The Regulation of STAT1 Nuclear Content by Mammalian Target of Rapamycin

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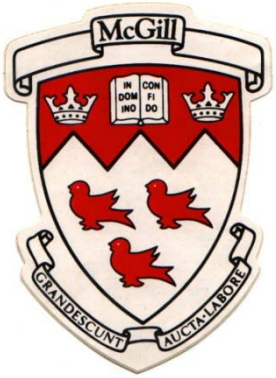

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ABSTRACT

Mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase that controls cell growth. In yeast, in addition to its regulation of cell growth, TOR lowers the nuclear content of specific transcription factors and thereby controls the expression of stress response genes. In mammalian cells, we previously identified a macromolecular protein complex containing mTOR, the kinase PKCδ, and the anti-proliferative transcription factor STAT1. We hypothesized that mTOR suppresses the nuclear content of STAT1 by a mechanism similar to that observed in yeast. Here, we showed that inactivation of mTOR by rapamycin induces nuclear accumulation of STAT1 and its kinase PKCδ. We also demonstrated that the STAT1 L407 residue, an importin α5-binding site, is required for this effect. mTOR-mediated inhibition of nuclear STAT1 levels required its associated phosphatase PP2A. The karyopherin importin α5 was required for the accumulation of STAT1 in the nucleus in response to mTOR blockade. Mutation of a highly conserved serine (S111) to alanine in importin α5 inhibited the effect of rapamycin. Finally, enhanced STAT1 nuclear localization in response to rapamycin did not correlate with apoptosis or markers of cell cycle arrest, despite the ability of rapamycin to reduce cell number *in vitro*. These results describe a novel role for mTOR in the regulation of STAT1 nuclear levels and suggest an impact on cell death other than apoptosis.

RÉSUMÉ

Mammalian target of rapamycin (mTOR) est une kinase sérine/thréonine fortement conservée qui contrôle la croissance des cellules. Chez le *Saccharomyces cerevisiae*, en plus de son rôle comme régulateur de croissance, TOR abaisse le contenu nucléaire de facteurs de transcription spécifiques et contrôle ainsi l'expression des gènes de réponse au stress. Dans les mammifères, nous avons déjà identifié un complexe macromoléculaire contenant la protéine mTOR, son kinase PKCδ et le facteur de transcription STAT1. Notre hypothèse est que mTOR contrôle le contenu nucléaire de STAT1 par un mécanisme similaire à celui observé chez *Saccharomyces*. Dans l'étude courante, nous avons démontré que l'inactivation du mTOR par rapamycin cause l'accumulation nucléaire de STAT1 et son kinase PKCδ. Nous avons également démontré que le résidu de STAT1 L407, un site d'interaction avec importin α5, est réquis pour cet effet. L'accumulation nucléaire de STAT1 a été trouvé d'être dépendante sur le phosphatase PP2A. L'importin α5 a été également trouvé d'être nécessaire pour l'accumulation nucléaire de STAT1 en réponse de l'inhibition de mTOR. Cependant, la mutation d'une sérine hautement conservée (S111) en alanine a renversé l'effet de l'inhibition de mTOR par rapamycin. Finalement, nous avons déterminé que la localisation nucléaire de STAT1 en réponse au rapamycin n'a pas augmenté l'apoptose, ni les marqueurs de l'arrêt du cycle cellulaire. Ces résultats décrivent un nouveau rôle pour la protéine mTOR dans la régulation des niveaux nucléaires de STAT1 et suggèrent un impact sur la mort des cellules autre que l'apoptose.

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Introduction

Cell growth is a complex process that entails the integration of numerous extrinsic stimuli into distinct cellular signalling pathways. Deregulation of cell growth control can lead to significant pathologies in the mammalian host, such as metabolic disorders, cancer, and other disease processes[1]. It is thus of relevance to understand the molecular interactions and mechanisms involved in regulating the cellular response to changes in the extracellular environment. In this vein, 'mammalian target of rapamycin' (mTOR, also known as FRAP and RAFT1), a ubiquitous protein kinase found in virtually all eukaryotic organisms, has been implicated as an important metabolic sensing protein, and acts as a central regulator in the cellular processes leading to cell growth. The ability of the cell to integrate cellular growth signals to regulate cell fate is of particular interest. This review will discuss the ability of mTOR to regulate the pro-inflammatory transcription factor 'signal transducer of activated transcription 1' (STAT1), thus potentially linking metabolism and the transcription of stress response genes involved in innate immunity.

Interferon Gamma (IFNγ) and the Immune Response

The mammalian immune system comprises numerous soluble mediators that communicate between different cells to modulate host defence. This communication is typified by the release of cytokines at sites of infection or injury, which, in turn, coordinate innate and adaptive immune responses. Many cytokines function through the ligation of membrane receptors, initiating complex intracellular signalling cascades that

lead to transcriptional activation and gene expression. The pro-inflammatory cytokine IFNγ is a critical mediator of innate immunity against infection as well as tumour control[2].

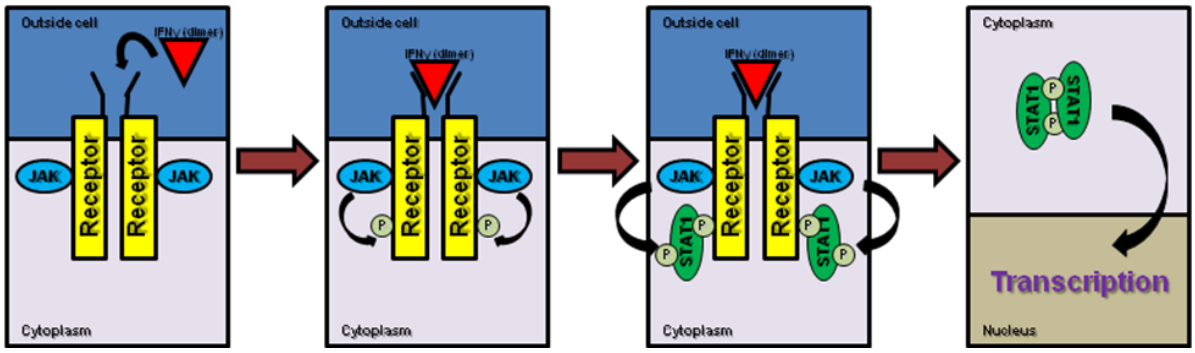


Diagram 1: IFNγ and JAK-STAT signalling

IFNγ initiates its cellular effects through its binding to a heterodimeric receptor consisting of interferon gamma receptor 1 (IFNγR1) and interferon gamma receptor 2 (IFNγR2). Upon ligation, the receptor undergoes numerous conformational and phosphorylation events, catalyzing the activation of the JAK-STAT signalling pathway[3]. Conformational changes in the receptor leads to activation of the receptor-associated tyrosine kinase 'Janus kinase 2' (JAK2). This kinase autophosphorylates and subsequently achieves transphosphorylation activity, leading to the phosphorylation of another receptor-associated kinase, JAK1. Once phosphorylated, JAK1 is able to phosphorylate tyrosine residues on the receptor, leading to the generation of phospho-tyrosine docking sites for Src-homology-2 (SH2) domain-containing proteins. STAT1 associates with the receptor docking site and is tyrosine phosphorylated by JAK2. Tyrosine phosphorylation of STAT1 by JAK2 leads to solubilisation of the STAT1 monomer, which then goes on to homodimerize. Dimerization of STAT1 exposes a

nuclear localization signal that allows it to interact with the nuclear translocation machinery, leading to its subsequent accumulation in the nucleus and transforming the latent transcription factor into a high-affinity DNA binding protein. Activated STAT1 is then free to drive transcription of its IFNγ-sensitive genes[4].

STAT Structure and Function

STAT1 is a member of the STAT family of transcription factors. Whereas STAT homologues have been found in all multicellular eukaryotes, STAT proteins are not found in yeast[5]. STATs commonly share similar structural characteristics, including an N-terminus that is involved in mediating its phosphorylation-dependent dimerization, a coiled-coil domain that is involved in the formation of protein-protein interactions, a central DNA binding domain that also encompasses both overlapping nuclear import and nuclear export sequences, a downstream SH2 domain, a single conserved tyrosine phosphorylation site, and a C-terminal transactivation domain[6-8]. This transactivation site, which is required for maximal transcriptional activation through the recruitment of histone acetyltransferases and other coactivators of transcription, is phospho-regulated by several factors, including IL-1, TNFα and UV irradiation[9]. S727 phosphorylation within the transactivation domain modulates the extent of STAT1-dependent gene expression, and has been shown to be involved in promoting efficient export of STAT1 from the nucleus[10]. STAT1 S727 phosphorylation also permits the interaction of STAT1 with p53, which together induce pro-apoptotic gene expression[11]. Tyrosine phosphorylation in the STAT family of proteins is a conserved process that leads to their homo- and hetero-dimerization through reciprocal phospho-tyrosine and SH2 domain interactions. Homo and heterodimerization of the different STAT molecules is typically considered to be a

requirement for their translocation to the nucleus[12]. The dimerization of STAT proteins is required for its interaction with specific DNA binding sequences, thus facilitating transcriptional activation of their associated genes.

