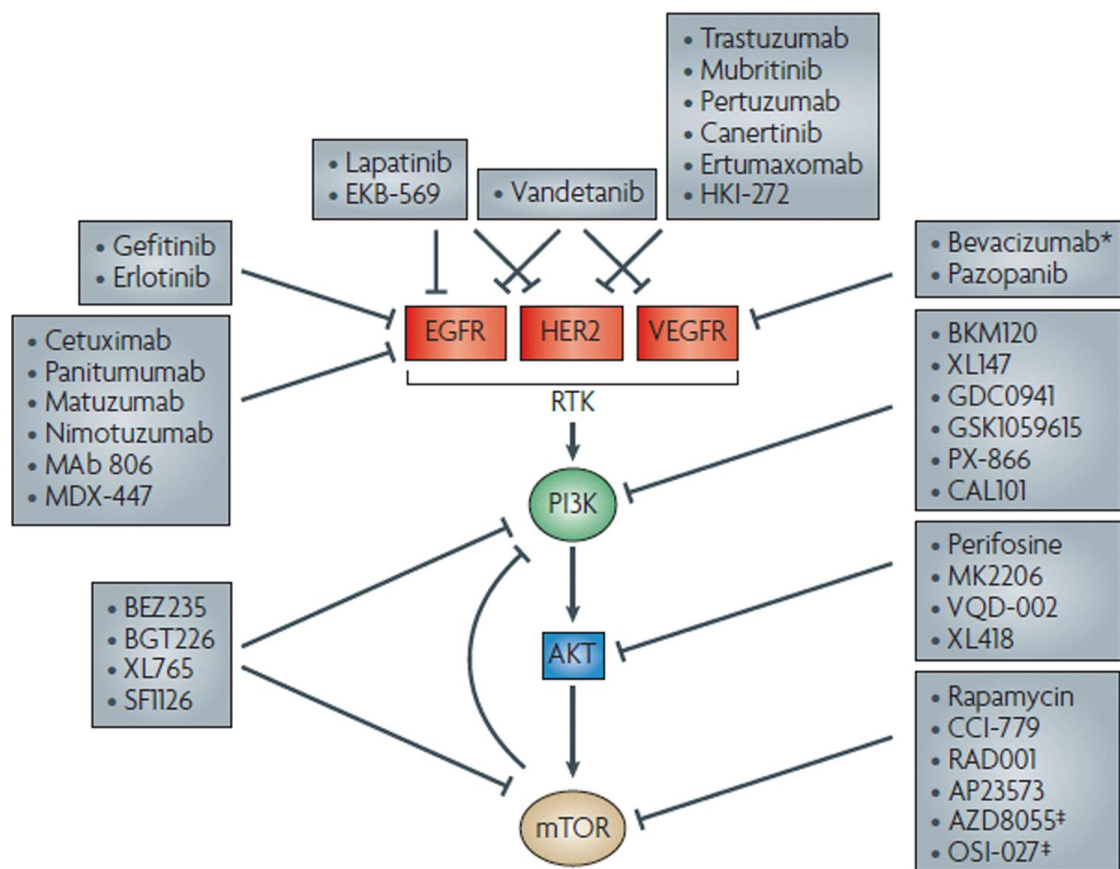


Diagram 4-2. Pharmacological targeting the PI3K pathway in cancer 2. Reproduced from Liu et al (2009b). Inhibitors in clinical development that target the PI3K pathway are shown. EGFR (epidermal growth factor receptor); HER2 (human epidermal growth factor receptor 2, also known as ERBB2); VEGFR (vascular endothelial growth factor receptor). (*)Bevacizumab targets VEGFA instead of VEGFR directly. ‡Both AZD8055 and OSI-027 are ATP-competitive mTOR inhibitors that target the mTOR complexes mTORC1 and mTORC2.

of *Akt1* (E17K) has been detected in human breast, colorectal, ovarian, and lung tumor samples, and shown to induce the ectopic membrane localization of Akt1 (**Carpenter et al 2007, Do et al 2008, Bleeker et al 2008, Malanga et al 2008, Shoji et al 2009**). The corresponding mutation in *Akt3* was also detected in clinical specimens of melanoma (**Davies et al 2008**). Furthermore, as mentioned in **Section 1-4.8**, in addition to the oncogenic alterations in the abundance and localization of Akt itself, changes in the expression levels of a number of genes encoding Akt-interacting proteins with various roles in the regulation of its activation, subcellular localization, and substrate specificity (including Tcl-1, CTMP, PIKE-A, and Hsp90/Cdc37) have been observed in a variety of tumors.

As described in the introductory chapter (**Section 1-4.3**), the disruption of TSC1/2-mediated Rheb inhibition can cause the familial (in the case of germline mutations) or sporadic (somatic mutations) forms of TSC (**Hodges et al 2001**). Although heterozygous mutants of either TSC1 or TSC2 are tumor prone in murine knockout models (**Onda et al 1999, Kobayashi et al 2001**), the human autosomal dominant TS hamartomas are generally benign focal neoplasms – that is, though they can grow abnormally (in some cases causing serious medical or cosmetic complications), their progression to malignancy is very rare (reviewed in **Kwiatkowski et al 2003**). Furthermore, The biochemical analysis of TSC1/2 functional deficiency further demonstrated that the TOR pathway was ectopically activated in TSC1- or TSC2-deficient cells (**Gao et al 2002, Goncharova et al 2002, Kwiatkowski et al 2002, Inoki et al 2002**), and could be suppressed by rapamycin treatment or TSC1/2 overexpression (**Kenerson et al 2002, Tee et al 2002**), suggesting (correctly) at the time that TSC1/2 may be an upstream-acting negative regulator of TOR/S6K1 signaling. The elucidation of the Akt/TSC/Rheb/mTORC1 signaling pathway in the mid-2000s, and the detection of mTORC1 hyperactivation in certain cancers made mTOR an attractive target of pharmacological inhibition in tumors bearing genetic deficiencies impinging upon its appropriate regulation, such as the loss of PTEN function which results in mTORC1 upregulation downstream of ectopically increased Akt activation (reviewed in **Guertin and Sabatini 2005, Sabatini 2006**). Furthermore, though initially characterized as rapamycin-insensitive, the demonstrated vulnerability of the mTORC2 complex to

prolonged rapamycin treatment (**Sarbassov et al 2006**), which in a clinical context (considering its direct role in Akt activation), was potentially as significant as its acute effects on classically rapamycin-sensitive mTORC1 activity (see **Section 1-3.3**). The vulnerability of mTORC2 activity to chronic rapamycin treatment implicated both mTOR complexes in rapamycin's recognized pre-clinical anti-tumor properties, and galvanized the examination of rapamycin (also known as sirolimus) and its analogs Torisel (CCI-779, temsirolimus); Afinitor (everolimus, RAD001, NCT01417559); and AP23573 (deforolimus); which along with various other small molecules targeting mTOR kinase activity, and Akt inhibitors such as perifosine (**Diagram 4-2**), are currently being assessed in numerous clinical trials (**Appendix II**). Although a statistically significant increase in the risk of metabolic complications has been ascribed to their clinical use (**Sivendran et al 2013**), and response rates in early clinical trials were modest, ranging from a low of less than 10% in GBM or advanced renal-cell carcinoma (RCC) patients (**Galanis et al 2005, Chang et al 2005, Atkins et al 2004**), to a high of ~40% in mantle-cell lymphoma (MCL) patients (**Witzig et al 2005**); temsirolimus has been approved for the treatment of RCC and MCL, and everolimus is set to be approved for pancreatic neuroendocrine tumors (reviewed in **Fasolo and Sessa 2012**).

Just as the latent (cell type-specific) inhibition of mTORC2 in response to prolonged rapamycin treatment underlies its increased relevance in the clinical setting of cancer pharmacotherapy, our experiments in *Drosophila* and cultured NIH-3T3 cells suggest that the inhibition of V-ATPase activity, though inhibitory of nutrient-dependent mTORC1 activation independently of Akt (**Zoncu et al 2011a**), may also result in the downregulation of both Akt and mTORC1 activity downstream of RTK activation. Our genetic and biochemical characterization of the interaction detected between *Dakt* and *Vha68-2* has demonstrated that *Vha68-2* is a positive regulator of Akt activity, acts downstream of the PTEN/PI3K junction in response to RTK activation, and importantly, the loss of its function can suppress the growth phenotypes associated with the loss of PTEN function in *Drosophila*. In addition, experiments with loss of function mutant alleles of *Vha68-2*, and the specific V-ATPase inhibitor bafilomycin A1 further suggest that the inhibition of V-ATPase activity results in a decrease in RTK-mediated Akt phosphorylation, with an attending decrease in viability as a result of apoptosis. In this

final chapter, the clinical relevance of these cytotoxic attributes attributed to V-ATPase inhibition is examined in two cancer disease models - multiple myeloma and glioblastoma - in which a number of characteristic mutations correlating with malignancy and poor prognosis are known to result in enhanced Akt activation, be it by gain of function mutations in Akt activators or loss of function mutations in negative regulators of Akt activation. A description of our preliminary *in vitro* experiments assessing V-ATPase inhibition in the therapeutic context of MM and GBM is preceded by a brief synopsis of the etiology, pathobiology, and pharmacotherapy of these two cancers (see below), and followed by a discussion of our findings with respect to a possible role for V-ATPase inhibitors in the current pharmacological arsenal ([Diagram 4-2](#)) targeting RTK-dependent signal transduction along the PI3K/Akt pathway in the clinical treatment of these two and other forms of cancer, either as single agents, or in combination with potentially synergistic pharmacological inhibitors of oncogenic signaling pathways.

Multiple myeloma (MM)

B cell lymphocytes are critical components of the highly adaptive humoral immune response, providing specific and long-lasting protection from the panoply of potential pathogens that can be encountered over the course of a human lifetime. The activation of B cells is initiated in response to antigen recognition through the B cell receptor, resulting in B cell proliferation and differentiation. Activated B cells can subsequently terminally differentiate into either plasma cells (PC) which are primed for active antibody secretion, or memory cells (MC) that archive the antigen encountered ([Diagram 4-3](#)), and provide protection against future recurrences of infection (reviewed in **Harwood and Batista 2010**). Following this terminal differentiation, normal PCs are non-proliferative, arrested in the G1 phase (G0), and typically undergo apoptosis following immunoglobulin secretion (reviewed in **Chen-Kiang 2005** and **Tarlinton et al 2008**). Multiple myeloma (MM), also known as plasma cell myeloma (PCM), is an incurable plasma cell malignancy resulting from the clonal expansion of malignant plasma cells in the bone marrow, often correlating with the excessive deregulated secretion of clonal immunoglobulins in the absence of activation. MM cells are characterized by a marked genomic instability, and carry complex and heterogeneous

cytogenetic abnormalities including ploidy status alterations, deletions/amplifications at different chromosomes, and chromosomal translocations, resulting in multiple oncogenic mutations that consequently deregulate the intrinsic biological function of the plasma cell (reviewed in **Kuehl and Bergsagel 2002, Hideshima et al 2007a**).

Among the cytogenetic abnormalities characteristic of MM cells, the deregulation of oncogenes by aberrant chromosomal translocations to the immunoglobulin heavy chain (IGH) locus (14q32) is a seminal event in the pathogenesis of B cell tumors (**Korsmeyer et al 1992, Bergsagel et al 1996**). The process of B cell activation and maturation requires somatic hypermutation (SHM) of the DNA encoding the hypervariable regions of the IGH locus in order to produce highly specific high-affinity antibodies to the particular antigen presented, and the functionality of these antibodies is increased by class switch recombination (CSR) which produces antibodies of different immunoglobulin isotypes. Mechanistically, both SHM and CSR are mediated by the generation of double strand DNA breaks (DSB) in the Ig loci (reviewed in **González et al 2007**). Aberrant chromosomal translocations occur when DSBs (particularly in switch regions) are not locally repaired following SHM or CSR, but are instead joined to DSBs occurring elsewhere in the genome. As such, since myeloma is a tumor of antibody-producing plasma cells, not only are these otherwise (in other cell types) rare aberrant mutations and translocations generated “routinely” as the result of recombination in a normal and highly specialized process, but they are even *tolerated* by their host, because in most individuals, these rearrangements actually improve immune function and short-term survival (though at a significant long-term cost). These non-random promiscuous translocations to the IGH locus (**Diagram 4-4A**), which occur in approximately 40% of MM tumors and nearly 90% of human MM-derived cell lines, result in the juxtaposition of various genes to a strong Ig enhancer that significantly increases their transcription. Accordingly, Ig translocations have been demonstrated to involve a large number of chromosomal loci including 11q13 (**Chesi et al 1996, Gabrea et al 1999**), 6p21 (**Shaughnessy et al 2001**), 4p16.3 (**Chesi et al 1997, Chesi et al 1998a**), 16q23 (**Chesi et al 1998b**), and 8q24 (**Avet-Loiseau et al 2001**), in which the putative target genes encoding the cell cycle regulators *cyclin D1* and *cyclin D3*, the fibroblast growth factor receptor *FGFR3* (as well as *MMSET*), and the proto-oncogenic transcription factors c-

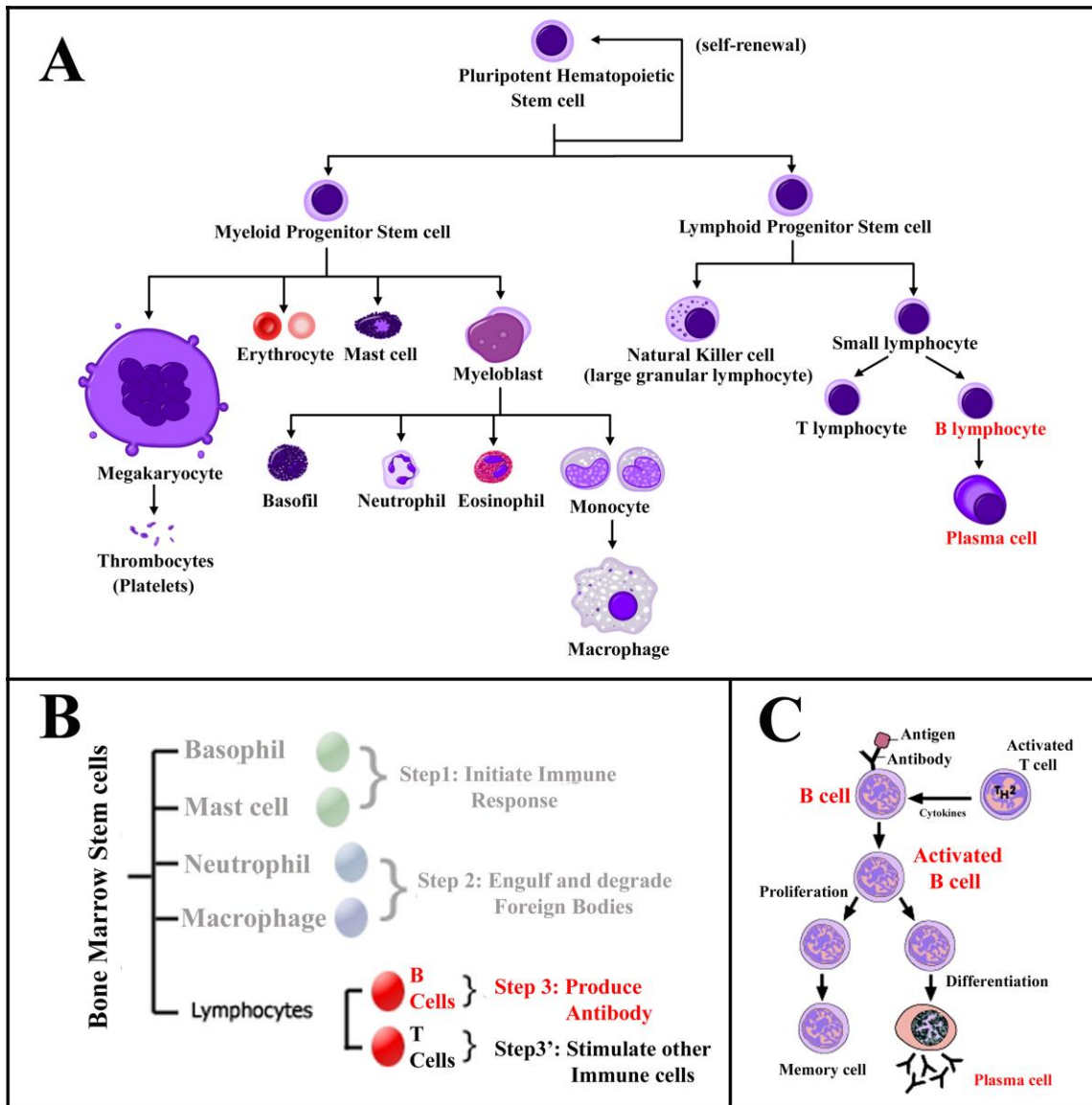


Diagram 4-3. Chain of hematopoietic events leading to B lymphocyte activation and plasma cell differentiation. (A) Schematic summary of hematopoietic B lymphocyte generation. During blood cell development, stem cells differentiate into lymphoid and myeloid lineages. With the exception of red blood cells (erythrocytes), these lineages differentiate into mature subsets of white blood cells which comprise the immune system. B lymphocytes are derived from small lymphocytes, themselves generated from the lymphoid precursor stem cells, which along with myeloid precursor stem cells, are progenitors of pluripotent self-renewing stem cells of the bone marrow. (B) Schematic representation of immune response amplification, whereby B cell activation stimulates antibody production. (C) Antigen- or T cell-activated B cells proliferate, and differentiate into either memory cells or antibody-producing plasma cells.

A

Locus	Oncogene	Incidence
11q13	<i>CCND1</i>	15%–20%
6p21	<i>CCND3</i>	5%
4p16.3	<i>FGFR3</i> and <i>WHSC1</i>	12%
16q23	<i>MAF</i>	5%–10%
8q24	<i>MYC</i>	< 10%
6p25	<i>MUM1/IRF4</i>	5%
20q11	<i>MAFB</i>	5%
1q21-34	<i>BCL9, IL6R, MCL1</i>	Frequent

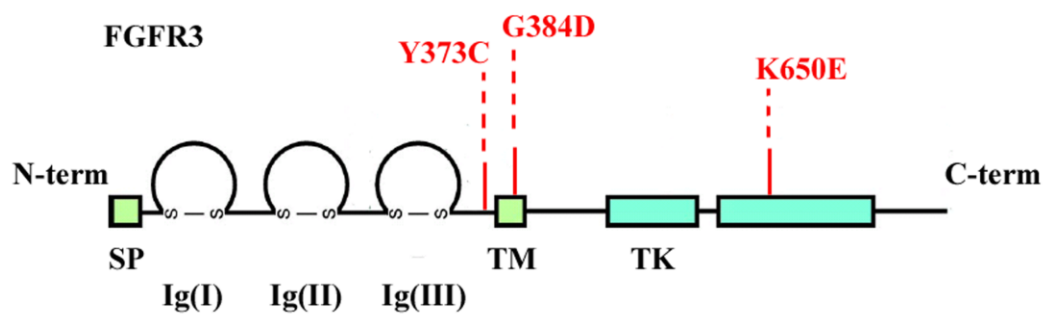
B

Diagram 4-4. The *FGFR3* gene is often overexpressed and/or mutationally activated in MM. (A) Table listing the genetic loci, affected oncogenes, and frequency of incidence in their aberrant chromosomal translocation to the immunoglobulin heavy chain (IGH) locus (14q32). (B) Schematic representation of the FGFR3 protein, including the relative locations of three activating somatic mutations frequently detected in the progression of MM. See text for details. SP (signal peptide), TM (transmembrane domain), TK (split tyrosine kinase domain), Ig (Immunoglobulin-like domains I, II, and III).

MAF and *c-MYC* are respectively located (reviewed in **Bergsagel and Kuehl 2001**). The various classes of translocations involving the *IGH* locus tend to be mutually exclusive, although in 25% of advanced MM cases, two independent translocations can be found in the same patient (**Bergsagel and Kuehl 2005**). The oncogenic consequences of these translocations lead to the ectopic survival and proliferation of malignant plasma cell clones within the bone marrow due to the loss of cell cycle controls, disabled pro-apoptotic mechanisms, or the elevated activity of growth- and survival-promoting signaling pathways.

For over 40 years, prior to the development of treatment regimens that incorporated the proteasome inhibitor bortezomib (**Richardson et al 2003, Richardson et al 2005, San Miguel et al 2008**) which received accelerated approval by the FDA in 2003, and the immunomodulatory drugs (IMiD) thalidomide (reviewed in **Palumbo et al 2008**) and lenalidomide (reviewed in **Falco et al 2008**), the standard of therapeutic care for patients newly diagnosed with multiple myeloma consisted of the combined administration of melphalan (trade name Alkeran, a nitrogen mustard alkylating agent initially investigated as a possible drug for use in melanoma treatment, and later found to be of use in myeloma) and prednisone (a synthetic corticosteroid drug that acts as a general immunosuppressant), with a median survival prognosis of 29-37 months. The current chemotherapeutic regimen for patients with newly diagnosed MM is influenced by a variety of factors (**Diagram 4-5**), including age, co-morbidities, and eligibility for standard high dose chemotherapy with autologous stem cell transplantation (reviewed in **Kumar and Rajkumar 2011, Mahindra et al 2012**). Along with advances in clinical practice predicated on a deepening understanding of the biology of myeloma clones and their interaction with the bone marrow, the adjunctive addition of more potent cytotoxic agents and immunomodulators like bortezomib and IMiDs (such as thalidomide and its derivatives lenalidomide and pomalidomide) to combinatorial therapeutic cocktails have improved prognosis with a survival period frequently exceeding five years (reviewed in **Latif 2012**). Specifically, these agents trigger caspase-mediated apoptosis, decrease the binding of tumor cells to bone marrow stromal cells, inhibit the secretion of survival-promoting cytokines from the bone marrow, inhibit angiogenesis, and stimulate immunity against myeloma cells (reviewed in **Mahindra et al 2010**). Unfortunately, drug

resistance and relapse occurs in the majority of patients with all currently available treatment options, and in addition, a subset of patients fails to respond to initial treatment altogether.

The t(4;14) translocation which is detected in ~20% of all MM tumors (**Finelli et al 1999, Malgeri et al 2000**) and its associated deregulation of *FGFR3* is particularly correlated to poor prognosis (**Moreau et al 2002**) in this genetic subtype of MM patients. Although the prognosis in t(4;14)-positive patients is poor irrespective of ectopic *FGFR3* expression (**Keats et al 2003**), the increased expression of *FGFR3* is often associated with this translocation, and activating somatic mutations (Y373C, and G384D in the transmembrane domain, K650E in the kinase domain, [Diagram 4-4B](#)) frequently occur during tumor progression, uncoupling FGFR3 activity from ligand binding-dependent activation, and thereby conferring constitutive activity to this already overexpressed RTK (**Chesi et al 1997, Ronchetti et al 2001, L'Hôte and Knowles 2005**). Downstream of the upregulated FGFR3 receptor, the aberrant activation of RTK signaling and the consequently constitutive recruitment of signal transducing adaptor complexes to the activated receptor can result in the ectopic activation of the Ras/MAPK, JAK/STAT and PI3K/Akt signaling pathways (reviewed in **Turner and Grose 2010**). As such, the inhibition of FGFR3 has been demonstrated to induce apoptosis (**Trudel et al 2004, Trudel et al 2006**) and inhibit tumor growth (**Xin et al 2006**) in t(4;14) multiple myeloma. Conversely, enhanced proliferation and resistance to apoptosis has been observed in response to the activation of the ERK (**Ogata et al 1997**), STAT (**Catlett-Falcone et al 1999**), and Akt (**Tu et al 2000, Hsu et al 2002**) signaling cascades in MM cells. In particular, Akt, which is activated in MM patient cells and correlates with advanced stage and poor prognosis (**Hsu et al 2001**), and the PI3K/Akt pathway have been ascribed a cardinal role in the proliferation, survival, and drug resistance of MM cell growth (**Hideshima et al 2007b**).

Over the past decade, a concerted effort towards the development and testing of small-molecule inhibitors of the PI3K/Akt pathway for the treatment of myeloma has produced encouraging results. *In vivo*, inhibition of Akt by the PI3K pathway inhibitor perifosine (KRX-0401) has been documented in xenograft mouse models of MM (**Hideshima et al 2006, Hideshima et al 2007b, Catley et al 2007**). In preclinical

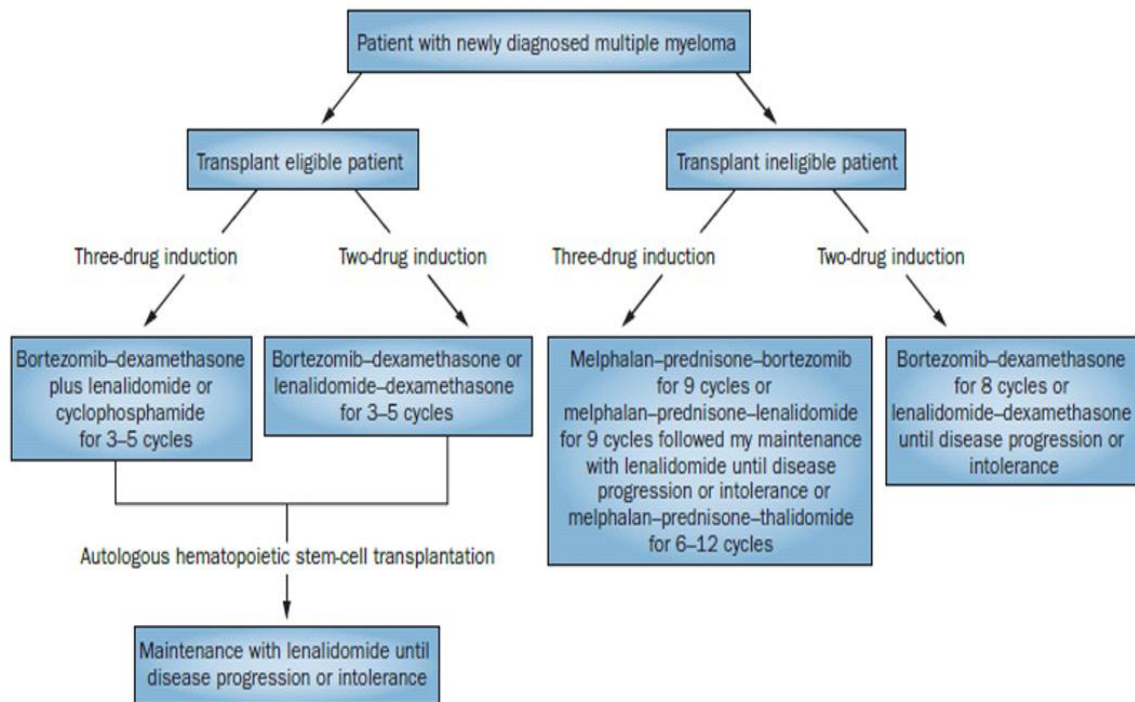


Diagram 4-5. Current treatment regimens for patients with newly-diagnosed multiple myeloma. Reproduced from Palumbo and Anderson (2011). Several of the listed drug regimens are currently being evaluated in investigational trials. These include combination induction therapy with bortezomib and dexamethasone plus cyclophosphamide or lenalidomide, maintenance therapy with thalidomide or lenalidomide in younger patients, and melphalan–prednisone–lenalidomide followed by maintenance therapy with lenalidomide in elderly patients. If autologous hematopoietic stem-cell transplantation is delayed until the time of relapse, bortezomib-based regimens should be continued for eight cycles, whereas lenalidomide-based regimens should be continued until disease progression or the development of intolerable adverse effects.

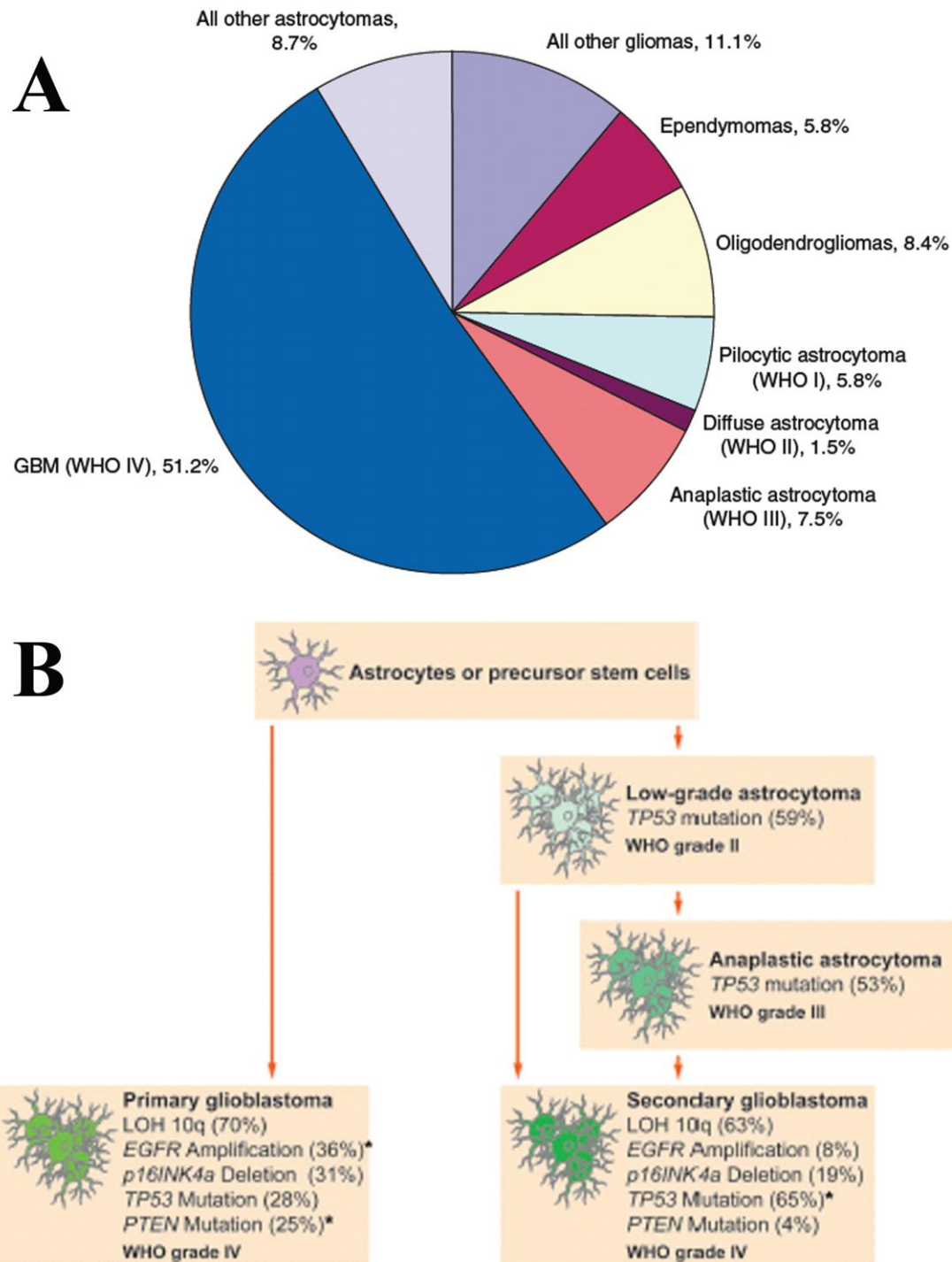


Diagram 4-6. Distribution of CNS gliomas and genetic pathways to primary and secondary GBMs. (A) Reproduced from Adamson et al (2009). Distribution of all primary CNS gliomas (n = 26,630). (B) Reproduced from Ohgaki and Kleihues (2007). (*) genetic alterations that differ significantly in frequency of occurrence between primary and secondary glioblastomas. See text for details.

models, the combination of bortezomib (which triggers Akt activation) with perifosine overcame resistance to bortezomib, and furthermore, perifosine was found to augment both melphalan- and bortezomib-induced MM cell cytotoxicity (**Hideshima et al 2006**, **Hideshima et al 2007b**). The results of Phase I-II trials with this combination therapy showed durable responses, even in the settings of resistance to (or relapse following) bortezomib treatment (**Richardson et al 2011**); and a Phase III clinical trial of bortezomib versus the combination of bortezomib and perifosine in patients with relapsed MM is ongoing (NCT01002248). In addition to the perifosine success story, a number of novel agents targeting Akt activation or activity are currently being tested in preclinical and Phase I-II clinical trials in combinatory therapeutic regimens including INK128 and AZD8055, which are dual inhibitors mTORC1/2 (**Cirstea et al 2010**); NVP-BEZ235, a composite inhibitor of mTORC1/2 and PI3K (**McMillin et al 2009**); the TORC2 inhibitor pp242 (**Hoang et al 2010**); and the mTOR-inhibiting rapamycin analogs Torisel (CCI-779, temsirolimus, **Farag et al 2009**), Afinitor (everolimus, RAD001, NCT01417559), and AP23573 (deforolimus, **Rizzieri et al 2008**).

Glioblastoma multiforme (GBM)

Malignant gliomas are the most common type of brain tumor (**Louis et al 2007**), and include astrocytomas and glioblastomas, which respectively comprise ~20-30% and 50% of cerebral gliomas ([Diagram 4-6A](#)). Glioma tumors are histologically classified into Grades I through IV according to the criteria of the World Health Organization (WHO), and carry corresponding prognostic and survival correlates (reviewed in **Kleihues et al 2002**, **Louis et al 2007**). Grade I tumors typically have a good prognosis, and most frequently occur in children (**Pollack et al 1995**). Grade II tumors (low grade diffuse astrocytomas) are histologically characterized by hypercellularity and correlate with a median survival of 5-8 years, while Grade III astrocytoma tumors (or anaplastic astrocytomas) are histologically hypercellular, exhibit nuclear atypia and mitotic figures, and correlate with a 3-year median survival (reviewed in **Kleihues et al 2002**, **Wen and Kesari 2008**).