There are seven known mammalian STAT proteins, which regulate a vast array of physiological processes, including immune responses, growth, and apoptosis[13-15]. Cytokine binding to cell surface receptors, such as IFNγ, IL-6, and IL-2, activate receptor-associated tyrosine kinases and feed into the JAK-STAT pathway to activate diverse members of the STAT family[16, 17]. Other exogenous factors, such as hormones like angiotensin II and chemokines like CCL5, bind to G-protein coupled cell surface receptors, which, in turn, activate other STAT proteins in a JAK-dependent manner[18, 19]. Finally, growth factor binding to receptor tyrosine kinases (*e.g.* EGF receptor) can initiate STAT tyrosine phosphorylation independently of JAK[20, 21].

The different STATs have varying functions. STAT1 and STAT2 mediate IFN-dependent cellular responses. This is typified by the regulation of genes involved in proliferation, differentiation, survival, and apoptosis[22-24]. Loss of STAT1 correlates with a loss of cellular apoptosis, loss of TNFα sensitivity, and increased incidence of malignant transformation[25]. STAT4 mediates T-helper 1 cell development in response to IL-2, while STAT6 mediates T-helper 2 cell development in response to IL-4. STAT5A and STAT5B share a high degree of homology, and are important for hematopoiesis[26, 27]. STAT3 is involved in normal cellular development, and regulates inflammatory responses, anti-apoptotic effects, and differentiation[28]. While there is a vast array of diversity in terms of the physiological roles played by the different STATs, the commonality is that the biological effect mediated by STAT proteins is universally dependent on their ability to translocate to the nucleus.

Nuclear Trafficking of STAT1

Because of the relatively large size of STAT1 molecules, STAT1 is unable to simply diffuse into the nucleus, but rather nuclear import occurs by a systematic energy dependent mechanism[29, 30]. Exogenous IFNγ receptor ligation leads to activation of the JAK-STAT pathway and subsequent Y701 phosphorylation of STAT1[31]. Tyrosine phosphorylation of STAT1 permits homo-dimerization and association with the nuclear import machinery. An atypical nuclear localization signal (NLS) that is generated through the dimerization of STAT1 leads to STAT1 association with a soluble karyopherin carrier protein called importin α5 (referenced interchangeably in the text as KPNA1, NPI-1 and SRP-1)[32]. Importin α5 has numerous armadillo helical repeats that mediate its association with classical NLS sequences. However, in the case of the STAT1 homodimer, importin α5 binds through its C-terminal[33]. In general, α karyopherin proteins act as adapters by detecting the basic NLS of its cargo, binding directly to its cargo and mediating its transport to the nuclear pore complex (NPC)-associated β-karyopherin importin β. Importin β recognizes the N-terminal importin β binding (IBB) domain of importin α, forming a ternary complex formed of importin β, importin α, and the cargo protein[34]. The translocation of molecules through the NPC occurs by an energy-dependent mechanism. RAN, a Ras-related GTPase, associates predominantly with GDP in the cytoplasm and GTP in the nucleus[35]. Binding of RAN-GDP to the ternary import complex causes nuclear translocation through the nuclear pore. Upon transit, the ternary complex binds with more affinity to RAN-GTP, causing a conformational change and subsequent release of STAT1 into the nucleus. The importin α5 binding site on STAT1 coincides with the STAT1 DNA binding domain and, thus, release of STAT1 by importin α5 both coincides with, and is required for, the subsequent binding of the STAT1 dimer to DNA[36].

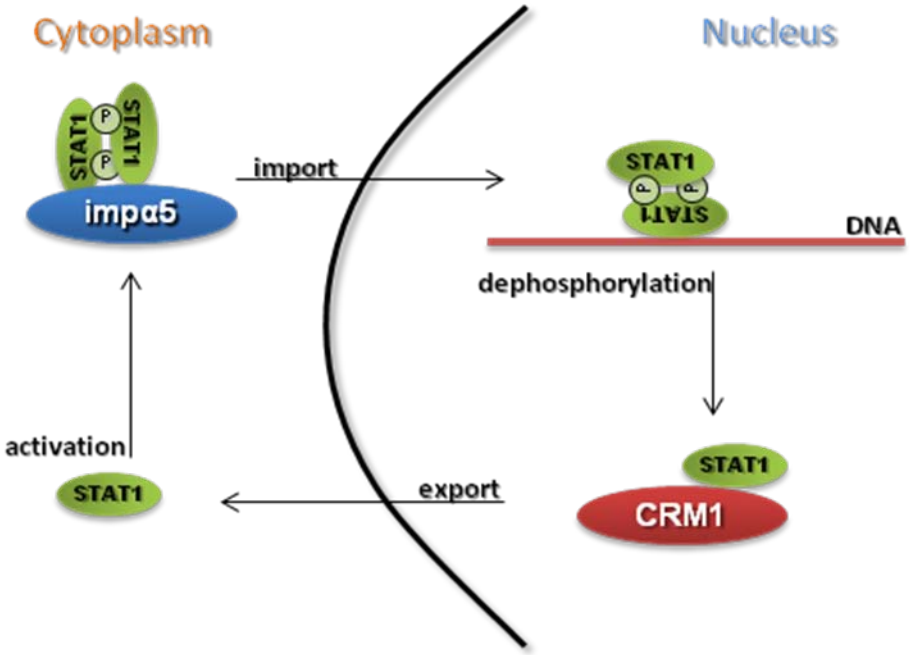


Diagram 2: STAT1 nuclear shuttling

Conversely, the nuclear export of STAT1 is driven by the presence of nuclear export sequences (NES) that are recognized by nuclear proteins called exportins. The NES is encoded by a sequence of hydrophobic and leucine rich residues that is recognized by an exportin called 'chromosome region maintenance 1' (CRM1)[30]. Binding of CRM1 to STAT1, and STAT1 nuclear export, requires STAT1 Y701 dephosphorylation[37]. Dephosphorylation of active STAT1 by the nuclear tyrosine phosphatase TC45 unmasks the STAT1 NES, allowing for the STAT1 dimer to dissociate from DNA, and associate with the nuclear export machinery[38]. The recognition of STAT1 by CRM1 requires the association with RAN-GTP to form a stable export complex. Upon assembly, the complex undergoes nuclear export, ultimately leading to the hydrolysis of GTP by RAN in the cytoplasm and subsequent release of its cargo proteins[35].

Numerous studies have shown that the NLS of STAT1 behaves atypically. STAT1 dimers and other proteins with classical basic NLS sequences have been shown to be able

to bind importin α5 simultaneously[39]. Additionally, STAT1-importin α5 binding requires a specific non-basic STAT1 residue (L407), which is uncharacteristic of typical NLS sequences[32]. STAT1 L407 mutations produce STAT1 molecules that respond to IFNγ in that they can become tyrosine phosphorylated, but do not undergo nuclear translocation[40]. STAT1 L407A mutants are unable to bind importin α5, though they can dimerize and bind DNA. Heterodimerization of wild-type STAT1 with STAT1 L407A restores nuclear import activity[33]. The exact relevance of the L407 residue in STAT1-importin α5 binding is unknown, though it is clear that the NLS function of STAT1 does not correspond simply to a basic stretch of amino acids but rather through structural changes that occur between STAT1 monomers following tyrosine phosphorylation.

There is evidence that low levels of unphosphorylated STAT1 accumulate in the nucleus. Previous studies have shown that STAT1 Y701F mutations do not block basal levels of nuclear STAT1[41]. In addition, inhibition of CRM1-mediated nuclear export by the export inhibitor leptomycin B did not lead to an increase in unphosphorylated STAT1 levels in the nucleus, indicating that latent STAT1 does not constitutively shuttle in and out of the nucleus[12]. It is possible that unphosphorylated STAT1 piggybacks across the nuclear membrane in association with another non-STAT protein. For example, unphosphorylated STAT1 has been shown to complex with interferon regulatory factor-1 (IRF-1) to recognize a site in the low molecular mass protein 2 (LMP2) gene[42], which is involved in proteosomal degradation and antigen processing. STAT1-deficient U3A cells have been shown to have reduced caspase 1, 2 and 3 expression. However, exogenous expression of STAT1-insensitive tyrosine phosphorylation/dimerization mutants reconstitutes caspase expression, indicating a physiological role of unphosphorylated STAT1. Other possible physiological effects of STAT1 and the mechanisms governing its

nuclear translocation and transcriptional activity are unclear. By surface plasmon resonance, STAT1 has been found to associate with numerous multimeric complexes in quiescent cells[43]. Also, STAT1 association with other proteins, such as transcription factors and co-activators, may permit unphosphorlyated STAT1 to bind DNA well enough to initiate transcription[25]. The mechanisms that control constitutive STAT1 nuclear trafficking are largely unknown.