The progressive malignant transformation starting with a series of initiation events in an astrocyte or astrocyte precursor cell leading to a benign astrocytoma (Grade

I) are followed by the accumulation of genetic defects causing anaplasia, and leading to the formation of the Grade IV tumors. This most malignant grade, also called glioblastoma multiforme (GBM), is characterized by hypercellularity, nuclear atypia, high mitotic rates, evidence of angiogenesis, and poor response to treatment (**Burger et al 1985**). Grade IV GBM tumors themselves can be further subdivided into primary and secondary tumors on the basis of the patient's age at presentation and the genetic alterations present in the malignancy. GBM tumors that arise from preexisting Grade II or III astrocytomas are rarer in occurrence (10% of all GBM tumors), generally occur in younger patients (mean age of 45 years), and are considered secondary tumors. Primary (or *de novo*) GBM tumors are morphologically indistinguishable from secondary GBM tumors, but present with a fulminant clinical course in older patients, typically >60 years of age, without a preexisting glioma of lower grade, and account for nearly 90% of all GBM tumors (reviewed in **Louis 2006**). The aggressive growth of *de novo* primary tumors precludes their incidental discovery at less malignant grades, and greatly contributes to the overall poor prognosis of survival for GBM patients. Despite recent advances in surgical and clinical oncology (and extensive treatment regimens including radical surgery, radiation, and chemotherapy), the median survival time is ~1 year from diagnosis (**Johnson and O'Neill 2012**).

In addition to poor prognosis, and despite a common clinical presentation, another pathological feature shared by MM, GBM, and most if not all other cancers is etiological heterogeneity (reviewed in **Ohgaki and Kleihues 2007**). Recent comprehensive genetic screens of GBM have confirmed that genetic alterations are scattered across the entire genome, affecting numerous chromosomes (**Parsons et al 2008**). Many of the genetic losses and mutations identified (**Diagram 4-6B**) result in the loss of function of specific tumor suppressor genes with direct effects on gliomagenesis. In particular, mutations or deletions in the *p53*, *PTEN*, and *p16^{INK4A}/p14^{ARF}* genetic loci on chromosomes 17p, 10q, and 9p, respectively, has been reported to occur with high frequency in GBM (detection rates of 41%, 40%, and 60%, respectively), leading to the loss of their tumor-suppressing functions (**Ohgaki et al 1995, Wang et al 1997, Tohma et al 1998, Ishii et al 1999**). Less frequently, gains of gene function have been demonstrated to be by-products of the duplication of entire chromosomes, intrachromosomal amplification of specific alleles,

and activating mutations. The most often-cited example is the amplification of the *EGFR* gene on chromosome 7 which occurs in ~40% of GBMs (**Wong et al 1987, Libermann et al 1985a, Libermann et al 1985b**), and its constitutively active variant III mutant (*EGFRvIII*) which is present in 20-50% of GBMs with EGFR amplification (**Steck et al 1988, Nishikawa et al 1994, Wong et al 1992**). Other instances include the amplification of the *MDM2* gene (see **Section 1-4.7**), an inhibitor of p53 located on chromosome 12q, which is present in >10% of GBMs (**Reifenberger et al 1993**) and is usually found strictly in GBMs that lack a *p53* mutation (**Biernat et al 1997, Ghimentì et al 2003**); as well as gain of function mutations of p110 α (*PIK3CA*, located on chromosome 3q), which is observed in 5-27% of newly diagnosed GBMs (**Broderick et al 2004, Samuels et al 2004**).

Cumulatively, these mutations in the EGFR/PTEN/PI3K pathway, the p53/MDM2/ p14^{ARF} pathway, and the p16^{INK4A}/RB1 pathway lead to signaling abnormalities that significantly contribute to the malignancy of GBM tumors (**Diagram 4-7**). Increased RTK signaling through EGFR, increased PI3K activity, and/or loss of PTEN function can all potentially result in the ectopic activation of Akt, with the consequent enhancement of Akt-mediated growth-, proliferation-, and survival-promoting signaling pathways; whereas the losses of p16^{INK4A}/p14^{ARF} and p53 function (or alternatively, *MDM2* amplification) result in the loss of G1 to S transitional inhibition in their respective pathways, further compounding the tumorigenic signaling context of enhanced PI3K/Akt activation by subverting the control of cell cycle progression, thereby facilitating proliferation, and enhancing survival.

The critical role of PI3K/Akt signaling in the pathological malignancy of GBM is further underscored by the differential detection frequency of the various common genetic alterations found in GBM between tumors of primary and secondary origin (reviewed in **Ohgaki and Kleihues 2007**). As depicted in **Diagram 4-6B**, in the case of secondary GBM tumors (which progress from preexisting lower-grade tumors), *p53* mutations are early and frequent genetic alterations (**Watanabi et al 1997**), and occur in approximately two-thirds of precursor low-grade diffuse astrocytomas (Grade II), anaplastic astrocytomas (Grade III), and the secondary glioblastomas (Grade IV) derived thereof (frequencies of 59%, 53% and 65%, respectively). However, despite the high

frequency of *p53* mutations in Grade II and III tumors, and the retention of these genetic defects over the course of tumor progression, the most malignant stage (Grade IV) is marked by the acquisition, albeit at lower frequencies, of additional genetic alterations, most commonly consisting of *EGFR* amplification (8%), loss of *PTEN* function (4%), and/or *p16^{INK4A}/p14^{ARF}* deletion (19%), suggesting a correlation between these crucial oncogenic mutations that upregulate growth and the high level of malignancy and poor prognosis associated with GBM Grade IV tumors (reviewed in **Ohgaki and Kleihues 2007, Gladson et al 2010**). In primary *de novo* GBM (Grade IV) tumors, which develop rapidly, and present at diagnosis as full blown tumors without any clinical, radiological or histopathological evidence of a less-malignant precursor lesion, *p53* mutations are far less prominent (28% frequency of detection) in comparison to secondary GBMs (65%). In contrast, *EGFR* amplification, loss of *PTEN* function and/or *p16^{INK4A}/p14^{ARF}* deletion, (which, as mentioned above, correlate with the malignancy of secondary Grade IV tumors), are predominant in primary GBMs, with comparatively high occurrence frequencies of 36-60%, 14-47%, and 31-60%, respectively (reviewed in **Ohgaki and Kleihues 2007, Gladson et al 2010**). This distinction suggests that pathological malignancy in GBM, irrespective of the etiological course of glioblastomagenesis, correlates with *EGFR*, *PTEN*, and/or *p16^{INK4A}/p14^{ARF}* mutations, and their attendant consequences on growth, proliferation, and cell cycle progression. In the case of secondary GBM tumors, these late alterations occur in a pathological genetic background predisposed to malignant transformation with a high frequency of *p53* mutations, whereas in primary GBMs, which account for the majority of newly diagnosed Grade IV GBMs, mutations in *EGFR*, *PTEN*, and/or *p16^{INK4A}/p14^{ARF}* occur at relatively high frequencies, and result in malignancy with or without concomitant mutations in *p53*, which is detected less frequently in primary GBMs.

Although its mechanism was not clearly understood at the time, radiation therapy (RT) was the first post-surgical adjuvant treatment that consistently demonstrated efficacy over the course of the 1960s and 1970s, typically doubling survival from 4-6 months to 10-11 months (reviewed in **Adamson et al 2009**). With respect to chemotherapeutic avenues, numerous studies with the alkylating agent carmustine (BCNU) spanning four decades suggested a marginal added benefit to survival of ~2

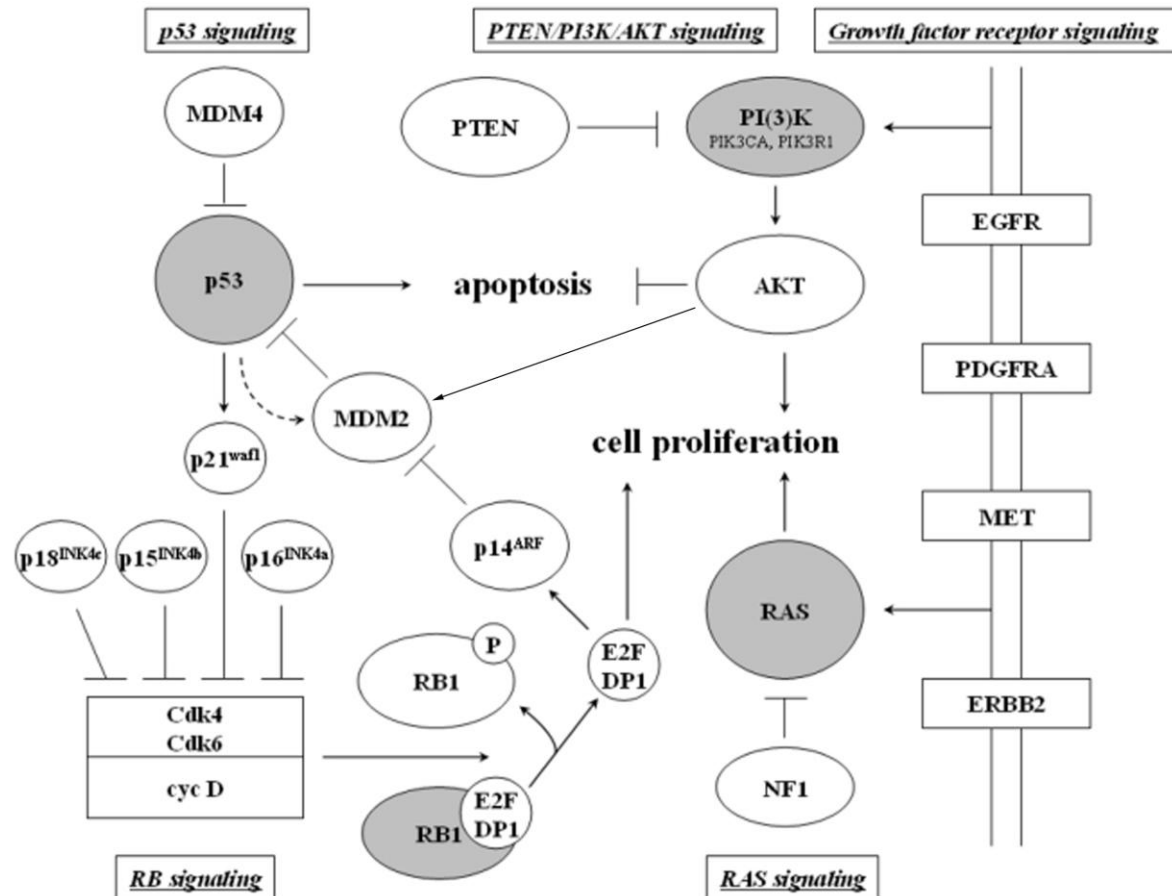


Diagram 4-7. Convergence of p53, Rb, and PI3K signaling in the pathogenesis of gliomas. Reproduced from Riemenschneider and Reifenberger (2009). While p53 mutation, amplification of MDM2/MDM4, or p14ARF deletion/methylation promote survival, alterations in p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p21^{waf1} subvert the inhibition of cell cycle progression at the G1/S-phase checkpoint by allowing the unfettered cyclin-dependent kinase-mediated phosphorylation of RB1, resulting in the release of E2F transcription factors and the promotion of proliferation. Amplification, overexpression or mutation of growth factor receptors stimulates cell proliferation and inhibits apoptosis through both the RAS as well as the PI3K/AKT signaling pathway.

Presentation	Treatment
Adult low-grade astrocytoma/oligodendroglioma	Surgery → RT or CT or observation
Recurrent, progressive low-grade disease	No prior RT: Surgery Surgery (if resectable) → RT ± CT Prior RT: Surgery → CT → RT at progression
Ependymoma	RT (limited field or craniospinal) or observation
Recurrent ependymoma	Surgery (if resectable) → RT (if no prior RT) → consider CT or BSC
High-grade glioma (anaplastic astrocytoma, anaplastic oligodendroglioma, glioblastoma multiforme)	Maximal excision ± BCNU wafer (Stereotactic or open biopsy if maximal excision not possible) → RT ± CT (concurrent and/or adjuvant)
Recurrent high-grade glioma, diffuse or multiple lesions	BSC (poor PS) or CT or palliative surgery
Recurrent high-grade glioma, localized disease	Surgery (if resectable) ± BCNU wafer → RT (highly conformal) or CT → BSC

Diagram 4-8. Treatment of gliomas by presentation. Summary of National Comprehensive Cancer Network (NCCN) guidelines. (RT) radiotherapy; (CT) chemotherapy; (BCNU) carmustine; (BSC) best supportive care; (PS) performance status.

months (**Stewart 2002**). Despite modest benefits, systemic administration of BCNU, and subsequently in the mid-1990s, locally administered BCNU (Gliadel, **Westphal et al 2003**), remained the universal standard of care for adjuvant chemotherapy. In 2005, a single study established a new systemic chemotherapy using another alkylating agent, temozolomide (TMZ), which demonstrated similar efficacy to that of BCNU, but with less toxicity (**Stupp et al 2005**). Unfortunately, despite decreased toxicity, the current standard regimen for GBM therapy (**Diagram 4-8**) consisting of a combination of surgical resection, followed by treatment with RT and TMZ only confers a median survival period of 14.6 months (**Stupp et al 2005**, reviewed in **Dresemann 2010**).

TMZ exhibits schedule-dependent antineoplastic activity by methylating DNA (predominantly at the O6-position of guanine), thereby interfering with DNA replication due to mismatching with thymine, resulting in cytotoxic lesions which, when left in disrepair, induce apoptotic cell death (reviewed in **Newlands et al 1997**, **Margison et al 2002**). One recognized reason for the failure of TMZ to act as an effective cytotoxic chemotherapeutic agent is the presence of DNA repair enzymes such as O⁶-methylguanine-DNA-methyltransferase (MGMT), which specifically removes pro-mutagenic alkyl groups from the O6-position of guanine in DNA, thereby counteracting the cytotoxic effect of alkylating chemotherapeutic agents like TMZ, and contributing to drug resistance (**Belanich et al 1996**). As such, epigenetic silencing of the *MGMT* promoter by methylation (**Watts et al 1997**), which has been observed in gliomas (**Nakamura et al 2001**), is a reliable predictive criterion of tumor response to TMZ treatment (**Hegi et al 2005**). With respect to the efficacy of chemotherapeutic agents with DNA damage-mediated cytotoxic effects such as TMZ, the influence of p53 status on the response to treatment is doubly complicated depending on signaling context and the presence or absence of its function. Functional p53 may contribute to drug resistance and survival if therapeutic DNA damage is preferentially repaired (**Fan et al 1995**); or conversely, to drug sensitivity if the tumor cells induce apoptosis in response to treatment (**Lowe et al 1993**). As such, the loss of p53 function may itself lead to drug sensitivity if apoptosis is induced p53-*independently* due to ineffective DNA repair and checkpoint failure; or conversely, to drug resistance if the p53-deficient tumor cells fail to apoptose despite DNA damage.

Conventional chemotherapeutic agents, such as TMZ in GBM chemotherapy (and melphalan in MM chemotherapy), were developed based on general cytotoxic properties correlating to efficacy in tumor treatment. Our deepening understanding of aberrant RTK signaling, its regulation of growth-, proliferation-, and survival-promoting pathways, and their relevance to GBM tumor pathology has led to the design of combinatorial clinical trials using target-directed small molecule inhibitors against various signaling components in the RTK/PI3K/Akt pathway (**Adamson et al 2009**), often in conjunction with RT and TMZ (reviewed in **Krakstad and Chekenya 2010**), and with encouraging results. A median survival of 19.3 months compared to 14.1 months in controls was observed in a Phase II clinical trial using a treatment regimen consisting of RT and a combination of TMZ and the EGFR inhibitor Erlotinib (**Prados et al 2009**). A number of combinatorial clinical trials with the Akt inhibitors perifosine (Phase II, NCT00590954) and nelfinavir (Phase I/II, NCT00694837); the mTOR inhibitors temsirolimus (Phase I, NCT00316849) and everolimus (Phase I/II NCT00085566); and the PI3K/mTOR dual inhibitor XL765 (Phase I, NCT00704080) are also planned, recruiting, or ongoing (reviewed in **Adamson et al 2009, Krakstad and Chekenya 2010**). Preliminary results in murine GBM disease models have also been promising. *In vivo*, the inhibition of Akt by perifosine, and its cooperation with TMZ to arrest the cell cycle was demonstrated in a glioma xenograft mouse model (**Momota et al 2005**); while *in vitro*, cell lines with activated Akt signaling were more sensitive to perifosine than those without, and the ability of perifosine to inhibit tumor growth *in vivo* correlated with decreases in Akt phosphorylation (**Hennessy et al 2007**). Furthermore, perifosine and temsirolimus were recently demonstrated to cooperate in the induction of cell death and decreased proliferation irrespective of PTEN status in a murine glioblastoma model (**Pitter et al 2011**), whereas a significant additive cytotoxicity was attributed to the PI3K/mTOR dual inhibitor XL765 in a GBM xenograft mouse model in response to combinatorial treatment with TMZ and XL765 (**Prasad et al 2011**).

Study rationale and objectives:

The preliminary findings described in the paragraph above suggest that the current flourish in clinical studies investigating target-directed PI3K/Akt inhibiting small

molecules may result in significant contributions to the current arsenal of GBM and MM chemotherapeutic agents. In the following three sections, our preliminary experiments examining the cytotoxic capacity of V-ATPase inhibition in MM-derived cell lines (in collaboration with Dr. Keith Stewart) and GBM-derived cell lines are described with respect to its efficacy in reducing viability, inducing apoptosis, and decreasing Akt phosphorylation in these cancer models associated with pathological levels of RTK/PI3K signaling. Our aim was twofold – our first objective was to assess the *in vitro* efficacy of bafilomycin as a cytotoxic agent in these tumor-derived cell lines in vitro, while our second objective, should the first be met, was to investigate the status of Akt phosphorylation in bafilomycin-sensitive tumor-derived cell lines in order to identify any potential correlation between bafilomycin-dependent cytotoxicity and bafilomycin-induced Akt downregulation in these two tumor models.

4-2 – Materials and Methods

Antibodies, and reagents.

Bafilomycin A1 was purchased from Sigma Aldrich (B1793), and dissolved in DMSO. Anti-CD138 microbeads were purchased by Dr. Stewart from Miltenyi Biotec Inc. The reagents used for immunodetection include: Akt (CST #9272), phospho-Akt S473 (CST, #4060), phospho-Akt T308 (CST #9275), HRP-linked secondary antibody (CST #7074), biotinylated anti-rabbit secondary antibody (Vector BA-1000), and streptavidin-FITC (Vector SA-5001). The fluorescent nuclear stain DAPI was purchased from Molecular Probes (D3571).

Cell lines and Patient samples.

All human MM cell lines were maintained by Dr. Stewart's staff in RPMI-1640 supplemented with 5% FBS, 100ug/mL penicillin and streptomycin (Hyclone). For patient samples, bone marrow aspirates were obtained by Dr. Stewart with written consent under a Mayo Clinic IRB approved protocol. Primary MM cells were isolated and purified by positive selection using anti-CD138 microbeads. The purified myeloma cells were cultured at 37°C (5% CO₂) in Iscove Modified Dulbecco Medium (IMDM, Invitrogen 12440-053) supplemented with 10% FBS. For my own experiments, the H929 cell line was purchased from ATCC (CRL-9068), and maintained in RPMI-1640/5% FBS. The U87MG, U343MG, and U373MG were maintained in DMEM/5% FBS, and kindly donated by Dr. Abhijit Guha.

Cell viability assays.

My assessment of cell viability in U87MG, U343MG, and U373MG cells was carried out as described in **Section 3-2**. Dr. Stewart and his colleagues measured cell viability in myeloma cells using the MTT cell proliferation kit according to the manufacturer's (Boehringer Mannheim) instructions. Cells were seeded in 96-well plates (20,000 cells/well), and incubated for 72 hours with vehicle or bafilomycin. The experiment was performed in triplicate.

FACS analysis.

Our assessment of apoptotic induction in H929, U87MG and U343MG cells was carried out as described in **Section 3-2**, with the exception of H929 cells, which were not trypsinized (as they are non-adherent cells grown in suspension), and 25,000 cells were gated for each treatment (as opposed to the 50,000 cells used for other cell lines).

Immunoblot analysis of cell lines and patient samples.

My Western blot analysis of U87MG cells was carried out with whole cell lysates of control and bafilomycin-treated samples harvested in RIPA buffer and processed as previously described. Western blot analysis by Dr. Stewart's laboratory was performed as described in Trudel et al (2005).

Immunofluorescence analysis of U87MG cells.

I cultured U87MG cells in DMEM/5% FBS on poly-L-lysine coated glass slides (Sigma Aldrich P0425) contained in non-treated sterile culture plates (in order to restrict cellular

adherence to the glass slide surface). Following vehicle or bafilomycin treatment, plates were rapidly rinsed with PBS at room temperature, fixed in 4% formaldehyde in PBS for 10 minutes, washed three times in PBS for 5 minutes, permeabilized with 0.1% Triton X-100 in PBS for 30 minutes, washed three times in PBS for 5 minutes, blocked in 5% BSA in PBS for 30 minutes, and thereafter incubated with the primary antibody (phospho-Akt S473 CST #4060) in blocking solution for an hour. Following the incubation period, the glass slides were washed three times in PBS for 5 minutes, and incubated with biotinylated anti-rabbit secondary antibody (Vector BA-1000) in blocking solution for an hour. After three final washes in PBS, the poly-L-lysine coated wells were covered in mountant with DAPI (~40 μ l) and streptavidin-FITC (Vector SA-5001) for analysis by confocal microscopy.

4-3 – Results

4-3.1 – Bafilomycin treatment reduces the viability of cultured human myeloma cell lines

In collaboration with my supervisor Dr. Manoukian, Dr. Keith Stewart and his colleagues investigated the effect of V-ATPase inhibition on cellular viability in a genetically heterogeneous and standardized panel of fourteen human MM-derived cell lines *in vitro* (see [Appendix III](#)). These myeloma cell lines were grown in culture and treated with a range of bafilomycin concentrations over a 72 hour time span. Following the incubation period, cellular viability was determined by MTT assay. Bafilomycin was found to be a highly potent cytotoxic agent for *all* fourteen assayed myeloma cell lines, as their residual *in vitro* viability after 72 hours of 40nM bafilomycin treatment ranged from 5% in the highly bafilomycin-sensitive H929 and KMS12PE cell lines to <50% in the least sensitive OPM2 cell line, as compared to untreated controls (**Figure 4-1**). The half-maximal effective bafilomycin concentration (EC_{50}) after 72 hours of treatment ranged from 2.5nM for the highly sensitive UTM2 cell line to 8.9nM for the moderately sensitive SKMM2 cell line (**Figure 4-1**, [Appendix III](#)). The least bafilomycin-sensitive cell line (OPM2) was an outlier to this range with a 72 hour EC_{50} of 20nM bafilomycin. We ourselves confirmed the contribution of apoptotic cell death to the decrease in viability demonstrated in the bafilomycin-sensitive cell lines by the FACS analysis of AnnexinV/PI-treated H929 cells. Following 48 hours of treatment with 50nM bafilomycin, we observed a ~60% decrease in the population of viable H929 cells, with a corresponding increase in the AnnexinV-positive dead or apoptotic H929 cell population (**Figure 4-2**).

Interestingly, of the fourteen MM-derived cell lines tested for reduced viability, six cell lines were positive for the t(4;14) IGH translocation (UTM2, KMS18, H929, KMS11, OPM1, and OPM2), four of which - UTM2, H929, KMS18 and KMS11 - were consistently among the most sensitive to bafilomycin treatment throughout the range of concentrations used (**Figure 4-1**). Considering the overexpression of FGFR3 in these four cell lines, some of which additionally harbor an activating mutation in the overexpressed

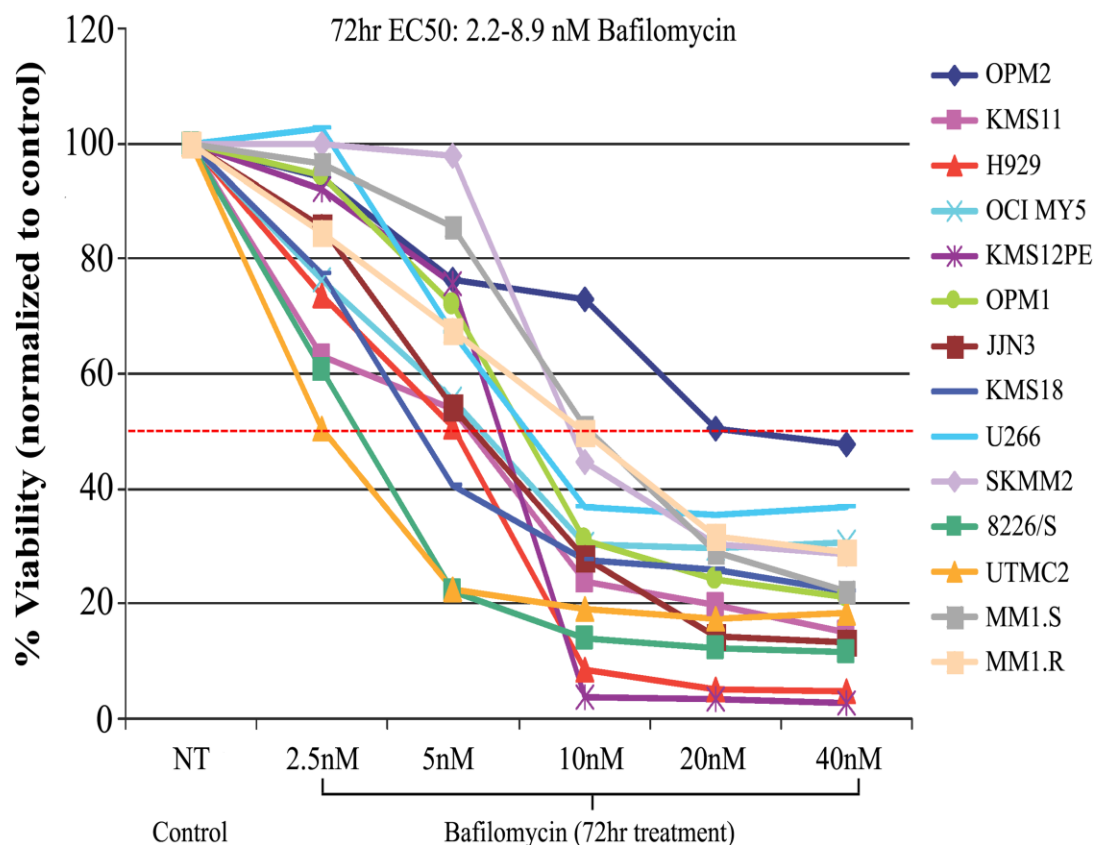


Figure 4-1. Bafilomycin treatment significantly reduces the viability of cultured human myeloma cell lines. Courtesy of Dr. Keith Stewart and colleagues. Fourteen cultured myeloma cell lines were treated with various concentrations (2.5-40nM) of bafilomycin. The viability of these cell lines following 72 hours of treatment was assessed with the MTT cell viability assay. Absorbance was read at 490nm and graphically depicted as a percentage value normalized to the corresponding non-treated control for each treated cell line. The half-maximal effective concentration (EC50) of bafilomycin for all fourteen cell lines was calculated using GraphPad Prism software.

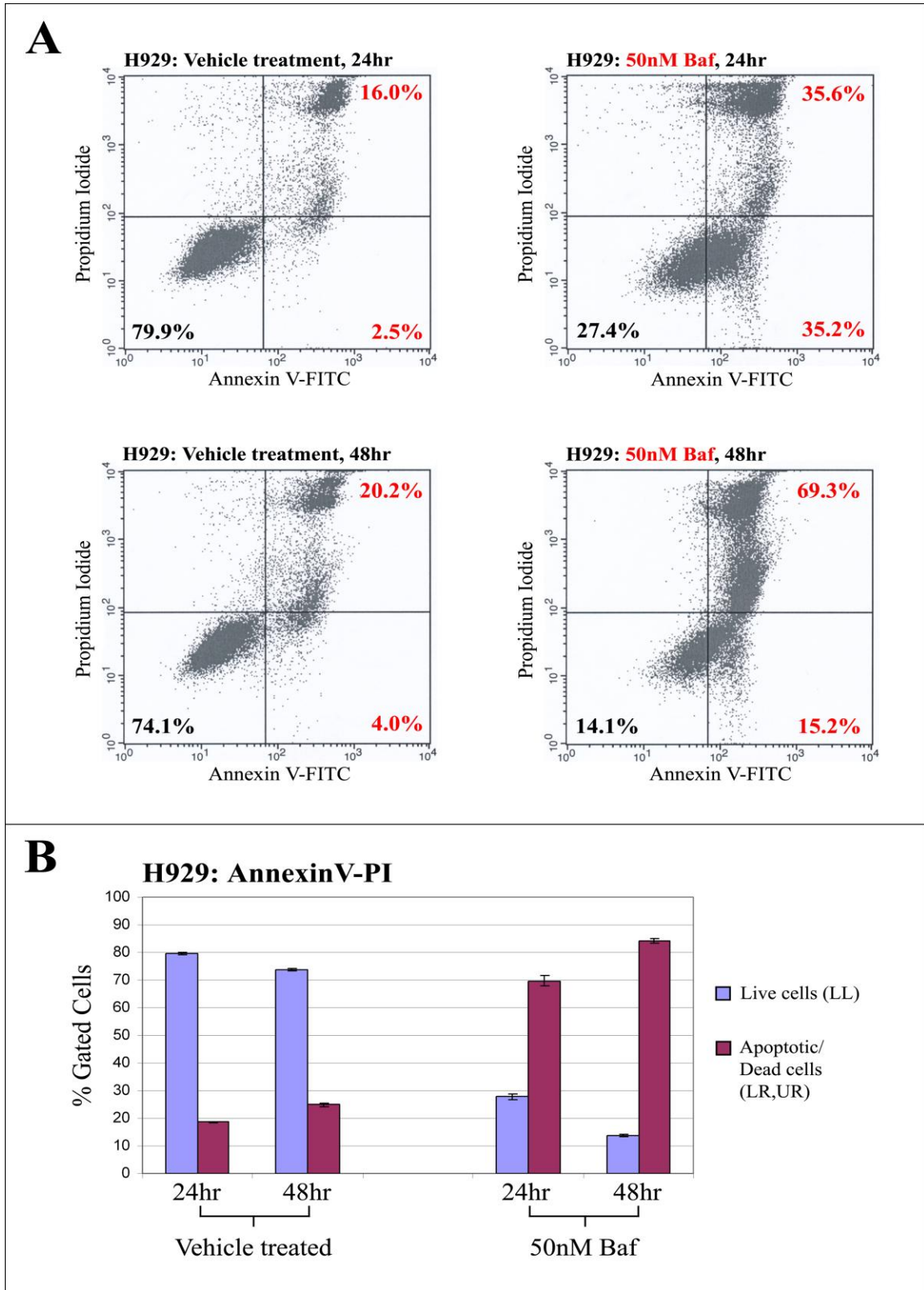


Figure 4-2. Bafilomycin treatment induces apoptosis in cultured H929 human myeloma cells. H929 human myeloma cells were cultured to a density of 5×10^5 cells/mL,

either vehicle (DMSO) or bafilomycin-treated the growing culture for up to 48 hours, and harvested at the indicated time intervals. Harvested cells were then labeled with AnnexinV- FITC (BioVision) and propidium iodide (PI, BioVision), and subjected to FACS analysis. The results of these experiments, done in triplicate, are presented as: **(A)** representative dual parameter scatter plots with indicated percentage values of viable cells (AnnexinV- and PI-negative, Lower Left quadrant, in black), apoptotic cells (AnnexinV-positive and PI-negative, Lower Right quadrant, in red), and dead cells (AnnexinV- and PI-positive, Upper Right quadrant, also in red) over the course of the bafilomycin treatment period as compared to vehicle-treated control cells over the same time span; and **(B)** a graphical depiction of the percentage distribution of viable and apoptotic/dead gated cells in response to vehicle and bafilomycin treatment over the same time span. LL-Lower Left, LR-lower right, UR- Upper Right.

gene ([Appendix III](#)), the presumed ectopic activation of RTK signaling as a direct consequence of this deregulation, and the increasingly appreciated role of the endocytic network in signaling downstream of multiple RTKs, the significant loss in viability observed in t(4;14)-positive cells in response to bafilomycin treatment suggests a possible predisposition of this upregulated signaling network to bafilomycin-mediated RTK-dependent downregulation, and an increased susceptibility to inhibitors of endocytic processes.

Incidentally, the two other t(4;14)-positive cell lines (OPM1 and OPM2) both overexpress an *FGFR3* mutant (K650E) with an activating mutation in the kinase domain (**Chesi et al 1997**) in addition to the putative deregulation of *FGFR3* caused by the t(4;14) translocation which they share (**Chesi et al 1998a**). However, unlike the OPM1 cell line, OPM2 also harbors a loss-of-function *PTEN* mutation (**Hyun et al 2000**), resulting in the elimination of a crucial negative regulator of RTK-based PI3K activity. This concomitant loss of PTEN activity thereby compounds the severity of the ectopic RTK signaling activity and has been demonstrated to result in significantly elevated levels of Akt phosphorylation in this cell line (**Hyun et al 2000, Shi et al 2002**). As such, OPM1 (wildtype *PTEN*) was found to be moderately sensitive to bafilomycin treatment (EC₅₀ of 7.6nM, only slightly lower than the highly sensitive KMS11 cell line, [Appendix III](#)), whereas the OPM2 cell line, was the least bafilomycin-sensitive cell line tested in the panel, with an EC₅₀ of 20nM after 72 hours of treatment (**Figure 4-1**). This disparity suggests that although ectopic activation of RTK-based signaling may sensitize a given cell line to bafilomycin-mediated cytotoxicity (as observed in the t(4;14)-positive UTM2, H929, KMS18, KMS11 and OPM1 cell lines), the *additional* loss of PTEN function in the OPM2 cell line may overwhelm the capacity of bafilomycin-mediated inhibition of V-ATPase activity to serve as an effective means of down-regulating ectopically elevated RTK-based signaling activation in a signaling context devoid of the negative regulation normally provided by PTEN activity, thereby resulting in the *relatively* low apparent sensitivity of the OPM2 cell line to bafilomycin-induced cytotoxicity.

4-3.2 – Bafilomycin treatment reduces the viability of cultured U87MG and U343MG human glioma cell lines

In addition to the investigation of bafilomycin cytotoxicity in the MM tumor model, which was spearheaded by Dr. Stewart, we ourselves also examined the cytotoxic capacity of bafilomycin in three established and well characterized glioma cell lines (in addition to our previously described FACS analysis of H929 cells, **Figure 4-2**). The glioblastoma U87MG and U373MG (or U251MG, see [Appendix IV](#) for explanation) cell lines, as well as the anaplastic astrocytoma-derived U343MG cell line have been routinely used in experiments examining the efficacy of various pharmacological cytotoxic agents. Genetic characterization studies have revealed that these three cell lines carry loss of function mutations and deletions in the *PTEN* and *p14^{ARF}/p16* tumor suppressor genes (**Ishii et al 1999, Sauvageot et al 2008**). However, whereas the U87MG and U343MG cell lines encode an intact *p53* gene, the U373MG cell line additionally carries a dominant negative mutation (R273H) in the *p53* coding sequence, resulting in the further loss of this crucial tumor suppressor's function (see [Appendix IV](#)). As such, the U87MG, U343MG and U373MG cell lines were grown in culture and treated with a range of bafilomycin concentrations over a 72 hour time span. Following the incubation period, cellular viability was determined by MTS assay at 24 hour intervals over a 72 hour period of bafilomycin treatment (**Figure 4-3**). Two of the three cell lines, U87MG and U343MG (*PTEN* and *p14^{ARF}/p16* mutant, *p53* wildtype), were found to be bafilomycin-sensitive, while the third cell line, U373MG (*PTEN*, *p14^{ARF}/p16* and *p53* mutant), was comparatively insensitive.