Studies in our laboratory have shown that inhibition of mTOR by rapamycin increases the induction of STAT1-dependent pro-apoptotic genes[44]. mTOR physically associates with, but does not phosphorylate STAT1, indicating a novel signalling mechanism in the control of STAT1 activity. Studies on yeast TOR and transcription factors suggest that mTOR might regulate the nuclear trafficking of STAT1.

Yeast TOR Overview

TOR proteins are highly conserved from yeast to man. TOR was first characterized in budding yeast. Yeast TOR was initially described in the context of the macrocyclic lactone drug rapamycin (sirolimus), which was found to have significant anti-fungal properties[45]. Rapamycin was also later found to possess powerful anti-tumour and immunosuppressive properties in mammals, driving interest in rapamycin and its mode of action. Much of what we know about the mammalian form of TOR derives from work previously done in yeast.

In yeast and mammalian cells, the mode of action of rapamycin has been determined. Rapamycin binds to a cellular protein called FKBP12, a cofactor needed for the effect of rapamycin[46]. Binding of rapamycin to FKBP12 induces formation of a complex that is able to associate with yeast cellular TOR proteins and inhibit their ability

to phosphorylate downstream targets. Yeast TOR genomes collectively encode two TOR homologues, called TOR1 and TOR2, which have 67% homology. TOR proteins in general resemble lipid kinases although they are known to behave like serine/threonine kinases belonging to the family of phosphatidylinositol kinase-related kinases (PIKK). TOR1 and TOR2 have different roles in yeast; whereas loss of TOR1 activity leads to temperature hypersensitivity and slower growth, loss of TOR2 is lethal, emulating G1 cell cycle arrest caused by rapamycin[47, 48]. TOR1 and TOR2 have been described to have both shared and individually unique cellular functions[49]. This fact is characterized, more precisely, by the distinct ability of each TOR protein to associate within separate multi-protein complexes[48].

Yeast TOR Complexes

TOR complex 1 (TORC1) contains either TOR1 or TOR2, as well as proteins 'lethal with sec thirteen 8' (LST8), 'TOR complex one 89' (TCO89), and 'kontroller of growth' (KOG1)[50-52]. TORC1 may be dimeric, though dimerization does not appear to be necessary for its function[53]. Rapamycin does not directly affect the stability of TORC1, though it is able to inhibit its cellular activity in an FKBP12-dependent manner[48]. TORC1 associates primarily with membrane structures, such as the plasma membrane, lysosomal membrane, and endosomal membrane, which is consistent with TORC1's role as a sensor of nutritional status[48, 51, 52]. TORC2, the TOR complex containing TOR2 only, LST8, AVO1, AVO2, AVO3, BIT2, and BIT61 may also be dimeric[48, 53]. TORC2 is unable to bind the FKBP12-rapamycin complex and is thus insensitive to rapamycin[48, 54]. TORC2 localizes primarily to the plasma membrane in discrete punctae[55]. The presence of LST8

in both TORC complexes may represent a mode of signalling crosstalk between the two different complexes[48].

Yeast TOR Downstream Functions

In yeast, TOR has numerous physiological functions. TOR regulates cell growth by acting as a coupling agent between nutritional cues and the cell growth machinery. Treatment with rapamycin blocks this, arresting cells in a G0-like state. Many cytological events that regulate growth are affected by TOR. TORC1 activity upregulates protein synthesis by promoting translation initiation, promoting the expression and synthesis of translational machinery, stabilizing mRNA turnover, and increasing the activity of nutrient transporters[56-58]. Conversely, TORC1 activity antagonizes cellular catabolic processes by downregulating those processes that relate to stress. For example, processes associated with nutritional or environmental stress, such as autophagy, are downregulated by TOR in nutritionally replete conditions[59]. TORC1 also negatively regulates growth arrest by antagonizing the expression of stress-responsive transcription factors. Genes involved in the assimilation of alternative nitrogen sources, in protein degradation, and in general stress protection, are upregulated upon inhibition of TORC1 activity[60, 61].

The regulation of stress response gene expression in yeast by TORC1 occurs in part largely through the sequestration of specific transcription factors in cytoplasm. By regulating the cellular localization of these transcription factors, TORC1 is able to modulate the transcriptional activity of these proteins. In terms of nitrogen discrimination, TORC1 activity inhibits the transcription of genes expressed following nitrogen deprivation by mediating the association of the transcription factor Gln3 with the cytoplasmic repressor protein Ure2[62]. In conditions of nitrogen limitation, or TORC1

inactivation, Gln3 is hypophosphorylated, which, in turn, leads to its dissociation from Ure2 and its nuclear import via the α karyopherin importin (SRP1). TORC1 has also been shown to prevent nuclear access of the GATA transcription factor GAT1[63], though this is likely mediated by a different mechanism than that seen with GLN3.

Other proteins whose cellular localization is mediated by TORC1 are the heterodimeric proteins Rtg1 and Rtg3[64]. Rtg1 and Rtg3 are signalling elements involved in the activation of genes involved in the biosynthesis and homeostasis of glutamate and glutamine. Rtg1 and Rtg3 cellular activity is inhibited by TORC1 through TORC1's ability to promote an association between Mks1, an upstream inhibitor of Rtg1/Rtg3, and Bmh1. Bmh1 positively regulates glutamine synthesis by binding and sequestering Mks1. Upon TORC1 inactivation, the protein Mks1/Bmh1 complex becomes dephosphorylated and dissociates from Rtg1/Rtg3, leading to translocation of the Rtg complex to the nucleus to promote the expression of associated genes.

TORC1 is further involved in regulating the cellular trafficking of the transcription factors Msn2 and Msn4[65]. These proteins are involved in promoting the expression of stress-response element (STRE)-dependent genes, whose expression occurs in response to numerous cellular stresses such as nutrient limitation, osmotic stress, low pH, heat-shock, and high ethanol concentrations. Under normal conditions, TORC1 activity promotes Msn2 and Msn4 accumulation in the cytoplasm. Inactivation of TORC1 activity by nutritional limitation or other cytological stresses causes Msn2 and Msn4 proteins to accumulate in the nucleus[66].

Effects of TORC1 on stress transcription factors are mediated by protein phosphatases. The yeast homologue of mammalian protein phosphatase 2A (PP2A) is the principal phosphatase whose activity is regulated by TORC1[67]. PP2A is a heterotrimeric

complex consisting of a catalytic subunit (PP2Ac), as well as a regulatory subunit (B) and scaffolding subunit (A). There are numerous PP2A catalytic subunits, affecting the functional relevance of the protein phosphatase complex. The diversity of downstream targets of the phosphatase complex is complicated by the diverse combinations of possible regulatory subunit-catalytic subunit complexes. TORC1-dependent cellular effects are mediated by PP2Ac and Sit4-containing catalytic complexes that associate with regulatory proteins such as Tap42 and phosphotyrosyl phosphatase activator (PTPA)[68]. TORC1 positively affects the phosphorylation status of Tap42, promoting the association of Tap42 with PP2Ac/Sit4 and release of the PTPA regulatory subunit[69]. Tap42 association with PP2Ac/Sit4 renders the protein catalytically inactive. Conversely, inactivation of TORC1 by rapamycin or stress leads to Tap42 hypophosphorylation and subsequent Tap42 dissociation from the PP2Ac/Sit4 catalytic subunit. Dissociated PP2Ac proteins are enzymatically active and their release into the cytoplasm enables them to act on their downstream targets[70, 71]. Loss of PP2A has been found to be detrimental to cell growth, indicating that PP2A may be mediating the downstream cellular effects of TOR[72].

mTOR Overview

The mammalian equivalent of yeast TOR (mTOR, FRAP, RAFT) is ubiquitously expressed and maintains 95% amino acid sequence homology between humans, mice, and rats[48]. As in yeast, mTOR responds to changes in the extracellular environment and mediates appropriate cellular responses. Mammalian genomes collectively encode for only one form of TOR, mTOR, as opposed to two in yeast[73].

The mTOR proteins are members of the PIKK group of kinases and, as such, contain a C-terminal catalytic domain that affords mTOR its serine/threonine kinase

activity. N-terminal to the kinase region is the FKBP12-rapamycin binding (FRB) domain. N-terminal to the FRB domain is the FAT domain, which is contained within all members of the PIKK family. C-terminal to the kinase domain is another FAT domain called FATC, which is required for proper mTOR function through its ability to sense redox status, as conferred by stabilizing disulfide bridges. These FAT domains may be involved in mediating protein-protein interactions between the different PIKKs and are generally regarded as protein scaffolds. At the N-terminal are tandem 'Huntington, elongation factor 3, PP2A, TOR' (HEAT) repeats that provide a large surface area for mediating protein interactions[1].

mTOR Complexes

As in yeast, mTOR regulates its downstream processes in association with two macromolecular protein complexes, mTORC1 and mTORC2[48, 52]. The first complex, mTORC1, containing mTOR, mLST8 and 'regulatory associated protein of mTOR' (Raptor, the mammalian orthologue of KOG1), regulates the temporal aspects of cell growth[74]. mTORC1 is inhibited by rapamycin treatment through the FKBP12-rapamycin complex, leading to the abrogation of mTORC1 kinase activity[75, 76]. The mTORC1-associated protein Raptor acts as a scaffolding protein that allows for the recruitment of substrates to mTOR. Raptor is the component of mTORC1 that confers rapamycin sensitivity. Reportedly, the interaction of mTOR with Raptor in mTORC1 can be inhibited by treatment with rapamycin, indicating a process whereby FKBP12-rapamycin blocks access of mTOR kinase activity to its substrates[77, 78]. Alternatively, FKBP12-rapamycin has been shown to block mTORC1 autophosphorylation at S2481, indicating that FKBP12-rapamycin affects mTOR kinase activity directly, rather than its access to

substrates[75]. The precise mechanism by which rapamycin blocks mTORC1 activity remains unclear. The other well-characterized component of mTORC1 is mLST8 (GβL). mLST8 is required for the full catalytic activation of mTOR. Previous studies have shown that upstream signals affect mTORC1 activity by signalling through mLST8, although a precise role of mLST8 in regulating mTORC1 is unknown[50, 79].

The other characterized mTOR-complex is mTORC2. mTORC2 contains mTOR, 'rapamycin-insensitive companion of mTOR' (Rictor, AVO3 in yeast), mLST8, and 'mitogen-activated protein kinase associated protein 1' (SIN1). mTORC2 is insensitive to rapamycin treatment, in part due to the absence of Raptor in the complex, and does not bind FKBP12-rapamycin[48]. mTORC2 forms multimeric mTORC2-mTORC2 structures that have the result of augmenting kinase activity above that which is seen in the monomeric form[53, 75]. Signalling through mTORC2 regulates the spatial aspects of cell growth by controlling actin polymerization and cell spreading, enabling the cell to coordinate growth into discrete loci. Although mTORC2 activity is insensitive to rapamycin, chronic exposure to rapamycin blocks the assembly of new mTORC2 complexes in some cell types[80].

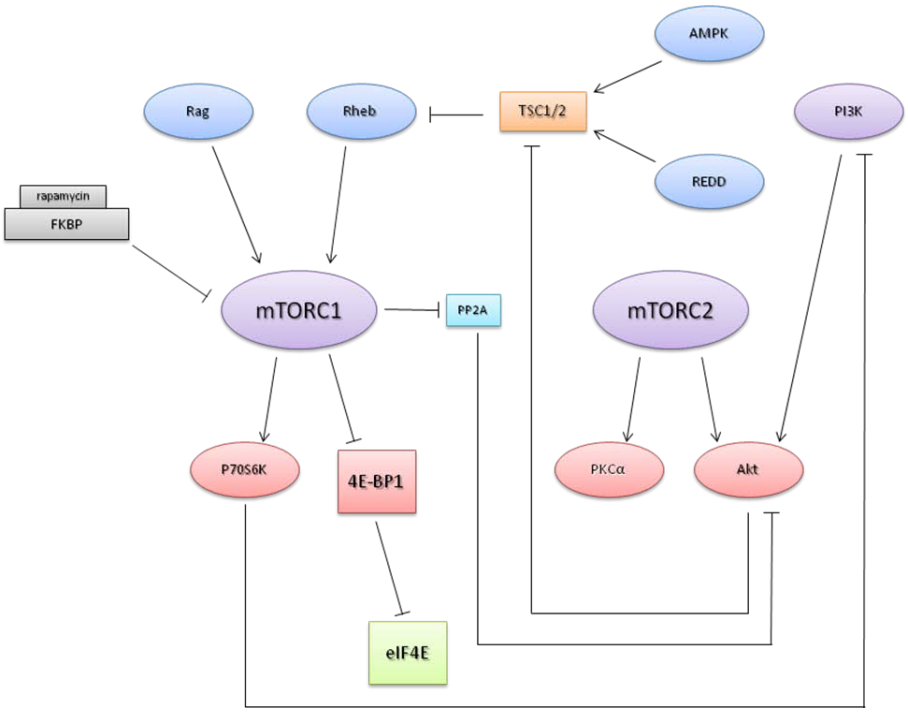


Diagram 3: mTOR complexes and signalling elements

mTOR Upstream Regulators

Four major inputs are known to affect TOR signaling[81]. Growth factors are known regulators of mTORC1 activity. Extrinsic factors feeding into the PI3K pathway, such an insulin and insulin-like growth factors (IGFs), induce phosphorylation events that affect the receptors themselves, leading to recruitment and tyrosine phosphorylation of ligands known as insulin receptor substrates (IRS). Tyrosine phosphorylation of IRS-1 leads to the association of PI3K with phosphorylated IRS-1 through its SH2 domain. PI3K mediates the conversion of membrane phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidyl-3,4,5-phosphate (PIP3) and, subsequently, induces the proteins pyruvate dehydrogenase kinase 1 (PDK1) and Akt (PKB) to localize to the membrane. PDK1 phosphorylates Akt, which then goes on to phosphorylate tuberin (TSC2), inactivating it. This phosphorylation of Akt is negatively regulated by the protein 'phosphatase and

tensin homolog' (PTEN), which, acting as a phosphatase, negatively regulates intracellular levels of PIP3. The heterodimeric tuberous sclerosis proteins TSC1-TSC2 negatively regulate mTOR signalling through acting as a GTPase-activating protein (GAP) on the GTPase Rheb[82]. Inactivation of TSC2 by Akt relieves the repression on mTOR activity by allowing Rheb to directly activate mTOR kinase activity in a GTP-dependent manner. Putatively, GTP loading of Rheb allows for an induced conformational change in mTORC1, leading to its activation and phosphorylation of downstream targets[83]. In addition, nutrients like amino acids play a significant role in regulating mTORC1 signalling. Though the precise mechanism is unknown, the presence of amino acids likely modulates mTORC1 activity either through inhibition of TSC1-TSC2 repression or by stimulation of Rheb activity[84]. Alternatively, hVPS34, a member of the PI3K family of kinases, can signal amino acid availability to mTORC1 directly, possibly altering the association of mTOR with Raptor and affecting mTORC1 activity[85]. Rag GTPases also regulate amino acid signalling to mTORC1. Rag proteins interact with mTORC1 in an amino acid-sensitive manner and their expression has been found to be required for the activation of mTORC1 by amino acids[86]. Amino acid repletion positively regulates Rag GTPase activity and mediates the relocalization of mTOR to the endomembrane system within the cell. Energy levels, as indicated by the concentration of AMP within the cell, further modulate mTORC1 activity. Cellular energy status is detected through 'AMP-activated protein kinase' (AMPK), which responds to high AMP levels within the cell. AMPK activation leads to direct catalytic phosphorylation of TSC2, upregulating its GAP activity and inhibiting mTORC1 function[87]. Finally, environmental stresses such as hypoxia and DNA damage are also negative regulators of mTORC1 activity. Hypoxia induces the activation of the transcription factor hypoxia-inducible

factor 1 (HIF-1)[88]. HIF-1 activation induces the expression of the proteins REDD1 and REDD2, which function between Akt and TSC2 to negatively regulate mTORC1 function. Reductive stress and DNA damage also signal through mTOR. DNA damage promotes p53 activation[89], which causes inactivation of mTORC1 activity through the AMPK-TSC2 pathway. Reductive stress may inhibit mTORC1 activity through the FATC domain of mTOR, which may act as a redox sensor for the environment[90, 91].