The U87MG cell line (**Figure 4-3A**) was found to be time- and dose-dependently sensitive at all examined concentrations of bafilomycin (5nM, 20nM, 50nM, and 100nM) and at all examined time intervals (24, 48 and 72 hours). A modest but tangible decrease in viability was evident only 24 hours after the initiation of bafilomycin treatment (10-20% decrease in viability over the range of concentrations used as compared to untreated control samples). After 72 hours of bafilomycin treatment, the maximal cytotoxicity was observed at a concentration of 20nM (~70% decrease in viability) as higher

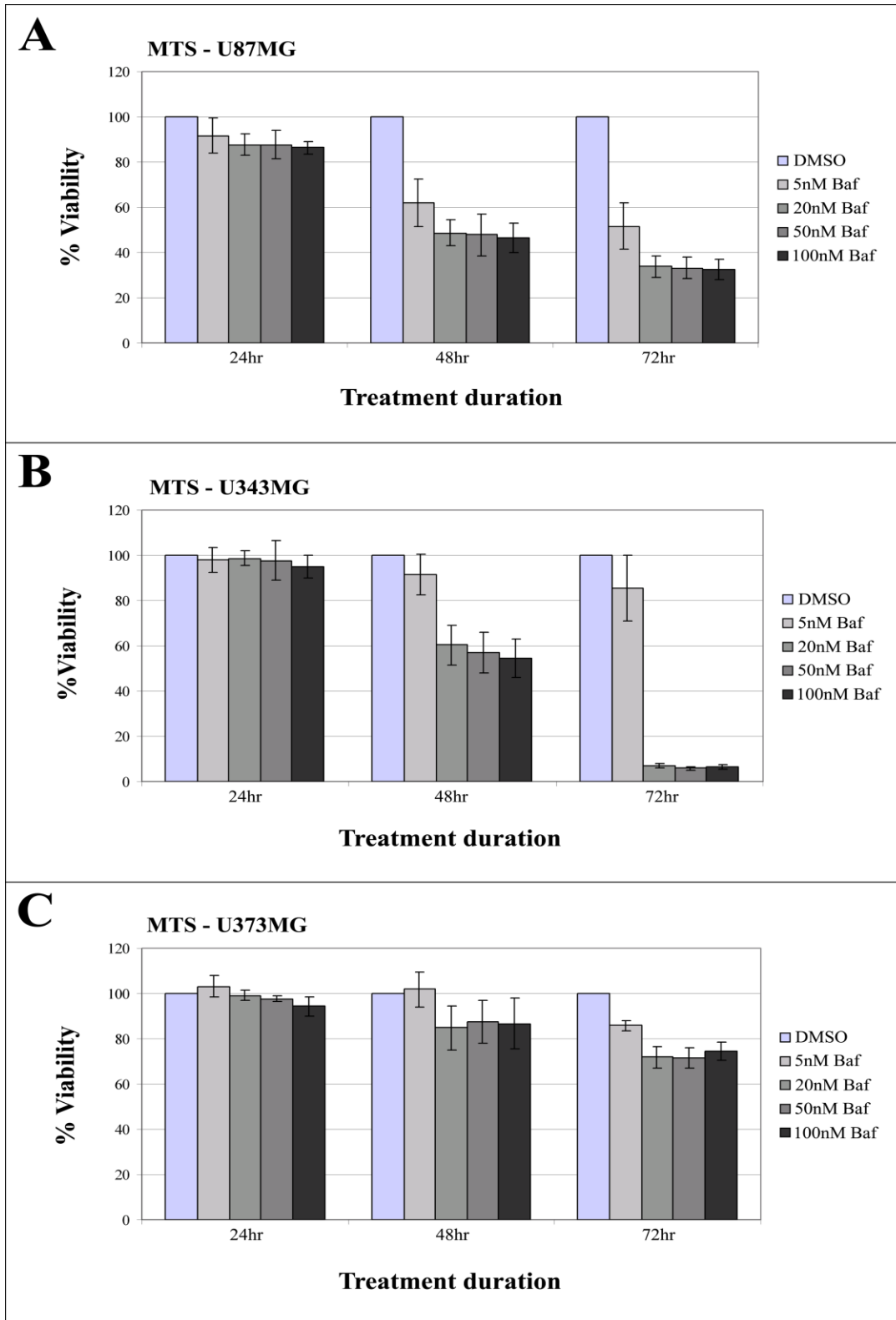


Figure 4-3. Bafilomycin treatment significantly reduces the viability of cultured human glioblastoma cell lines. (A-C) Cultured U87MG (A), U343MG (B), and

U373MG (C) human glioblastoma cell lines were grown to near confluence, and treated with either vehicle (DMSO) or bafilomycin (at indicated concentrations) in triplicate for 24, 48, and 72 hours. The viability of vehicle and bafilomycin-treated cells was assessed at each indicated time interval with the MTS cell viability assay. Absorbance was read at 490nm and graphically depicted as a percentage value normalized to the vehicle-treated control at each time point arbitrarily assigned a value of 100%.

concentrations of bafilomycin (50nM and 100nM) did not result in an appreciable further decrease in viability. The EC_{50} was approximated at 5nM after 72 hours of treatment, which is roughly comparable to the EC_{50} of the highly bafilomycin-sensitive H929 MM-derived cell line (**Figure 4-3**, **Appendix III**) over an identical time interval, although the maximal bafilomycin-induced cytotoxicity observed with the latter was significantly higher (~95% decrease in viability, **Figure 4-1**) compared to the ~70% maximal decrease in viability observed in U87MG cells at a similar concentration (20nM) and time interval (72 hours).

Whereas U87MG cells were found to have: (a) a relatively early cytotoxic response (within the first 24 hours of treatment), (b) a moderately high (~70%) maximal decrease in viability (20nM, 72 hours), and (c) a relatively high sensitivity (EC_{50} of 5nM, 72 hours) to bafilomycin treatment; in the U343MG cell line, which was also found to be time- and dose-dependently sensitive to bafilomycin at all examined concentrations (**Figure 4-3B**), the onset of cytotoxicity was not detected until the 48 hour time interval. Despite this relative latency of cytotoxicity, the maximal decrease in viability after 48 hours of bafilomycin treatment was comparable in both U87MG and U343MG cell lines at concentrations above 20nM. The relative insensitivity of U343MG to the lowest concentration of bafilomycin (5nM) after 48 hours of treatment, as compared with the significant decrease in viability observed at the same concentration and time interval in U87MG cells, suggested a correspondingly higher EC_{50} value for U343MG cells. In fact, after 72 hours of treatment, only a discrete reduction (15%) in viability was observed in U343MG cells treated with 5nM bafilomycin, as compared to the ~50% decrease in viability observed in identically treated U87MG cells. However, despite the latency of cytotoxicity in U343MG cells and the relative insensitivity of this cell line in the lower range of bafilomycin concentrations, the maximal cytotoxicity of ~95% observed after 72 hours of treatment at concentrations of 20nM or higher (also comparable to the *maximal* cytotoxicity observed in H929, **Figure 4-1**) was significantly more elevated than that observed in the relatively more (at least with respect to the EC_{50} value) bafilomycin-sensitive U87MG cells (~70% maximal cytotoxicity) at identical concentrations.

The contribution of apoptotic cell death to the decrease in viability demonstrated in the bafilomycin-sensitive U87MG and U343MG cell lines was further confirmed by

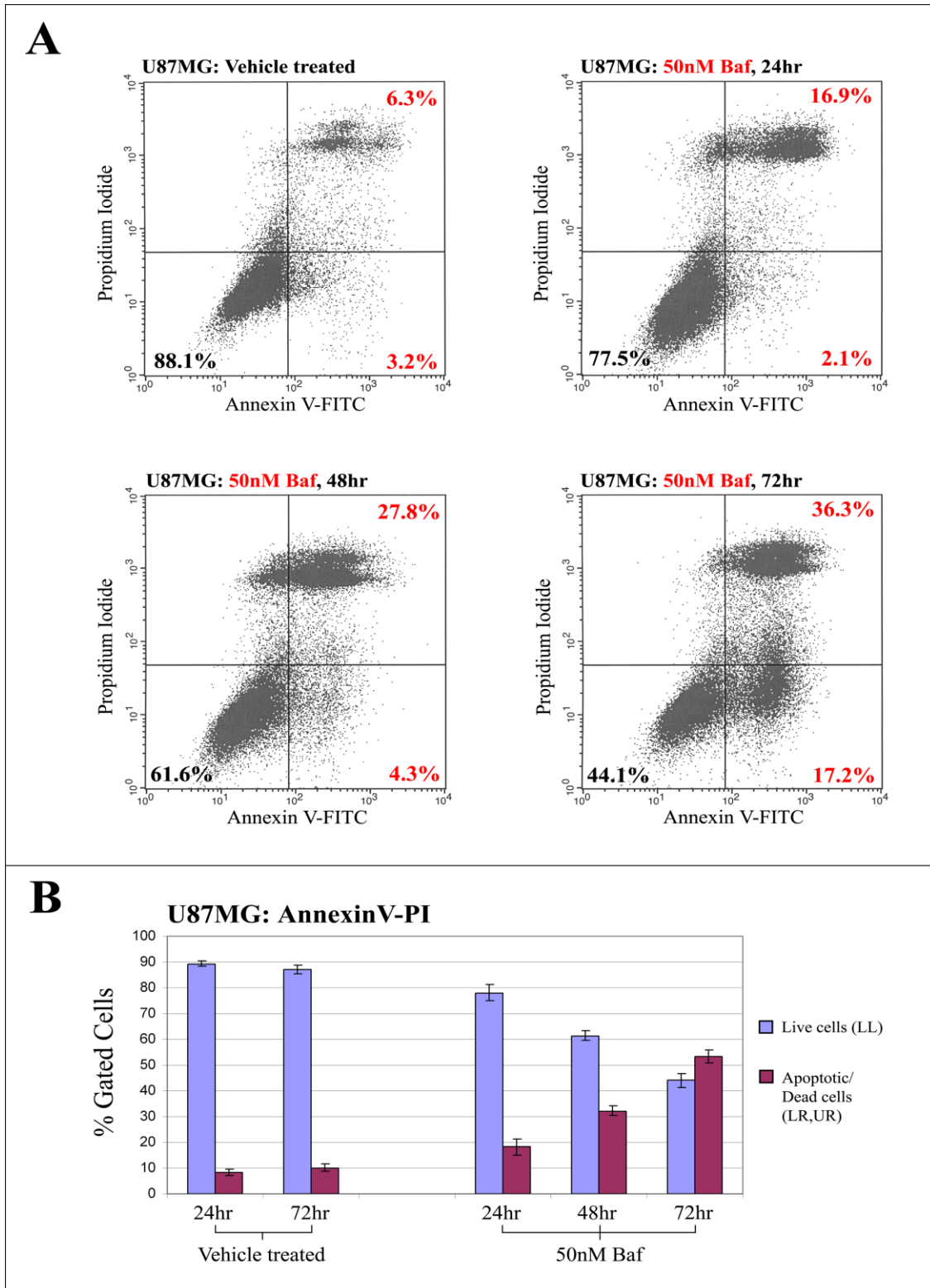


Figure 4-4. Bafilomycin treatment induces apoptosis in cultured U87MG human glioblastoma cells. We cultured U87MG human glioblastoma cells to near confluence, treated them with either vehicle (DMSO) or bafilomycin (50nM) for up to 72 hours, and

harvested at the indicated time intervals. Harvested cells were then labeled with AnnexinV- FITC (BioVision) and PI (BioVision), and subjected to FACS analysis. The results of these experiments, done in triplicate, are presented as: **(A)** representative dual parameter scatter plots with indicated percentage values of viable cells (AnnexinV- and PI-negative, Lower Left quadrant, in black), apoptotic cells (AnnexinV-positive and PI-negative, Lower Right quadrant, in red), and dead cells (AnnexinV- and PI-positive, Upper Right quadrant, also in red) over the course of the bafilomycin treatment period as compared to vehicle-treated control cells over the same time span; and **(B)** a graphical depiction of the percentage distribution of viable and apoptotic/dead gated cells in response to vehicle and bafilomycin treatment over the same time span. LL-Lower Left, LR-lower right, UR- Upper Right.

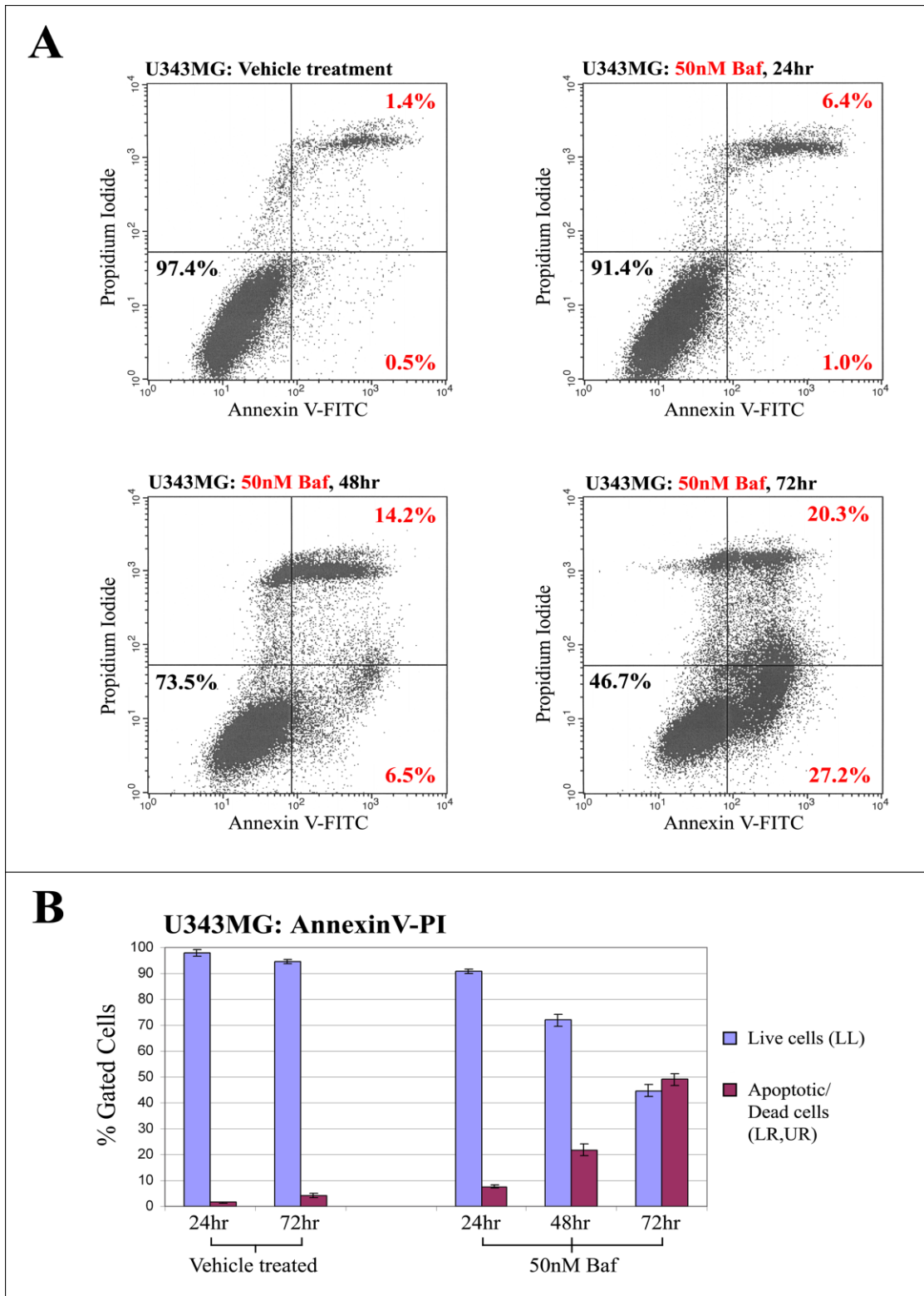


Figure 4-5. Bafilomycin treatment induces apoptosis in cultured U343MG human glioblastoma cells. Grown, treated, and analyzed as described for U87MG cells in **Figure 4-4**. See text for discussion.