mTOR Downstream Function

As well as possessing numerous upstream regulators, mTOR signalling affects a multitude of downstream targets. mTORC1 regulates translational activation by modulating the phosphorylation status of 4E-BP1 and S6K1[92]. Phosphorylation of 4E-BP1 by mTORC1 promotes cap-dependent translational activation by enabling the release of the translation factor eIF4E, which, along with eIF4G, stimulates translation initiation by binding to the 5' untranslated of mRNA. Overexpression of 4E-BP1 has also been shown to stabilize the expression of cell cycle inhibitor KIP1 (p27), denoting a possible mechanism of cell growth arrest in response to rapamycin treatment[93]. In addition, mTORC1 targets S6K1. S6K1 is a kinase that phosphorylates the 40S ribosomal protein S6[1]. Phosphorylation of S6 leads to increased translation of mRNAs containing 5' oligopyrimidine tracts (TOP), whose encoded proteins represent ribosomal proteins and translational elongation factors. Complete activation of S6K1 requires two phosphorylation events; phosphorylation at the T loop in the kinase domain is mediated by PDK1, whereas mTORC1 targets the T389 site in the C-terminal hydrophobic motif. TOR signalling (TOS) motifs in S6K1 and 4E-BP1 mediate their binding to the scaffolding protein Raptor[77, 94]. siRNA-mediated knockdown of Raptor has been shown to

block phosphorylation of downstream targets S6K1 and 4E-BP1, indicating that binding of mTOR substrates to Raptor is critical in order for them to become phosphorylated[95].

mTOR also regulates ribosome biogenesis, which is a tightly controlled process that correlates with energy levels and the presence of monomeric building blocks within the biosynthetic machinery of the cell. mTOR controls the biosynthesis of ribosomes by regulating the transcription of numerous RNA polymerase associated genes[96]. Inhibition of mTORC1 by rapamycin has been shown to inhibit TIF1A activation[97]. TIF1A, an RNA polymerase I-associated transcription factor, is required for the formation of a transcription initiation complex[98]. RNA polymerase II-dependent expression of ribosomal protein genes is similarly inhibited by mTORC1 inhibition. RNA polymerase II activity is regulated by the transcription factor FHL1, which is constitutively bound to its ribosomal protein promoter sequences[99]. FHL1 transcriptional activity is regulated by the co-activator protein IFH1 and the co-repressor protein CRF1. Inactivation of mTORC1 activity leads to CRF1 nuclear localization from the cytoplasm, which then goes on to compete with IFH1 for FHL1 binding[100]. Displacement of IFH1 by CRF1 leads to inactivation of FHL1 transcriptional activation, thus inhibiting ribosomal protein expression.

Other cellular processes regulated by mTOR signalling include autophagy, actin cytoskeleton polymerization, and the regulation of metabolism. Autophagy is a catabolic process whereby cytoplasmic contents, including organelles, are enclosed by double-membrane structures called autophagosomes and delivered to the vacuole for degradation. Autophagy is activated when cellular nutrients are deficient. Previous studies have shown a negative correlation between autophagic protein degradation and the degree of phosphorylation of S6K1, a direct mTORC1 target[59]. Rapamycin treatment alone has

been shown to produce a partial block in protein synthesis and to stimulate autophagic processes by a mechanism that occurs independently from the presence or absence of amino acids[101]. The process of recycling cellular contents for ensuring survival is another process regulated by mTORC1 activity. mTORC1 activity controls the trafficking of amino acid and glucose transporters, promoting the uptake of metabolites and nutrients from outside the cell[102]. mTORC1 thus not only senses the presence or absence of nutrients, but controls the cellular intake of these products. mTORC2, as in yeast, also modulates the actin cytoskeleton. The exact mechanism by which mTOR signals to the actin cytoskeleton remains unknown, though this process likely involves stimulation of F-actin stress fibres, and regulation of paxillin, Rho, Rac, and protein kinase α activity[75, 76]. Finally, fat metabolism has been shown to be affected by mTOR activity. The nuclear receptor PPARγ, which is involved in regulating efficient fatty acid storage and glucose metabolism, has been demonstrated to be inhibited by rapamycin treatment[103]. This effect of mTOR inhibition on fatty acid metabolism may also occur through S6K1, which has been shown to control fat accumulation and in mice.

Numerous regulatory loops exist in the TOR signalling pathways, allowing for intricate control on cellular anabolic processes. In particular, increasing amino acid concentrations within the cell has been shown to limit signalling through PI3K[104]. Inhibition of mTORC1 by rapamycin reverses the inhibition on PI3K signalling, indicating that mTORC1 or downstream effectors impart a negative feedback loop on insulin responsiveness of the cell[81]. mTORC1 activity also negatively regulates PI3K pathway activation through its downstream substrate S6K1. S6K1 directly phosphorylates IRS-1, impairing its ability to recruit PI3K to the membrane and catalyze Akt activation[105]. Akt activation is mediated by two phosphorylation events: PDK1

phosphorylation of T308 in the activation loop and phosphorylation by mTORC2 at S473 in the hydrophobic motif[90]. mTORC2 thus plays a role in the activation of Akt and mediates crosstalk between mTORC1 and mTORC2-associated pathways. Finally, S6K1 has also been shown to phosphorylate mTOR at T2446 and S2448 directly, though the functional relevance of these phosphorylation events in terms of regulating mTOR signalling is still unknown[106].

Mammalian PP2A

As in yeast, mammalian PP2A is an ubiquitous cellular serine/threonine phosphatase that has broad substrate specificity and numerous cellular functions. PP2A is also very structurally similar to its yeast counterpart, containing structural (A), catalytic (C), and regulatory (B) subunits. PP2A is involved in promoting the dephosphorylation of Bcl2, Bad, and p53, steering the cell towards cell death[107]. PP2A activity has also been shown to be regulated by mTOR. Phosphorylation of the PP2A regulatory subunit α4 by mTOR prevents the dephosphorylation of mTORC1 targets S6K1 and 4E-BP1 and leads to a pro-survival phenotype. Rapamycin treatment of mTOR decreases cell proliferation by displacing α4 from PP2Ac, activating its catalytic function[108]. The regulatory role of α4 in the cell is significant for cell survival as cells lacking α4 undergo apoptotic cell death[109].

SECTION 2: HYPOTHESIS AND PROJECT OUTLINE

The cytokine interferon-gamma (IFNγ) regulates host immunity, the onset of inflammation, and apoptosis, in part via the transcription factor STAT1. Regulators of STAT1 transcriptional activity or nuclear content are thus targets for modulation of lung inflammatory, immune, and neoplastic processes. We previously identified a macromolecular complex that contains STAT1, its kinase PKCδ, and the serine/threonine kinase 'mammalian target of rapamycin' (mTOR)[110]. In yeast, TOR suppresses the nuclear content of Msn2, a stress response transcription factor, via an associated phosphatase. Preliminary data suggest that in mammals a homologous phosphatase, PP2A, associates with the mTOR-STAT1 complex. We hypothesize that, similar to yeast, inactivation of mTOR enhances the nuclear localization of STAT1 and that the phosphatase PP2A mediates this effect.

We first characterized the ability of mTOR to regulate cellular localization of STAT1. We determined the effect of mTOR inhibition on nuclear localization of STAT1 by immunostaining and fluorescence confocal microscopy. Then we addressed the ability of rapamycin to regulate cellular localization of numerous STAT1 mutants, in order to elucidate a possible mechanism of mTOR-mediated STAT1 trafficking. Following this, we determined the role of PP2A in mediating the effect of rapamycin on STAT1 nuclear content by expressing its constitutively active and dominant negative isoforms of the protein. To further elucidate the import mechanisms surrounding STAT1 nuclear localization, we assessed the ability of inhibition of mTOR activity to induce association of mTOR with STAT1 and the karyopherin protein importin α5. By confocal imaging we determined how overexpression of wild-type importin α5, and of importin α5 S111A,

would affect STAT1 cellular localization. Using the PP2A inhibitor okadaic acid, we studied the role of PP2A in mediating importin α5 binding to mTOR in response to rapamycin treatment. Finally, we looked for functional roles of mTOR-mediated STAT1 nuclear localization in terms of regulation of cell fate using cell viability assays and immunoblotting to determine levels of cell cycle inhibitors.

SECTION 3: MATERIALS AND METHODS

Materials

SV40-transformed African green monkey kidney cells (COS7) were generously donated by Dr. S. Laporte. Adenovirus type 5-transformed human embryonic kidney cells (HEK 293T) were generously donated by Dr. S. Lemay. MDA-BM-231 cells were donated from Dr. S. Ali. Immortalized human alveolar basal epithelial cells (A549) were obtained from the American Type Cell Culture Collection (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), F-12 Nutrient Mixture, Fetal Bovine Serum, Penicillin-Streptomycin, Lipofectamine™ LTX, Lipofectamine™ 2000, Alexa Fluor® 488 and 568 were obtained from Invitrogen Canada Inc. (Burlington, ON); Plasmid Plus Midi Kit was obtained from Qiagen (Mississauga, ON); rapamycin was obtained from EMD Biosciences (Mississauga, ON); human recombinant IFNγ was obtained from Roche Applied Science (Laval, QC); okadaic acid was obtained from BioMol (Plymouth, PA); SuperSignal West Pico Chemiluminescent Substrate (ECL) was obtained from Thermo Fisher Scientific (Rockford, IL); Bio-Rad Protein Assay Dye Reagent was from Bio-Rad (Mississauga, ON); Protein A sepharose beads, and Amersham Hybond™-ECL nitrocellulose paper was obtained from GE Healthcare (Montreal, QC); Vectashield® SoftSet Mounting Medium was obtained from Vectorlabs (Burlington, ON); rabbit anti-mTOR antibodies were obtained from Upstate (Burlington, MA); mouse anti-STAT1 antibodies for immunofluorescence were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA); mouse anti-importin α5 (NPI-1) antibodies were obtained from Zymed (San Francisco, CA); rabbit anti-cleaved caspase-3, and rabbit anti-STAT1 antibodies for Western blot were obtained from Cell Signaling (Danvers, MA); rabbit anti-V5 antibodies

were obtained from Chemicon (Burlington, MA); mouse anti-V5 antibodies were obtained from Invitrogen (Burlington, ON); mouse anti-p27 antibodies were obtained from BD Biosciences (Mississauga, ON); anti-rabbit and anti-mouse horseradish-peroxidase-conjugated antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). All other chemicals, reagents, and biological agents were obtained from Sigma-Aldrich Canada (Mississauga, ON) or Bioshop (Montreal, QC), unless otherwise indicated.

Vectors

cDNAs used were STAT1, STAT1 Y701F, STAT1 L407F, STAT1 S727A, PP2Ac L199P, PP2Ac Y307F, KPNA1 WT, and KPNA1 S111A. Clones were generated using the Invitrogen **Gateway**® cloning system. Source cDNAs were cloned into pDONR221 vectors followed by transfer by recombination into pcDNA3.1/V5/ECFP-DEST and pcDNA3.1/V5/DEST mammalian expression vectors under the control of a CMV promoter. Vectors were propagated in *Escherichia coli* with selectivity for ampicillin-resistance followed by plasmid isolation and purification using a Qiagen Plasmid Plus Midi Kit.

Protein complementation assay (PCA) vectors used were VF1-STAT1, and VF2-PKCδ. These were derived from cDNAs of yellow fluorescent protein fragments YF1 (amino acids 1-158) and YF2 (159-239) that were cloned into the **Gateway®** cloning system (donated by Dr. S. Michnick). STAT1 and PKCδ cDNAs were cloned by PCR into pDONR221 vectors followed by transfer to pcDNA3.1-based mammalian expression vectors containing yellow fluorescence protein fragments.

Cell culture and treatments

HEK 293T and COS7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic. A549 cells were maintained in F-12 Nutrient Media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic. All cell lines were grown at 37°C and 5% CO_2 in vented tissue culture plates. Cells were re-fed prior to experimentation with appropriate growth media. For IFNγ treatments, cells were incubated with 100U/mL human recombinant IFNγ in serum-containing media for 1h. Rapamycin treatments were performed by the addition of 50ng/mL rapamycin in serum-containing media, for times specified. Okadaic acid was used at a concentration of 50nM in serum-containing media. Doxorubicin was used at a concentration of 1uM in serum-containing media.

Expression of recombinant proteins for microscopy

Transient transfections of fluorescence-tagged STAT1 constructs (ECFP-STAT1, ECFP-STAT1 Y701F, ECFP-STAT1 L407F, and ECFP-STAT1 S727A) were performed using the liposomal reagent Lipofectamine LTX, as per the manufacturer's recommendations, with only minor changes. Briefly, A549 cells were plated in Lab-Tek 4-chamber culture slides at 25,000-40,000 cells/well. Cells were grown overnight then transfected at approximately 70% confluency. Cells were transfected in 500uL serum-free media (SFM), using mixtures consisting of 800ng of the appropriate plasmid DNA and 2uL of Lipofectamine LTX per 1.8cm^2 surface area. Liposomal complexes were dissolved in 100uL of serum-free media and added dropwise to each well. Cells were

incubated at 37°C with transfection reagents for 5h before replacement with fresh, serum-containing culture media. 48h following transfection, cells were treated with the appropriate experimental conditions then washed in 1X PBS and fixed in 4% paraformaldehyde solution for 15 minutes at room temperature. Following fixation, cells were washed three times and mounted in Vectashield® Soft-Set Mounting Medium with DAPI for visualization.

VF1-STAT1/VF2-PKCδ, ECFP-STAT1/PP2A L199P and Y307F, and ECFP-STAT1/KPNA1 WT and S111A co-expression experiments were performed using similar experimental parameters to those used in the singly transfected transfectants. Cells were seeded in Lab-Tek 4-chamber culture slides at 25,000-40,000 cells/well. Upon reaching 70% confluency, cells were liposomally transfected in serum-free media. PCA vectors were co-expressed in a 1:1: ratio (VF1-STAT1:VF2-PKCδ), while ECFP-STAT1/PP2A and ECFP-STAT1/KPNA1 were co-expressed at a 3:1 ratio. Transfections were performed using a total of 800ng of plasmid with 2uL Lipofectamine LTX per well. After transfection, cells were incubated at 37°C for 5 hours before replacement with fresh culture media. 48h following transfection, cells were treated with appropriate experimental conditions followed by washing and fixation in 4% paraformaldehyde. Cells were then mounted for visualization.

Endogenous STAT1 immunostaining for microscopy

A549 cells were plated in 8-chambered culture slides at 25,000 cells/well. Following treatment, cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature prior to permeabilization by 0.25% Triton X-100. Cells were washed then incubated on mouse anti-STAT1 antibody at a 1:400 dilution for 2 hours at room temperature. Anti-STAT1 antibody was then removed and cells were incubated with fluorescence tagged anti-mouse Alexa Fluor® 568 conjugated antibody at a 1:400 dilution for 1 hour at room temperature. Cells were washed then mounted in Vectashield® Soft-Set Mounting Medium with DAPI for visualization.

Imaging by confocal microscopy

Imaging was performed using a Zeiss LSM 510 META scanning confocal microscope with a Plan-Neofluar 40x/1.3x oil DIC objective. Images were acquired at room temperature using a Zeiss AxioCam HR digital sensor at a 4 megapixel resolution and Zeiss LSM 510 software. Fluorescence was detected by multi-track image acquisition and excitation was obtained using a blue diode laser (excitation at 405nm), an argon laser (excitation at 458nm, 477nm, 488nm, and 514nm), and a helium/neon laser (excitation at 543nm). Fluorophores detected were ECFP (excitation: 458nm, emission: 475nm), YFP (excitation: 512nm, emission: 529nm), Alexa Fluor® 568 (excitation 568nm, emission: 603nm), and DAPI (excitation: 405nm, emission: 475nm). Measurements were performed using ImageJ software (version 1.38x, NIH) and fluorescence intensity was assessed by obtained pixel density values (pixels/area). Images were displayed using a Zeiss LSM

Image Browser (version 4.2.0.121, Carl Zeiss MicroImaging). Relative pixel density is expressed as a ratio of nuclear to cytoplasmic pixel densities. Results are measured from three cells per condition, from three separate experiments.

Transfection of HEK 293 cells by calcium phosphate

HEK 293T cells were seeded at 600,000 cells per 10cm dish and grown overnight. The next morning, media were changed with 8mL of fresh serum-containing media. A separate calcium phosphate transfection reagent was prepared by adding 0.5mL 2X HBS to 440µL sterile distilled water, 8µg DNA, and 61µL 2M $CaCl_2$. Complexes were given time to form, followed by dropwise addition to cells after 20 minutes. Media were swapped after 6 hours of transfection and cells were grown for 48h to allow for transgene expression. Cells were then treated and lysed as per protocol for generation of whole cell lysates.

Generation of whole cell lysates

Endogenous or recombinant protein HEK 293T cell lysates were generated using 0.3% CHAPS non-denaturing detergent. HEK293T cells were first washed in cold 1x DPBS before being mechanically scraped from the plates (for generation of whole cell lysates for detection of apoptosis, cells were scraped in culture media without prior washing). Cells were centrifuged for one minute at 3,000RPM before resuspension in three times the packed cellular volume of lysis buffer (20M Tris pH 8.-, 0.3% CHAPS, 1mM EDTA, 10mM β-glycerophosphate, 2.5µg/mL aprotinin, 2.5ug/mL leupeptin, 1mM

PMSF, 50mM NaF, 100μM $NaVO_4$). Cells were incubated on ice for 15 minutes on the lysis buffer, then cell suspensions were frozen over night at -80°C. The following day, samples were thawed at room temperature and subsequently homogenized by vortexing for 30 seconds. Samples were then centrifuged at 13,000RPM for 30 minutes. The resulting debris pellet (containing membrane structures) was discarded and the soluble fraction-containing supernatant stored for future use.