FACS analysis. Following 72 hours of treatment with 50nM bafilomycin, a time-dependent decrease in the population of viable cells was observed in U87MG (**Figure 4-4**) and U343MG cells (**Figure 4-5**), with corresponding increases in the AnnexinV-positive apoptotic cell populations. The U87MG and U343MG cell lines were both demonstrated to be sensitive to bafilomycin-induced cytotoxicity over the investigated time span of treatment, despite differences in the temporal onset of cytotoxicity and the effective dosage for maximal cytotoxicity. In contrast, the U373MG cell line (which was not included in the FACS analysis) was found to be relatively insensitive to bafilomycin treatment over the same experimental time span and range of bafilomycin concentrations (**Figure 4-3C**). In bafilomycin-treated U373MG cells, there was no discernable decrease in viability at the first 24 hour interval of bafilomycin treatment for all tested concentrations, and a modest (though variable) decrease in viability after 48 hours of treatment (~15%) at concentrations of bafilomycin greater than 20nM. Furthermore, at the experimental end point, after 72 hours of treatment, > 70% of bafilomycin-treated U373MG cells remained viable (maximal cytotoxicity of <30%, no quantifiable EC₅₀ value) throughout the range of concentrations used, as compared to the <40% and <10% residual viability observed in U87MG and U343MG cells respectively, at concentrations of bafilomycin greater than 20nM.

4-3.3 – Bafilomycin treatment downregulates Akt phosphorylation in human myeloma and glioblastoma cells

As described in the previous chapter, the apoptotic response to bafilomycin treatment observed in NIH-3T3 fibroblasts was temporally (if not causally) preceded by a significant decrease in the levels of Akt phosphorylation. Having ascribed cytotoxic and pro-apoptotic properties to bafilomycin treatment in the MM and GBM disease models, the correlation of these effects with the endogenous presence and bafilomycin-induced downregulation of Akt phosphorylation was investigated. As such, the *in vitro* effects of bafilomycin treatment on Akt phosphorylation were examined in both multiple myeloma cultured cells (3 cell lines, and primary mononuclear cell samples from 5 patients) and the U87MG glioblastoma cell line. From the genetically heterogeneous panel of 14 MM-

derived cell lines treated with bafilomycin and assayed for viability (**Figure 4-1**, **Appendix III**), the highly sensitive t(4;14)-positive (*FGFR3* overexpressing) H929 and KMS11 cell lines (EC_{50} of 5nM and 6.5nM, respectively) and the moderately sensitive t(4;14)-negative JJN3, OCI MY5, and U266 cell lines (EC_{50} of 6.5nM, 6.7nM and 7.7nM, respectively) were treated by Dr. Stewart's group with 20nM bafilomycin for a period of 24 hours, and subsequently purified protein samples from each treated cell line were Western blotted for total Akt protein as well as Akt phosphorylated at S473. Although a robust total Akt protein signal was evident in the samples from U266 and OCI MY5 cells, Akt phosphorylation at S473 was not detected in either vehicle-treated or bafilomycin-treated samples from these 2 cell lines (data not shown). Conversely, in the highly bafilomycin-sensitive KMS11 and H929 cell lines in which a strong phospho-S473 signal was evident prior to treatment, a significant reduction in phospho-S473 signal intensity was observed in response to bafilomycin (**Figure 4-6A**). A near-maximal loss of phospho-S473 signal was seen within 12 hours of treatment in H929 cells, and after 24 hours of treatment in KMS11 cells. Incidentally, the JJN3 cell line, which was found to be less bafilomycin-sensitive than KMS11 and H929 cells, but more sensitive than OCI MY5 and U266 cells based on the MTS viability assay, accordingly showed an intermediate response with respect to Akt phosphorylation, whereby the trace levels of P-S473 detectable in this cell line prior to treatment became undetectable following 24 hours of bafilomycin administration.

These results suggested a correlation between the cytotoxic efficacy of bafilomycin treatment and the endogenous levels of Akt phosphorylation in a given cell line, whereby cell lines intrinsically exhibiting a robust activation of Akt prior to bafilomycin treatment (as in the *FGFR3*-overexpressing KMS11 and H929 cell lines) display a higher sensitivity to bafilomycin-induced decreases in viability, whereas cell lines exhibiting little or no endogenous Akt phosphorylation prior to bafilomycin treatment were comparatively less sensitized to the cytotoxic effects of V-ATPase inhibition. This suggested correlation was further tested in the mononuclear cells (MNCs) of five MM patients. MNCs isolated from patients 1, 6, 7 and 8 (high responders to bafilomycin-induced cytotoxicity, Dr. Keith Stewart, personal communication) and patient 2 (low responder to bafilomycin-induced cytotoxicity, Dr. Keith Stewart, personal

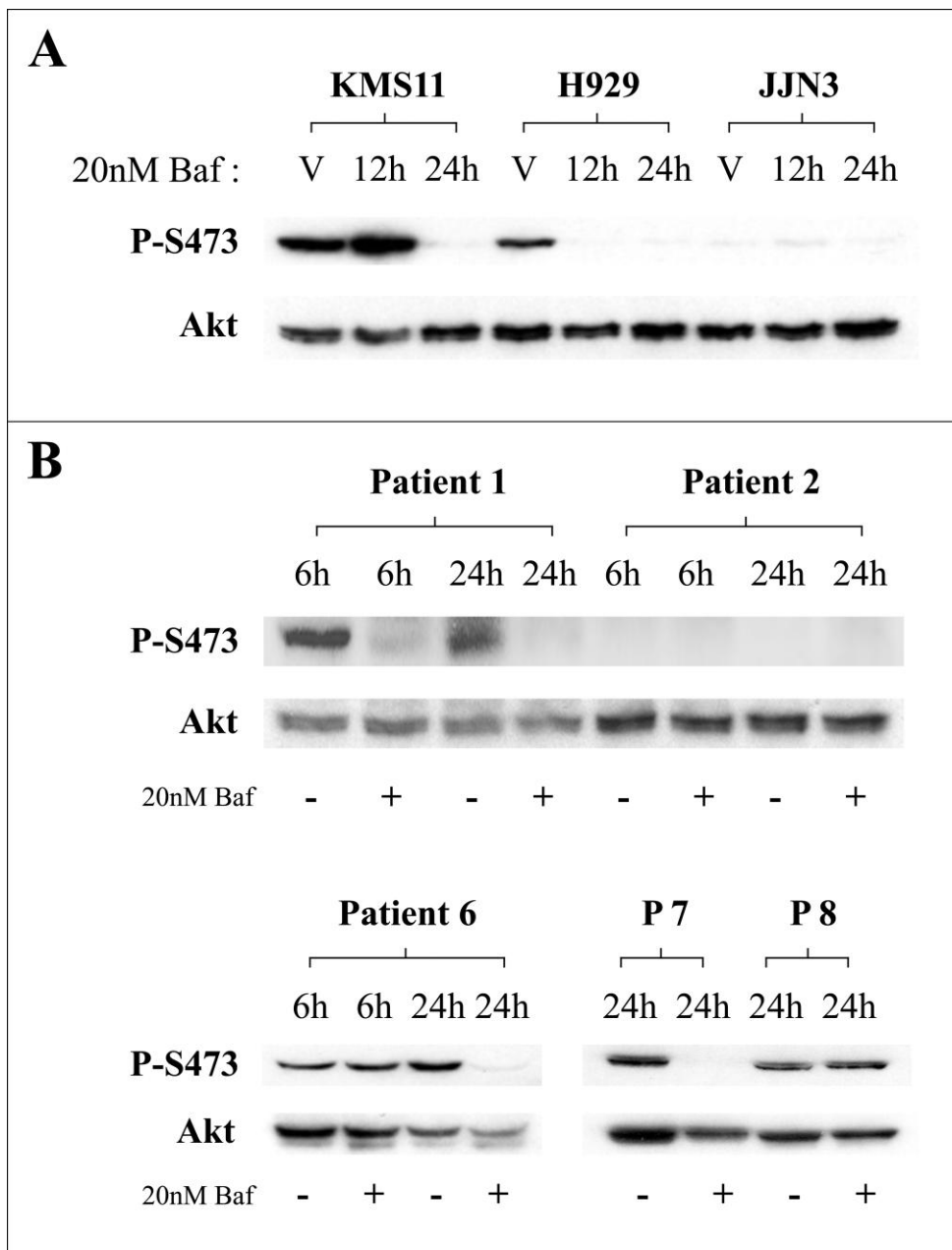


Figure 4-6. Bafilomycin downregulates Akt phosphorylation in some myeloma cell lines and cultured myeloma patient primary cell samples. Courtesy of Dr. Keith Stewart and colleagues. (A) The KMS11, H929, and JJN3 human myeloma cell lines and (B) Primary cell samples from the bone marrows of 5 myeloma patients were cultured, and either vehicle-treated or treated with 20nM bafilomycin for up to 24 hours. Whole-cell lysates were analyzed by Western-blotting for changes in the levels of Akt phosphorylation at S473 over the 24 hour incubation period, with total Akt protein as the loading control.

communication) were cultured and treated with 20nM bafilomycin for a period of 24 hours, and subsequently purified protein samples from the MNCs of each patient were Western blotted for total Akt protein as well as Akt phosphorylated at S473 (**Figure 4-6B**). Consistent with the trend observed in the MM-derived cell lines, the endogenous phosphorylation of Akt at S473 was not detectable in patient 2 (low responder), whereas a robust phospho-S473 signal was detected in samples from all 4 high responders (patients 1, 6, 7, and 8). Furthermore, the phospho-S473 signal in three out of the four high responders (patients 1, 6, and 7) was significantly diminished following 24 hours of bafilomycin treatment, and interestingly, in patient 8 (the least sensitive of the high responders), the levels of endogenously phosphorylated Akt at S473 were relatively unaffected by bafilomycin treatment.

Lastly, considering our demonstration of the suppression of the *dPTEN*-deficiency phenotype in *dPTEN/Vha68-2* mutant clones of *Drosophila* salivary gland cells (see Chapter 2) we ourselves investigated the consequences of bafilomycin treatment on the phosphorylation of Akt in the *PTEN*-mutant U87MG glioblastoma cell line. Whereas the ectopic phosphorylation of Akt in MM-derived t(4;14)-positive cell lines may be attributable to *FGFR3* overexpression and increased RTK activity, the elevated levels of Akt activity in the U87MG cell line have been shown to be a direct consequence of the loss of PTEN function in these GBM cells (**Myers et al 1998, Haas-Kogan et al 1998, Li and Sun 1998, Maier et al 1999**). Having demonstrated their sensitivity to bafilomycin-induced apoptosis (**Figure 4-4**), U87MG cells were cultured and treated with up to 50nM bafilomycin for a period of 12 and 24 hours, and subsequently purified protein samples were Western blotted for total Akt protein, as well as Akt phosphorylated at S473 and T308 (**Figure 4-7A**). At all examined concentrations (5nM, 20nM, and 50nM), bafilomycin treatment was found to decrease the levels of both S473 and T308 Akt phosphorylation within 12 hours of bafilomycin treatment, an effect also observed in NIH-3T3 cells (see **Sections 3-3.1, 3-3.3**), and the H929 MM-derived cell line mentioned above (**Figure 4-6A**). The decrease in phospho-S473 levels observed by immunoblot analysis of whole lysates was further confirmed by immunofluorescence confocal microscopy experiments in which the abundance of the phospho-S473 signal was found

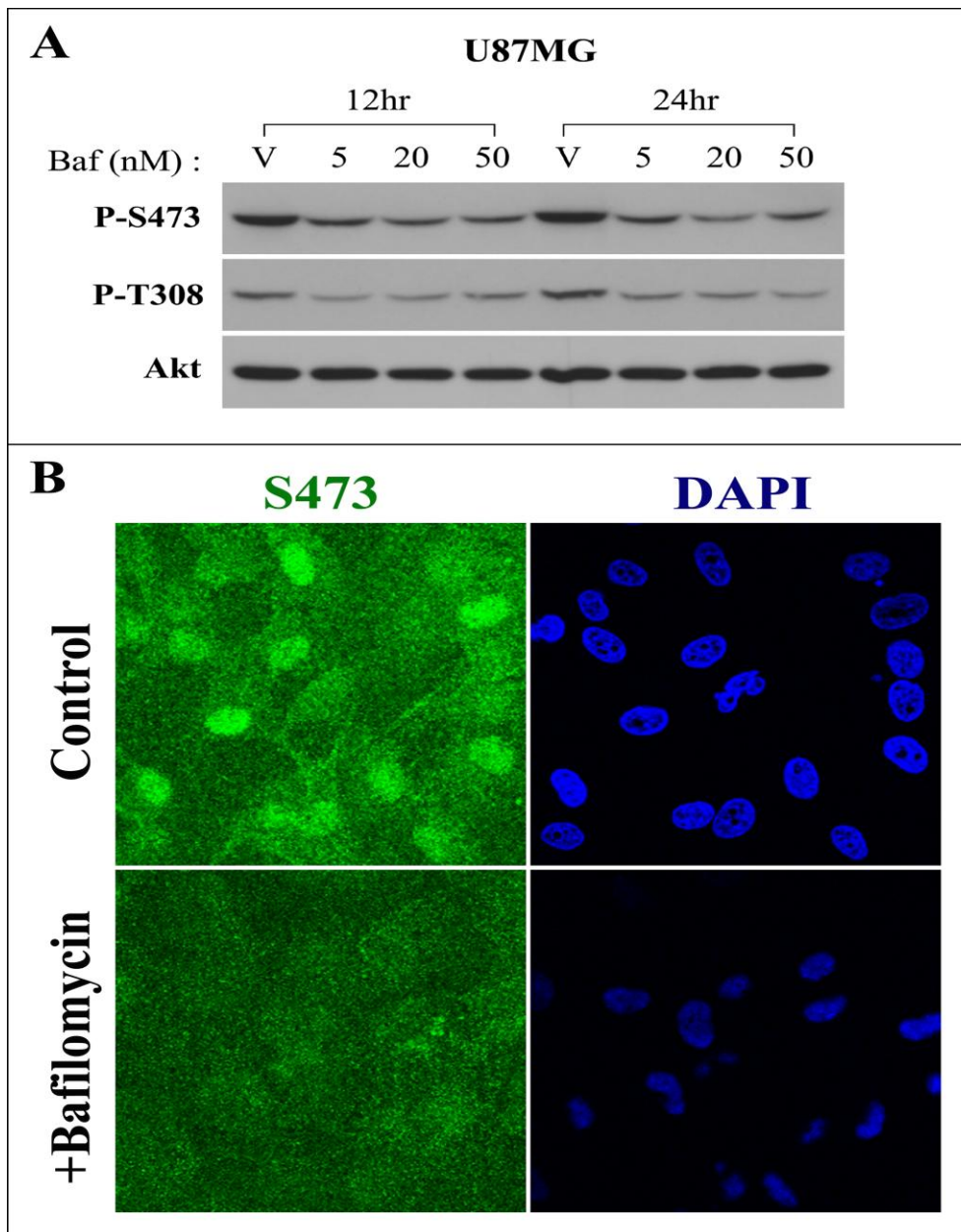


Figure 4-7. Bafilomycin downregulates Akt phosphorylation in cultured U87MG human glioblastoma cells. (A) U87MG human glioblastoma cells were cultured to near confluence, treated them with various concentrations of bafilomycin for up to 24 hours and harvested them at 12 hour intervals, with a parallel vehicle-treated sample for each time interval. Whole-cell lysates from each time point were analyzed by Western blotting for changes in the levels of Akt phosphorylation at S473 and T308 over the 24 hour incubation period, with total Akt protein as the loading control. Immunoblots shown are representative of the experiments carried out in triplicate. (B) Cultured U87MG human glioblastoma cells were grown to near confluence, and either vehicle-treated or treated with 50nM bafilomycin for 24 hours. Following the incubation period, the cells were analyzed by confocal microscopy for phospho-S473 fluorescence (in green) in control and bafilomycin-treated samples. Nuclei are stained blue with DAPI in both samples.

to be significantly decreased in bafilomycin-treated U87MG cells, particularly with respect to the abolishment of the nuclear localization of this signal (**Figure 4-7B**), which is consistent with the depletion of phospho-S473 signals in bafilomycin-treated PKB-HA expressing *Drosophila* salivary glands (**Figure 2-11**) and NIH-3T3 cells (**Figures 3-6, 3-14**). Furthermore, these results in the U87MG glioblastoma cell line reiterate the correlation seen in MM-derived cell lines as well as in MM patient samples, between the presence of robust endogenous Akt activity and its susceptibility to bafilomycin-induced downregulation, and the severity of the concomitant cytotoxic response and the subsequent apoptotic decrease in viability.

4-4 – Discussion

Having identified and characterized V-ATPase as a positive regulator of Akt activity in *Drosophila*, including roles in embryonic survival and larval growth downstream of PTEN/PI3K, and demonstrated the downregulation of Akt activation and the induction of apoptosis in response to the pharmacological inhibition of V-ATPase activity in cultured mammalian cells, we set out, in collaboration with Dr. Keith Stewart's research group, to investigate the relevance of V-ATPase inhibition and its clinically useful cytotoxic properties in the context of cancer pharmacotherapy *in vitro*, using cancer cell lines known to bear oncogenic mutations that result in ectopic levels of PI3K signaling (FGFR3 overexpression/mutational activation in MM, PTEN-deficiency in GBM).

The assessment of bafilomycin-induced toxicity in fourteen genetically heterogeneous MM cell lines revealed it to be cytotoxic in all cell lines tested, with cell lines positive for the t(4;14) IGH translocation (UTMC2, H929, KMS18 and KMS11), which results in the overexpression of FGFR3 (itself mutationally activated in KMS11), consistently ranking among the highest responders to the bafilomycin regimen, suggesting a correlation between ectopic RTK signaling (in this case FGFR3) and susceptibility to bafilomycin-induced apoptotic cell death (**Figure 4-1, 4-2**). Moreover, in cultured glioblastoma cells, the PTEN-deficient U343MG and U87MG cell lines were both found to be bafilomycin-sensitive (comparable to H929, a high responder among the MM cell lines), with maximal cytotoxicities of ~95% and ~70%, respectively, at 72 hours of treatment (**Figures 4-3, 4-4, 4-5**).