Immunoprecipitation

Immunoprecipitation was performed using HEK 293T lysates. 1mg of clarified protein was incubated with 5μg rabbit control IgG or rabbit anti-mTOR overnight on rotation at 4°C. The following day, 20μL of washed 1x Protein A Sepharose beads was added and immunoprecipitation continued for 1 hour. The Protein A Sepharose beads were then spun down and washed 3 times with PBS containing 0.3% CHAPS and protease inhibitors. Beads were washed one additional time in PBS with protease inhibitors alone, before being boiled in 1x SDS sample buffer for 5 minutes at 99°C. Solubilized proteins were then separated by SDS-PAGE followed by detection by Western blot analysis.

Western blotting

Endogenous and recombinant proteins, from whole cell lysates and from immunoprecipitates, were detected by Western blotting using antibodies, as outlined in "Materials". Equal quantities of protein were separated by SDS-PAGE under reducing

conditions and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 1 hour in 5% fat-free dry milk, followed by incubation overnight with primary antibody at 4°C. The following day, membranes were washed and incubated with secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. Proteins were detected using enhanced chemiluminscence reagent (ECL). Protein band densities (densitometry) were performed using the Discovery Series Quantity One 1-D Analysis Software® Version 4.6.7.

Crystal violet staining

Cells were seeded at 150,000cells/well in 6-well dishes. Following treatment, cells were washed once in PBS before staining with 0.05% crystal violet solution for 5 minutes. Cells were then washed 3 times before stain was solubilised in 1% SDS. Samples were taken and read by spectrophotometer at 570nm.

SECTION 4: RESULTS

mTOR suppresses STAT1 nuclear localization. Canonically, STAT1 is a pro-inflammatory transcription factor associated with IFNγ signalling. Cellular effects mediated by STAT1 typically include dimerization and nuclear translocation events. To investigate the role of mTOR in regulating STAT1 cellular localization, the pharmacological inhibitor rapamycin was used. Rapamycin is a known inhibitor of mTOR activity in mammalian cells. Endogenous immunostaining of STAT1 exhibited a marked increase in nuclear STAT1 levels in A549 cells in response to rapamycin as compared to unstimulated controls (figure 1A). Serum starvation alone caused a similar, but lesser, effect. The nuclear accumulation of STAT1 in response to rapamycin treatment was not as striking as the increase in nuclear STAT1 levels induced by IFNγ, suggesting that mTOR might regulate nuclear STAT1 accumulation by a different or parallel mechanism to IFNγ signalling. Consistently, separate experiments, whereby fluorescence-tagged STAT1 constructs were transiently expressed in A549 cells, demonstrated similar rapamycin-sensitivity in terms of regulating STAT1 nuclear levels. Exogenously transfected mammalian cells expressing recombinant ECFP-STAT1 that were treated in the presence of rapamycin showed increased nuclear levels of STAT1 (figure 1B). Together, these results indicate that inhibition of mTOR activity by rapamycin is sufficient to induce nuclear accumulation of STAT1.

Discrete loci in STAT1 are required for sensitivity to mTOR. To define how mTOR regulates STAT1 localization mechanistically, fluorescence-tagged STAT1 constructs were generated. ECFP-STAT1 constructs exhibiting point mutations in various loci of

interest were expressed in A549 cells and the ability of rapamycin or IFNγ to induce nuclear localization of these fluorescence-tagged STAT1 mutants was assessed. The STAT1 S727 residue has been described as being required for the maximal expression of STAT1-dependent genes, as well as required for the efficient clearance of dephosphorylated STAT1 from the nucleus. Expression of the STAT1 S727A mutant did not markedly affect nuclear STAT1 levels, although it increased nuclear STAT1 slightly under both basal and rapamycin-treated conditions (figure 2A). Expression of the fluorescence-tagged STAT1 Y701F mutant, which is unable to become phosphorylated and undergo dimerization events in response to IFNγ, expectedly showed a lack of sensitivity in response to IFNγ treatment (figure 2B). However, expression of this mutant was unable to block the effect of rapamycin on nuclear STAT1 levels, indicating that STAT1 homo-dimerization events may not be required for rapamycin-induced promotion of STAT1 nuclear accumulation. In the context of IFNγ signalling, STAT1 dimerization is followed by nuclear import that is mediated by karyopherin proteins. This import is dependent on the L407 residue, which enables the proper ionic interaction between STAT1 and importin α5. To assess whether this L407 residue was required for the promotory effect of rapamycin on STAT1 nuclear accumulation, fluorescence-tagged STAT1 L407A was expressed in mammalian cells. As expected, expression of the STAT1 L407A mutant blocked the ability of IFNγ to induce nuclear import of STAT1 (figure 2C). As well, expression of this L407A mutant blocked the ability of rapamycin to increase nuclear STAT1 levels, indicating that an association between STAT1 and importin α5, mediated through the L407 residue, is required for the effect of rapamycin.

PP2A mediates mTOR suppression of STAT1 nuclear content. It was hypothesized that the ability of mTOR to regulate STAT1 nuclear levels is mediated by the protein phosphatase PP2A. To determine whether PP2A, in the context of regulating STAT1 localization, acts downstream of mTOR, PP2A mutants were generated. These mutants were subsequently co-expressed in A549 cells with fluorescence-tagged STAT1 and the subcellular localization of STAT1 was determined. Expression of a constitutively active PP2A mutant, Y307F, blocked the ability of rapamycin to induce nuclear STAT1 accumulation, as compared to empty vector controls (figure 3A). Conversely, overexpression of a dominantly negative acting form of PP2A, L199P, was found to mimic the effect of rapamycin under baseline conditions. Expression of PP2A L199P alone was sufficient to increase nuclear STAT1 levels, thus indicating that the nuclear accumulation of STAT1 in response to inhibition of mTOR by rapamycin is at least partly dependent on the activity of the phosphatase PP2A (figure 3B).

mTOR regulates the cellular localization of a STAT1-PKCδ-containing complex. Previous work has suggested that mTOR associates with STAT1 and its kinase PKCδ in a macromolecular protein complex. We hypothesized that mTOR activity may regulate the nuclear localization of a STAT1 and PKCδ-containing complex. Constructs encoding full-length STAT1 and PKCδ tagged with complementary fragments of the fluorescent protein YFP were transiently transfected into A549 cells. Co-expression of these two recombinant proteins was sufficient to reconstitute YFP fluorescence activity under basal conditions, indicating that the two proteins were interacting (figure 4). Under unstimulated conditions, this association was found to be primarily cytoplasmic. Treatment of cells with the mTOR inhibitor rapamycin was able to induce nuclear

accumulation of the reconstituted YFP signal, indicating that inhibition of mTOR was sufficient to increase nuclear levels of the STAT1-PKCδ-containing complex.

Importin α5 demonstrates significant homology to other alpha-karyopherins in the cargo-binding domain. Previous studies have demonstrated that phosphorylation and acetylation events of α karyopherin proteins are required for their activation and recognition of their specific cargo. Importin α1, an adaptor protein involved in nuclear import, has been shown to become phosphorylated by AMPK on its S105 site and that mutation of this site inhibits the ability of importin α1 to mediate the nuclear import of its cargo HuR[111]. We hypothesized that inhibition of mTOR by rapamycin directly or indirectly mediates the nuclear accumulation of STAT1 by regulating the phosphorylation status of this serine site. As we had implicated a distinct alpha karyopherin protein, importin α5, as being involved in the nuclear import process of STAT1, we wished to determine whether a homologous site to S105 existed in importin α5. We performed an *in silico* sequence alignment experiment using the ClustalW2 web-based multiple sequence alignment utility on importin α1, importin α2, importin α3, importin α4, importin α5, importin α7, and importin α8. Multiple sequence alignment of these proteins found significant homology between all the importins, and, most importantly, found that the S105 site of importin α1 aligned with the S111 site of importin α5.