The presumably ectopic activation of PI3K/Akt signaling in these MM and GBM cell lines was also investigated, and the correlation observed in the MM cells lines between FGFR3 overexpression and susceptibility to bafilomycin was recapitulated by the demonstration that the highly bafilomycin-sensitive KMS11 and H929 cell lines, in which a strong phospho-S473 signal was evident prior to bafilomycin treatment, a significant reduction in phospho-S473 signal intensity was observed in response to bafilomycin (**Figure 4-6A**), while the least bafilomycin-sensitive cell lines (with respect to viability) were found to contain very little phospho-S473 Akt prior to bafilomycin

treatment. A similar trend was also found in primary cultures of MM patient mononuclear cells (**Figure 4-6A**), while our own experiments demonstrated that bafilomycin diminished baseline levels of phospho-S473 Akt in the PTEN-deficient U87MG cell line, and furthermore, downregulated phospho-S473 immunofluorescence throughout the cell, including the nuclei (**Figure 4-7**).

Equally interesting to the responsive cell lines, however, are the lowest responders to bafilomycin – OPM2 in the MM panel, U373MG in the GBM panel. As mentioned in **Section 4-3.1**, whereas high responders such as KMS11 overexpress mutationally active FGFR3, an oncogene addiction that presumably renders the cell susceptible to bafilomycin-mediated Akt downregulation, the OPM2 cell line, though also overexpressing mutationally-activated FGFR3, additionally carries a loss-of-function PTEN mutation, suggesting that the combined effects of FGFR3-mediated PI3K overactivation and the loss of PTEN-dependent PI3K downregulation likely overpower the Akt-inhibitory capacity of bafilomycin administration in the range of doses and treatment periods used in the viability assays. In our evaluation of GBM cell lines, the disparity we observed between the cytotoxic response of the bafilomycin-sensitive (and p53 wildtype) U87MG and U343MG cell lines and the relative insensitivity of the (p53 mutant) U373MG cell line could arise from a number of endogenous factors in these glioma lines. First, genetic and biochemical studies of these GBM cell lines have demonstrated that the bafilomycin-insensitive U373MG line has very low levels of EGFR expression (**Sauvageot et al 2009**), the ectopic activation of which is a common source of oncogene addiction in GBM (**Pillay et al 2009, Yan et al 2011**), and which is moderately expressed in the bafilomycin sensitive U87MG and U343MG cell lines. Accordingly, though Akt phosphorylation can be detected in all three GBM cell lines (**Myers et al 1998, Sauvageot et al 2009**), the U373MG line is least phosphorylated of the three GBM lines (consistent with its negligible EGFR expression), and (presumably) largely invulnerable to further decreases in Akt phosphorylation. Furthermore, *in vitro* experiments with TMZ have demonstrated a similar p53 status-dependent differential sensitivity (**Roos et al 2007, Blough et al 2011**), suggesting that the loss of p53-dependent apoptotic signaling, along with the relatively low levels of baseline Akt

activation likely contribute to the observed resistance to bafilomycin-induced apoptotic induction in U373MG cells.

Concluding Remarks

As discussed in the prologue of the introductory chapter, the evolution of ancestral metazoan lineages largely coincides with the establishment of the RTK “tool kit” in premetazoan unicellular organisms like the choanoflagellate *Monosiga brevicollis*. This RTK machinery, which integrated largely pre-existing intracellular signaling networks (such as the Ras/MAPK pathway, which is involved in the GPCR-mediated pheromone response of yeast, or protozoan AGC kinases such as the *D. discoideum* Akt homologs AKT and PKBR1), thereby allowed the attainment of multicellularity by providing an intracellular communication network which could support the rapid and efficient communication of developmental and growth-promoting signals, resulting in the “centralized” control of pre-established cell-autonomous functions, and an increased capacity for higher forms of organization. In higher metazoans, including mammals, signaling downstream of RTKs is largely relied upon for the communication of developmental, metabolic, proliferative, and survival-promoting inputs. Accordingly, the dysfunction of negative regulatory elements, and/or the deregulation of positive regulatory elements acting downstream of these growth-promoting inputs can subvert the requisite and endogenously imposed checks and balances, thereby resulting in pathologies typified by transformation and tumor formation in the case of cancer. It is then perhaps no coincidence that our contemporary understanding of both RTK-dependent signaling processes and the disease state of cancer can be traced back to Michael Bishop and Harold Varmus’ Nobel prize winning collaboration in their identification of the viral Src oncogene (v-Src) in the mid-1970s. On the one hand, its subsequent characterization by Tony Hunter as a tyrosine kinase in the late 1970s set off the avalanche of studies that would trace the molecular machinery operating downstream of RTKs, while Dominique Stehelin and Beborah Spector’s contemporaneous identification of the proto-oncogene c-Src, the normal cellular counterpart of v-Src, paved the way for the identification of numerous other viral-oncogenes such as Ras, whose normal cellular counterparts are integral components of RTK-borne mitogenic signals. As our understanding of signaling networks operating downstream of RTKs has expanded in order to account for cross-talk, feedback,

compensation, compartmental restriction, and temporal regulation, so has our approach to the pharmacotherapy of cancer correspondingly expanded from the earlier “sledge-hammer” approaches using general cytotoxic agents with narrow windows of clinical tolerability such as melphalan, to regimens of increasingly sophisticated combinations of target-directed small molecule inhibitors such as perifosine and rapamycin analogues. With respect to tumors in which aberrant activation of the PI3K/Akt pathway is typically observed, the loss of PTEN tumor suppressor function, which is difficult to treat pharmacologically, has proven to be a formidable pharmacotherapeutic obstacle, resulting in a high level of interest in druggable targets acting downstream of the PI3K/PTEN junction in the positive regulation of Akt activity.

Our genetic and biochemical experiments in *Drosophila* and cultured NIH-3T3 cells suggest that the inhibition of V-ATPase function may represent a tractable means of targeting ectopic Akt activity downstream of both PI3K and PTEN activity, and our preclinical *in vitro* studies demonstrated a significant response to bafilomycin in MM and GBM cells, but the question to be answered is: what benefits can be drawn from the pharmacological inhibition of V-ATPase, vis-à-vis its purported role as a positive regulator of Akt signaling, in the context of target-directed cancer therapy in tumors displaying ectopically elevated levels of transduction through the RTK/PI3K/Akt pathway? Prior to any assessment of this interaction’s relevance in an oncogenic setting, a formulation of its role in normal signaling contexts must be formulated. The term “normal”, when used as a synonym for “appropriate” in describing the level of signaling through a particular (for our purposes, RTK-dependent) pathway, can vary greatly depending on cell type, developmental stage, nature of stimulating signal, and the cellular response(s) to be elicited. Our experiments addressing the relationship between Akt and V-ATPase were carried out predominantly in two vastly different cell types – *Drosophila* larval tissues (both *in vivo* and *in vitro*), and cultured murine NIH-3T3 cells. Be it (1) in *Drosophila* larval ERTs, which undergo an intense increase in PI3K/Akt-dependent growth; and larval imaginal disc cells, which undergo a similarly intense proliferation program that is cell-autonomously regulated by the PI3K/Akt pathway; or (2) in NIH-3T3 cells, which, when maintained under standard *in vitro* culture conditions (5% FBS), are constitutively stimulated at intermediate levels (near-starvation would consist of

maintenance with 0.5% serum, while over-stimulation, or serum-shock, is frequently induced with medium consisting of 50% serum); we have demonstrated two distinct bafilomycin-dependent effects irrespective of cell-type and developmental stage. First we have shown that the loss of V-ATPase activity downregulates Akt phosphorylation at *both* S473 and T308, resulting (in NIH-3T3 cells) in the subsequent downregulation of mTORC1 signaling; and second, we have demonstrated the depletion of phosphorylated Akt from the nucleus, which based on our fractionation experiments in NIH-3T3 cells and the increasingly appreciated role of the endocytic network in nuclear traffic, we have hypothesized to occur as a result of the diminished recruitment of Akt to early-endosomal compartments. We propose that it is precisely these two potentially significant attributes of bafilomycin that may translate the use of small molecule inhibitors of V-ATPase into tangible clinical benefits in the pharmacological treatment of cancer.

The heightened interest in the clinical use of rapamycin and its analogs was generated by the demonstration that the prolonged administration of rapamycin also inhibited (in some cell types) mTORC2 catalytic activity (**Sarbassov et al 2006**), which had previously been thought of as rapamycin-insensitive (see **Section 1.3.3**), thereby elevating their status as promising agents for the targeted inhibition of both mTORC complexes, and by extension, mTORC2-mediated Akt activation, which is a crucial proximal node downstream of the RTK/PI3K/PTEN junction, and therefore a high-priority therapeutic target. The cell type-dependence of rapamycin-induced mTORC2 inhibition notwithstanding, however, a number of mitigating factors combine to limit the efficacy of rapamycin and its derivatives as inhibitors of ectopic Akt signaling. First, though Rictor deficiency results in the loss of Akt HM phosphorylation at S473, its effect on Akt substrates, as described in **Section 1-3.3**, is not uniform, and decreases the phosphorylation of some substrates (such as FoxO1 and FoxO3, while leaving the phosphorylation of two other important substrates with respect to growth and survival – TSC2 and GSK3 – unaffected (**Guertin et al 2006**). Second, as demonstrated by Dario Alessi's seminal study (**Alessi et al 1996a**), S473A mutant molecules, which cannot be phosphorylated at the HM site, retain a considerable level of activity when phosphorylated at T308, a distinction whose paradigmatic consequences are exaggerated in *Drosophila*, in which S505A mutant flies are viable, and only display minor

impairments of growth, suggesting that HM phosphorylation at S505 (unlike T-loop phosphorylation) is dispensable for development, and that its loss is largely compensated for during growth (**Hietakangas and Cohen 2007**). Third, as elegantly explained by David Sabatini himself (**Sabatini 2006**), since rapamycin universally inhibits mTORC1 signaling, its effects on apoptosis, which vary among cell types, may correlate with its varying effects on Akt activation, whereby cell types in which rapamycin effectively inhibits mTORC2 (and by extension Akt) may induce apoptosis in response to its administration, while in cells in which rapamycin is an ineffective mTORC2 inhibitor, the inhibition of mTORC1 can actually lead to Akt *activation* through the downregulation of its mTORC1-dependent negative feedback. As such, the use of rapamycin or its analogs have not shown anti-tumor activity in a number of cancers in which its capacity as an mTORC2 inhibitor would be of clinical benefit, such as glioblastomas (**Lee et al 2012, Lassen et al 2013**), which frequently harbor PTEN mutations. Significantly therefore, the downregulation of Akt phosphorylation at both S473 *and* T308 which we observed in response to V-ATPase inhibition may prove to be an important and distinctive property of V-ATPase inhibitors in tumorigenic contexts featuring ectopic Akt stimulation.

Lastly, several Akt inhibitors have been developed, which can be classified into various groups, including lipid-based phosphatidylinositol (PI) analogs, ATP-competitive inhibitors, and allosteric inhibitors. The most clinically advanced inhibitor, perifosine, is a lipid-based PI analog that targets the Akt PH domain, and prevents its PIP₃-mediated membrane localization (**Hilgard et al 1997**). However, though perifosine can therefore inhibit both T308 and S473 phosphorylation, there are no indications in the literature suggesting that either perifosine or rapamycin analogs can impinge on the nuclear localization of Akt, which may represent a highly significant dimension of its survival and growth-promoting functions. The EGFR and FGFR RTKs, the overexpression or mutational activation of which is commonly detected in GBM and MM (respectively), have significant nuclear (and in the case of EGFR at least, endosomal) signaling programs. For example, in kidney- and mammary carcinoma-derived cell lines, endosomal EGFR signaling has been demonstrated to be sufficient for the activation of the major signaling pathways leading to survival and proliferation, and for the

suppression of apoptosis induced by serum withdrawal (**Wang et al 2002**); while the nuclear localization of the EGFR family of receptors has been established for over two decades, and demonstrated to be crucial for EGFR-mediated transcriptional regulation (reviewed in **Wang et al 2010**). Similarly, the investigation of activated FGFR3 mutants commonly detected in MM revealed a distinct perinuclear accumulation of the molecules (**Legeai-Mallet 1998, Ronchetti et al 2001**). Considering the prominent nuclear localization of these crucial RTKs, and the established presence of PI3K signaling components in the nucleus (see **Section 3-1**), including PI3K, PIKE-A, PDK1, PTEN and Akt itself, the capacity of V-ATPase inhibition to block or diminish the nuclear localization of Akt may provide a crucial dimension to the therapeutic downregulation of ectopic RTK/PI3K/Akt-signaling programs.

Ultimately, our capacity to pharmacologically combat cancer in a clinical setting is limited by (1) our understanding of its molecular and physiological underpinnings, and (2) our ability to translate this understanding into a method of selectively eradicating cancer cells. Although V-ATPase inhibition may provide an attractive means of inhibiting tumor growth and survival, the challenge in clinically applying the principle in question has been predominantly centered on the physiological tolerability of early V-ATPase inhibitors like bafilomycin and concanamycin, whose high toxicity was deemed to be prohibitive for clinical use (reviewed in **Dröse and Altendorf 1997**). More recently identified or developed specific V-ATPase inhibitors, such as the macrolactone Archazolid (**Huss et al 2005**), and the indole derivative NiK-12192 (reviewed in **Farina et al 2001**), have shown promise as adjuvant components of pharmacotherapeutic cocktails, whereby archazolid has been recently shown to overcome trastuzumab (a drug targeting the HER2 oncogene) resistance in the treatment of breast cancer (**von Schwarzenberg et al 2013**), while in human colon cancer carcinomas, NiK-12192, which targets DNA topoisomerase I, has been shown to potentiate the cytotoxic effects of camptothecins (**Petrangolini et al 2006**). In addition to enhancing the cytotoxicity of currently used pharmacotherapeutic agents, V-ATPase function itself has been demonstrated to play a crucial role in the metastatic invasiveness of human breast cancer cells (**Hinton et al 2009, Capecchi and Forgac 2013**), further increasing the potential efficacy of V-ATPase inhibition in the treatment of mammary tumors. Considering the

multi-faceted contribution of V-ATPase function to cellular growth, survival, and motility, the therapeutic benefits of its inhibition in the context of cancer may prove to be of tangible (and much needed) clinical benefit.

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APPENDICES

Genetic alteration	Cancer type	Incidence of tumors with alterations
p110α (PIK3CA)		
Mutations	Breast	27% (468/1766)
	Endometrial	24% (102/429)
	Colon	15% (448/3024)
	Upper digestive tract	11% (38/352)
	Gastric	8% (29/362)
	Pancreas	8% (8/104)
	Ovarian	8% (61/787)
	Liver	6% (19/303)
	Brain	5.9% (59/996)
	Oesophageal	5% (13/239)
	Lung	3% (28/962)
	Melanoma	9% (24/278)
	Urinary tract	17% (28/162)
	Prostate	2% (1/57)
	Thyroid	2% (7/394)
Amplifications	Lung (squamous cell)	53% (40/75)
	Lung (adenocarcinoma)	12.5% (15/120)
	Lung (small cell)	21.4% (3/14)
	Lung (non-small-cell)	12.0% (11/92)
	Cervical	69% (11/16)
	Breast	8.7% (8/92)
	Head and neck	32.2% (52/161)
	Gastric	36% (20/55)
	Thyroid	9% (12/128)
	Oesophageal	6% (5/87)
	Cervical	9% (2/22)
	Endometrial	10% (3/29)
	Ovarian	11.9% (16/134)
	Glioblastoma	6.1% (21/344)
p110β (PIK3CB)		
Amplifications	Ovarian	5%
	Breast	5%
Increase in activity and expression	Colon	70% (7/10)
	Bladder	89% (8/9)

Appendix I. Incidence of oncogenic alterations in the PI3K signaling pathway.

Reproduced from Liu et al (2009b) and continued on the next page. PDPK1 (3-phosphoinositide-dependent protein kinase 1); PIK3CA (PI3K catalytic subunit α isoform, p110 α); PIK3CB (PI3K catalytic subunit β isoform; PIK3R1 (PI3K regulatory subunit α , p85 α); PTEN (phosphatase and tensin homologue).

Genetic alteration	Cancer type	Incidence of tumors with alterations
PDPK1		
Amplifications and overexpression	Breast	20%
AKT		
AKT1 mutation (E17K)	Breast	3.7% (31/845)
	Colon	2.8% (4/139)
	Ovarian	2% (1/50)
	Lung	1.9% (2/105)
AKT1 amplifications	Gastric	20% (1/5)
AKT2 amplifications	Ovarian	14.1% (30/213)
	Pancreas	20% (7/35)
	Head and neck	30% (12/40)
	Breast	3% (3/106)
AKT3 mutation (E17K)	Skin	1.5% (2/137)
AKT3 amplifications	Glioblastoma	2% (4/205)
p85a (PIK3R1)		
Mutations	Glioblastoma	9.9% (9/91); 8% (8/105)
	Ovarian	4% (3/80)
	Colon	2% (1/60)
PTEN		
Loss of heterozygosity	Gastric	25.3% (84/332)
	Breast	24.9% (99/398)
	Melanoma	37% (53/143)
	Prostate	30% (70/230)
	Glioblastoma	28% (113/404)
Mutations	Endometrial	38% (604/1569)
	Brain	21% (611/2913)
	Skin	17% (96/555)
	Prostate	14% (51/371)
	Colon	13% (53/416)
	Ovary	9% (55/645)
	Breast	6% (34/561)
	Haematopoietic and lymphoid tissue	6% (54/866)
	Stomach	6% (28/499)
	Liver	5% (20/372)
	Kidney	5% (14/294)
	Vulva	65% (17/26)
	Urinary tract	9% (13/142)
	Thyroid	5% (27/591)
	Lung	9% (48/548)

Appendix I (continued). Incidence of oncogenic alterations in the PI3K signaling pathway. Reproduced from Liu et al (2009b). See text for details, and cited article for references.

Drug category	Drug or agent name	Company	Phase of clinical trials	Cancer type
Pan-PI3K inhibitors	GSK1059615	GlaxoSmithKline	Phase I terminated	Solid tumors; metastatic breast cancer; endometrial cancer and lymphoma
	ZSTK474	Zenyaku Kogyo Co, Ltd	Phase I	Advanced solid malignancies
	CH5132799	Chugai Pharma Europe Ltd	Phase I	Advanced solid malignancies
Selective PI3K inhibitors	BKM120	Novartis	Phase I – II	Solid tumors
	XL147	Exelixis	Phase I – II	Lymphoma; breast cancer; endometrial cancer; NSCLC; ovarian cancer; glioblastoma; astrocytoma
	GDC0941	Genentech	Phase I	Advanced solid tumors; non-Hodgkin's lymphoma; locally recurrent breast cancer; metastatic breast cancer; NSCLC
	PX866	Oncothyreon, Inc.	Phase I – II	Incurable metastatic colorectal carcinoma; incurable progressive, recurrent, or metastatic squamous cell carcinoma of the head and neck; advanced solid tumor; prostate cancer; glioblastoma
PI3K (α isoform)	BYL719	Novartis	Phase I	Advanced solid tumors with a mutation of the <i>PIK3CA</i> gene
PI3K (δ isoform)	CAL-101	Calistoga	Phase I – II	Non-Hodgkin's lymphoma; chronic lymphocytic leukemia; small lymphocytic lymphoma; acute myeloid leukemia; multiple myeloma; allergic rhinitis
Akt Inhibitors	Perifosine	Keryx	Phase I – III	NSCLC; solid tumors; metastatic breast cancer; sarcoma; childhood solid tumors; malignant gliomas; lymphomas; colon cancer; multiple myeloma; solid tumors
	MK-2206	Merck	Phase I – II	Advanced solid tumors; breast cancer; lymphoma; colorectal neoplasms; leukemia; ovarian cancer; fallopian tube cancer
	GSK690693	GlaxoSmithKline	Phase I (terminated)	Relapsed or refractory hematologic malignancies; solid tumors; lymphoma
mTOR Kinase Inhibitors	AZD8055	AstraZeneca	Phase I – II	Advanced solid malignancies; glioblastoma multiforme; anaplastic astrocytoma; anaplastic oligodendroglioma; malignant glioma; brainstem glioma; advanced hepatocellular carcinoma
	OSI027	OSI Pharmaceuticals	Phase I	Solid tumors; lymphoma
	INK-128	Intellikine	Phase I	Breast cancer; advanced solid malignancies; non-Hodgkin's Lymphoma; relapsed or refractory multiple myeloma

Appendix II. Drugs targeting the PI3K/Akt/mTOR pathway currently being assessed in clinical trials. Reproduced from Bartholomeusz and Gonzalez-Angulo (2012) and continued on the next page. Information obtained from clinicaltrials.gov.

Drug category	Drug or agent name	Company	Phase of clinical trials	Cancer type
Rapamycin and Rapamycin Analogues (Rapalogues)	Rapamycin	Wyeth/Pfizer	Phase I – II	Glioma; renal cell carcinoma; advanced solid cancers; bladder cancer; head and neck cancer; renal cell carcinoma
	Temsirolimus	Wyeth/Pfizer	Phase I – III	Metastatic cancers; advanced solid tumors; breast cancer; glioma; NSCLC; renal cell carcinoma; head and neck cancer; bladder cancer; colorectal cancer; thyroid cancer; squamous cell cancer; prostate cancer; non-Hodgkin's lymphoma; multiple myeloma; malignant melanoma; endometrial cancer; ovarian cancer; glioblastoma multiforme; sarcoma
	Everolimus	Novartis	Phase I – III	Bladder cancer; advanced solid tumor; head and neck cancer; gliomas; renal cell carcinoma; lymphoma; kidney cancer; endometrial cancer; thyroid cancer; breast cancer; NSCLC; metastatic breast cancer; hematologic malignancies
	Deforolimus	Merck/Ariad	Phase I – II	Solid tumor; hematological malignancies; endometrial cancer; breast cancer; brain tumors; prostate cancer; sarcoma
Dual PI3K-mTOR Inhibitors	BEZ235	Novartis	Phase I – II	Metastatic breast cancer; advanced solid tumors
	BGT226	Novartis	Phase I – II	Advanced solid tumors; advanced breast cancer; Cowden's syndrome
	XL-765 GDC0980	Exelixis Genentech	Phase I – II Phase I	Solid tumors; malignant gliomas; breast cancer Solid tumors; non-Hodgkin's lymphoma; breast cancer
Multimodal Inhibitor (Pan-PI3K, mTORC1/2, DNA-PK, HIF-1 α)	SF1126	Semafore Pharmaceuticals	Phase I	Advanced or metastatic solid tumors

Appendix II (continued). Drugs targeting the PI3K/Akt/mTOR pathway currently being assessed in clinical trials. Reproduced from Bartholomeusz and Gonzalez-Angulo (2012). Information obtained from clinicaltrials.gov.

Appendix III. Origin and selected characteristics of a genetically heterogeneous panel of human MM cell lines

MM Cell Line	Origin	IGH Translocations	Unique gene alterations/ aberrant gene expression	EC ₅₀ (Baf, 72hr)
UTMC2	- A monoclonal plasma cell line established from the pleural effusion of a 72 year old female multiple myeloma patient (Ozaki et al 1994)	- t(4;14) translocation, deregulation of <i>FGFR3</i> and <i>WHSC1/MMSET</i> (Chesi et al 1998a)	- <i>FGFR3</i> mRNA expression (Ozaki et al 1994)	2.5 nM
8226/S	- Established from the peripheral blood of a 61 year old male multiple myeloma patient (Matsuoka et al 1967)	- c-myc insertion on t(16;22) (Lombardi et al 2007); - t(14;16) translocation, overexpression of the <i>MAF</i> oncogene (Chesi et al 1998b)	- p53-defective (ex8) LOH (Mazars et al 1992); - activating K-ras mutation (Portier et al 1992); - MDM2 protein overexpression (Teoh et al 1997); - undetectable p16(INK4A) protein expression (Urashima et al 1997)	3.5 nM
KMS18	- Established from a 58 year old male with multiple myeloma associated with hyperammonemia (Otsuki et al 1998)	- t(4;14) translocation, deregulation of <i>FGFR3</i> and <i>WHSC1/MMSET</i> ; - t(2;8)(qter;24) translocation, deregulation of the <i>MYC</i> oncogene (Lombardi et al 2007)	- mutant <i>FGFR3</i> (G384D) overexpression (Ronchetti et al 2001)	4.2 nM
H929	- Established from the pleural effusion in a 62 year old female myeloma patient	- t(4;14) translocation, deregulation of <i>FGFR3</i> and <i>WHSC1/MMSET</i> (Chesi et al	- germline/rearranged c-myc (Gazdar et al 1986); - <i>FGFR3</i> mRNA	5 nM

	(Gazdar et al 1986)	1998a)	overexpression (Chesi et al 1997)	
KMS11	- Established from the pleural effusion in a 67 year old female myeloma patient (Namba et al 1989)	- t(4;14) translocation, deregulation of <i>FGFR3</i> and <i>WHSC1/MMSET</i> (Chesi et al 1998a) ; - t(14;16) translocation, overexpression of the <i>MAF</i> oncogene (Chesi et al 1998b); - t(8;14) translocation, deregulation of the <i>MYC</i> oncogene (Lombardi et al 2007)	- <i>c-myc</i> mRNA overexpression (Ohtsuki et al 1991); - <i>FGFR3</i> mRNA overexpression (Chesi et al 1997); - mutant activated <i>FGFR3</i> (Y373C) overexpression (Chesi et al 1997, Richelda et al 1997)	6.5 nM
JJN3	- Derived from the JJN-1 cell line which was established from the bone marrow of a 57 year old female with plasma cell leukemia (Jackson et al 1989)	- t(14;16) translocation, overexpression of the <i>MAF</i> oncogene (Chesi et al 1998b); - t(8;14) translocation, deregulation of the putative <i>MYC</i> oncogene (Lombardi et al 2007)	- selective expression of one <i>c-myc</i> allele (Kuehl et al 1996)	6.5 nM
OCI MY5	- Established from the peripheral blood of a patient with advanced multiple myeloma (Wandl et al 1988)	- t(14;16) translocation, overexpression of the <i>MAF</i> oncogene (Chesi et al 1998b)	- MDM2 protein overexpression ((Teoh et al 1997); - germline <i>c-myc</i> insertion (Shou et al 2000); - undetectable p16(INK4A) protein expression (Urashima et al 1997)	6.7 nM

KMS12PE	- Established from the pleural effusion in a 64 year old female myeloma patient (Namba et al 1989)	- t(11;14) translocation, deregulation of the <i>CCND1</i> (<i>cyclin D1/ bcl-1</i>) oncogene; - t(11;9) translocation, deregulation of the <i>c-abl</i> oncogene (Namba et al 1989)	- <i>Cyclin D1</i> mRNA overexpression (Chesi et al 1996); - <i>c-myc</i> mRNA overexpression (Ohtsuki et al 1991); - <i>bcl-2</i> gene amplification and overexpression (Lombardi et al 2007)	7.2 nM
OPM1	- Established from the peripheral blood of 56 year old female myeloma patient (Katagiri et al 1985)	- t(4;14) translocation, deregulation of <i>FGFR3</i> and <i>WHSC1/MMSET</i> (Katagiri et al 1985)	- germline <i>c-myc</i> insertion (Shou et al 2000); - mutant activated <i>FGFR3</i> (K650E) overexpression (Chesi et al 2001)	7.6 nM
U266	- Established from the peripheral blood of a 53 year old male myeloma patient (Nilsson et al 1970)	- t(11;14) translocation, deregulation of the <i>CCND1</i> (<i>cyclin D1/ bcl-1</i>) oncogene (Lombardi et al 2007)	- <i>L-myc</i> mRNA expression (Jernberg-Wilklund et al 1992); - <i>Cyclin D1</i> mRNA overexpression (Gabrea et al 1999); - Biallelic loss of <i>Rb1</i> (Corradini et al 1994); - <i>bcl-2</i> gene amplification and overexpression in early passage cells (Pettersson et al 1992); - PTEN deletion, 12% clonal involvement (Chang et al 2006); - undetectable p16(INK4A) protein expression (Urashima et al 1997)	7.7 nM

SKMM2	- Established from the peripheral blood of a 54 year old male plasma cell leukemia patient (Eton et al 1989)	- t(11;14) translocation, deregulation of the putative <i>CCND1</i> (<i>cyclin D1/ bcl-1</i>) oncogene (Chesi et al 1996)	- <i>Cyclin D1</i> mRNA overexpression (Chesi et al 1996);	8.9 nM
MM1.R	- Derived from the MM.1 myeloma cell line (Goldman-Leiken et al 1989); - Glucocorticoid (Dexamethasone)-resistant cell population (Moalli et al 1992)	- t(14;16) translocation, deregulation of the putative <i>MAF</i> oncogene (Chesi et al 1998b)	- c-maf mRNA overexpression (Chesi et al 1998b)	10 nM
MM1.S	- Glucocorticoid (Dexamethasone)-sensitive cell population (Moalli et al 1992)			10.1 nM
OPM2	- Established from the peripheral blood of 56 year old female myeloma patient (Katagiri et al 1985)	- t(4;14) translocation, deregulation of <i>FGFR3</i> and <i>WHSC1/MMSET</i> (Chesi et al 1998a); - t(20;?) translocation not involving IGH locus, correlates with overexpression of the putative oncogene <i>MAFB</i> ; - t(8;14) translocation, deregulation of the putative <i>MYC</i> oncogene (Lombardi et al 2007)	- selective expression of one c-myc allele (Kuehl et al 1996); - germline c-myc insertion (Shou et al 2000); - mutant activated FGFR3 (K650E) overexpression (Chesi et al 1997); - ATP1B1 gene amplification and overexpression (Lombardi et al 2007); - Loss of function PTEN mutation; Increased phosphorylation of Akt (Hyun et al 2000)	20 nM

Appendix IV. Origin and selected characteristics of human glioblastoma (GBM) cell lines

GBM Cell line	Origin	<i>PTEN</i> status	p53 status	p16 status	p14 ^{ARF} status	EC ₅₀ (Baf, 72hr)
U87MG	- Established from a primary <i>de novo</i> GBM tumor in the occipital lobe of a 44 year old male (Pontén and Macintyre 1968)	- exon 3 in frame deletion (Furnari et al 1997); - intron 3 G1T splicing variant (Steck et al 1997); - exon 3 splice acceptor mutation GT to TT (Sauvageot et al 2009); - 49 base pair frame shift deletion in codon 54 of exon 2 (Li et al 1997); - PTEN protein deficient (Li and Sun 1998, Myers et al 1998)	- WT/WT (Ishii et al 1999)	- Homozygous <i>p16</i> gene deletion (Ishii et al 1999, Gomi et al 1995, He et al 1994)	- Homozygous <i>p14</i> gene deletion (Ishii et al 1999)	5 nM

U343MG	- Established from a primary <i>de novo</i> AA in the right temporal lobe of a 54 year old male (Westermarck et al 1973)	- TGT(Cys) to TCT(Ser) point mutation in codon 124 (Ishii et al 1999); - C124S mutant is catalytically inactive (Maehama and Dixon 1998)	- wildtype p53 (Ishii et al 1999 , Sauvageot et al 2009)	Homozygous <i>p16</i> gene deletion (Ishii et al 1999)	Homozygous <i>p14</i> gene deletion (Ishii et al 1999)	5-20 nM
U251MG	- Established from a primary <i>de novo</i> GBM in the left parietal lobe of a 75 year old male (Pontén and Macintyre 1968)	- 2 base pair (TT) frame shift insertion at codon 241 (Steck et al 1997);	- CGT(Arg) to CAT(His) point mutation in codon 273 (Ishii et al 1999); - R273H is a DNA contact site mutant (Cho et al 1994 , Rolley et al 1995); - R273H is a dominant negative mutation (Willis et al 2004)	- Homozygous <i>p16</i> gene deletion (exons 1-3) (Ishii et al 1999 , Gomi et al 1995 , He et al 1994)	- Homozygous <i>p14</i> gene deletion (Ishii et al 1999)	N/A *
U373MG **	- Established from a primary <i>de novo</i> GBM in	- 2-base pair (TT) frame shift insertion at	- CGT(Arg) to CAT(His) point mutation in	- wildtype <i>p16</i> (Ishii et al 1999 , He et al 1994)	- wildtype <i>p14</i> (Ishii et al 1999)	N/A *

	the right temporal lobe of a 61 year old male (Pontén and Macintyre 1968)	codon 241 (Li et al 1997); - PTEN protein deficient (Myers et al 1998)	codon 273 (Ishii et al 1999); - R273H is a DNA contact site mutant (Cho et al 1994, Rolley et al 1995), dominant negative mutation (Willis et al 2004)	- Homozygous gene deletion (Arap et al 1995, Gomi et al 1995)		

GBM: glioblastoma, AA: anaplastic astrocytoma.

* N/A: not applicable, these cell lines were relatively insensitive to bafilomycin treatment (>50% viable) at the tested concentrations.

** This table is largely based on Table 1 in **Ishii et al (1999)**, to which the authors added a note in proof stating that independent work by Jonas Fuxe and Ralf F. Peterson (Ludwig Institute for Cancer research, Stockholm, Sweden) had come to the conclusion that the GBM cell line U373MG (described above) distributed by ATCC (American Type Culture Collection) is likely identical to the U251MG cell line, while the original U373MG cell line established in Sweden has different genetic alterations than those reported in the table above. Subsequently, ATCC and ECACC (European Collection of Cell Cultures) found that the stock held as U373MG was identical to the U251MG stock based on short tandem repeat (STR)-PCR profiling. As a result, the errant U373MG cell line (catalogue number 89081403) has been recently (spring 2012) renamed as U251MG (catalogue number 09063001), while the designation of U373MG (Uppsala, ECACC catalogue number 08061901) has been given to a new deposit of the original U373MG cell line obtained from the originator's laboratory in Uppsala, Sweden. Unfortunately, since the U251MG cell line was sold or circulated as U373MG for over a decade (a conservative estimate), it has been used and as such misidentified in numerous studies including our own, and many of the studies referenced herein. Therefore, in order to avoid confusion and remain consistent with the nomenclature used in the cited literature, the erroneous designation of the U251MG cell line as U373MG has been kept throughout, unless stated otherwise.