The importin α5 S111 site is required for the effect of rapamycin on nuclear STAT1 levels. We wished to determine the relevance of the importin α5 S111 site in regulating nuclear STAT1 levels. In order to determine whether the S111 site was required for the effect of rapamycin on STAT1 trafficking, importin α5 S111A mutants were co-

expressed with fluorescence tagged STAT1 in COS7 cells. In control transfectants, cells treated with rapamycin exhibited increased STAT1 nuclear staining (figure 5A). Co-expression of ECFP-STAT1 with wild-type importin α5 not only increased nuclear STAT1 levels in response to rapamycin treatment, but simulated the effect of rapamycin under basal conditions (figure 5B). Conversely, visualization of cells dually expressing ECFP-STAT1 and importin α5 S111A constructs showed that STAT1 localized primarily to the cytoplasm in unstimulated cells (figure 5C). When cells were treated with rapamycin, however, no increase in nuclear STAT1 accumulation was observed. The lack of sensitivity of the importin α5 S111A-expressing cells to rapamycin indicates that the S111 residue is required for the effect of rapamycin on STAT1 cellular localization.

mTOR activity mediates the association of mTOR with importin α5 in a PP2A-dependent manner. To further elucidate the mechanism of STAT1 nuclear accumulation in response to mTOR inactivation by rapamycin, protein complex immunoprecipitation experiments were performed. 293T cells were treated with rapamycin for 30 minutes before lysis in non-denaturing buffer. Endogenous mTOR was immunoprecipitated from cell lysates and immunoprecipitates were separated by SDS-PAGE. Following transfer, samples were analyzed by Western blotting. STAT1 was found to associate with mTOR constitutively at baseline, and rapamycin treatment did not significantly affect this association. mTOR was not found to associate significantly with importin α5 under basal conditions, although rapamycin treatment was able to induce an interaction between the two proteins (figure 6A). This association was found to be maximal at 30 minutes post rapamycin treatment. To clarify the role of PP2A in regulating mTOR-dependent STAT1 localization, further protein complex immunoprecipitation studies were performed. 293T

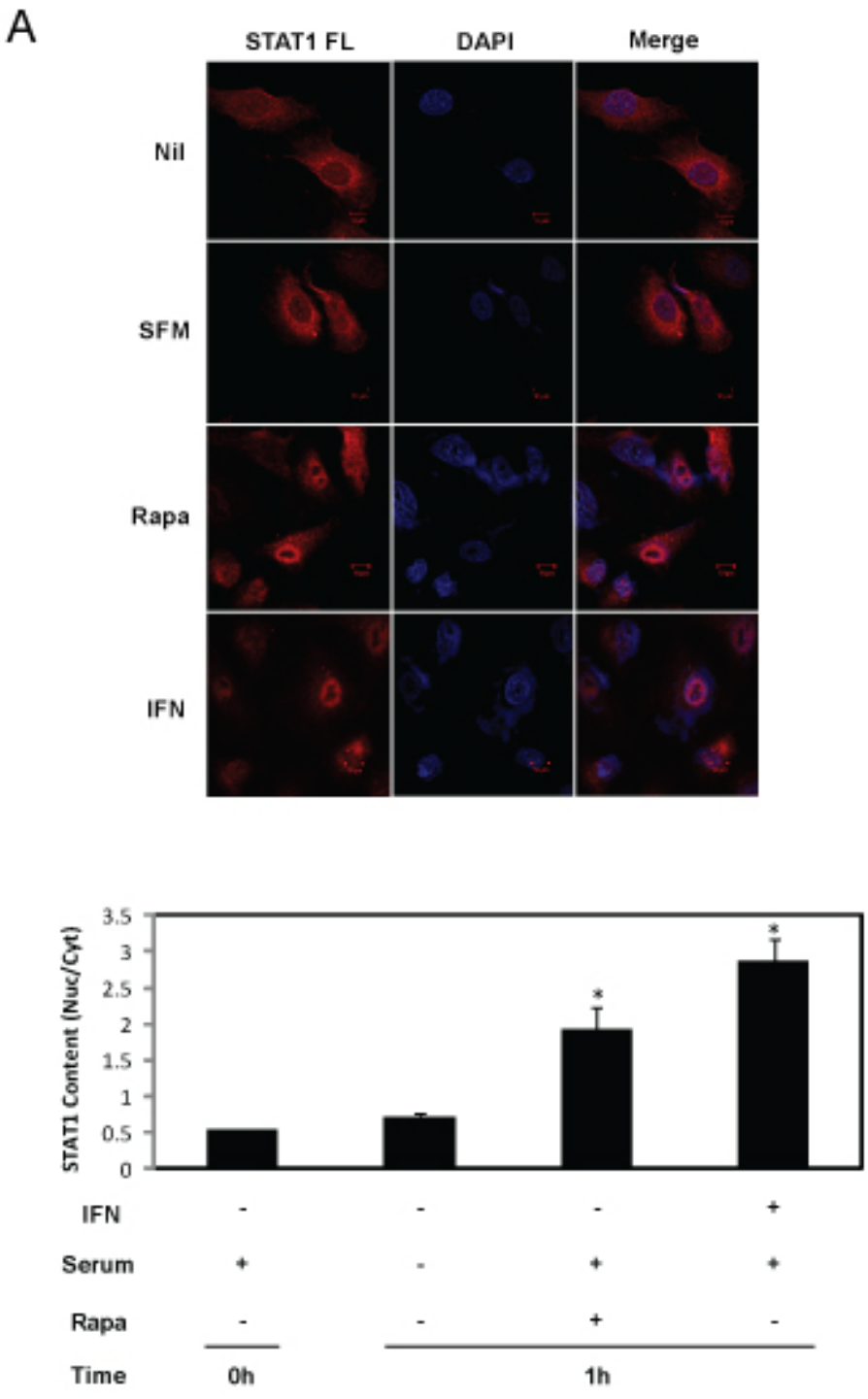
cells were pre-treated in the presence or absence of the phosphatase inhibitor okadaic acid for 1 hour, followed by treatment with rapamycin for an additional hour. Cells were then lysed in non-denaturing buffer. Endogenous mTOR was immunoprecipitated from cell lysates and immunoprecipitates were separated by SDS-PAGE. Following transfer, samples were analyzed by Western blotting. As expected, rapamycin treatment caused mTOR to associate with importin α5 in the absence of okadaic acid (figure 6B). Pre-treatment with okadaic acid blocked the ability of rapamycin to induce an association between mTOR and importin α5, thus indicating that the effect of mTOR inhibition by rapamycin on nuclear STAT1 levels is mediated by the activity of the phosphatase PP2A.

mTOR-dependent nuclear accumulation of STAT1 does not increase apoptotic cell death. In order to determine a functional role in rapamycin-induced nuclear STAT1 accumulation, the pro-apoptotic agent doxorubicin was used. Doxorubicin is a known STAT1-dependent potentiator of apoptosis[112]. We hypothesized that mTOR, as a potential regulator of STAT1 nuclear content, may modulate the expression of pro-apoptotic genes and apoptosis by the same pathway. MDA-MB-231 cells were stimulated in the presence or absence of doxorubicin and/or rapamycin for 24h, and 48h, followed by crystal violet staining to determine the presence of healthy, viable cells. Doxorubicin visibly decreased cell viability, as indicated by decreased crystal violet readings (figure 7A). Rapamycin alone was shown to have a similar, but lessened effect on cell viability. Treatment of cells with both doxorubicin and rapamycin did not incrementally decrease cell viability as measured by the crystal violet assay. Detection of cleaved caspase-3 levels in lysates (marker of apoptotic cell death) indicated high caspase-3 cleavage in doxorubicin treated samples as compared to untreated controls (figure 7B). Rapamycin alone did not increase

caspase-3 cleavage. When added with doxorubicin, rapamycin did not augment, but dampened the effect of doxorubicin on apoptosis. Overall, rapamycin blocked doxorubicin-induced apoptosis as seen with decreased cleavage of caspase-3, but did not block the effect of doxorubicin on cell viability (crystal violet staining). We then hypothesized that because rapamycin was unable to induce apoptosis, that the effect of mTOR on cell physiology may be on the ability of the cell to proliferate rather than undergo apoptotic cell death. We looked at the ability of doxorubicin and rapamycin to affect the expression of the cyclin-dependent kinase inhibitor protein p27. Treatment of cells with doxorubicin at 24h increased p27 levels significantly (figure 8). However, pre-treatment with rapamycin decreased the effect of doxorubicin on p27 expression levels. Rapamycin alone was found to have no effect on p27 expression at 24h, indicating that the effect of rapamycin on inhibiting cell growth does not occur through the p27 cell cycle inhibitor.

Overexpression of wild-type importin α5 reverses the effect of rapamycin on cell proliferation. COS7 cells were transiently transfected with empty vector or wild-type importin α5 followed by stimulation in the presence or absence of rapamycin for 24h. Cells were stained with crystal violet, and read by spectrophotometer to determine cell viability. Rapamycin decreased cell proliferation in empty vector-transfected cells, as expected (figure 9). Overexpression of wild-type importin α5 did not significantly increase the basal proliferation of COS7 cells, but did reverse the ability of rapamycin to block proliferation at 24h.

Figure 1A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*
*
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Serum + - + +
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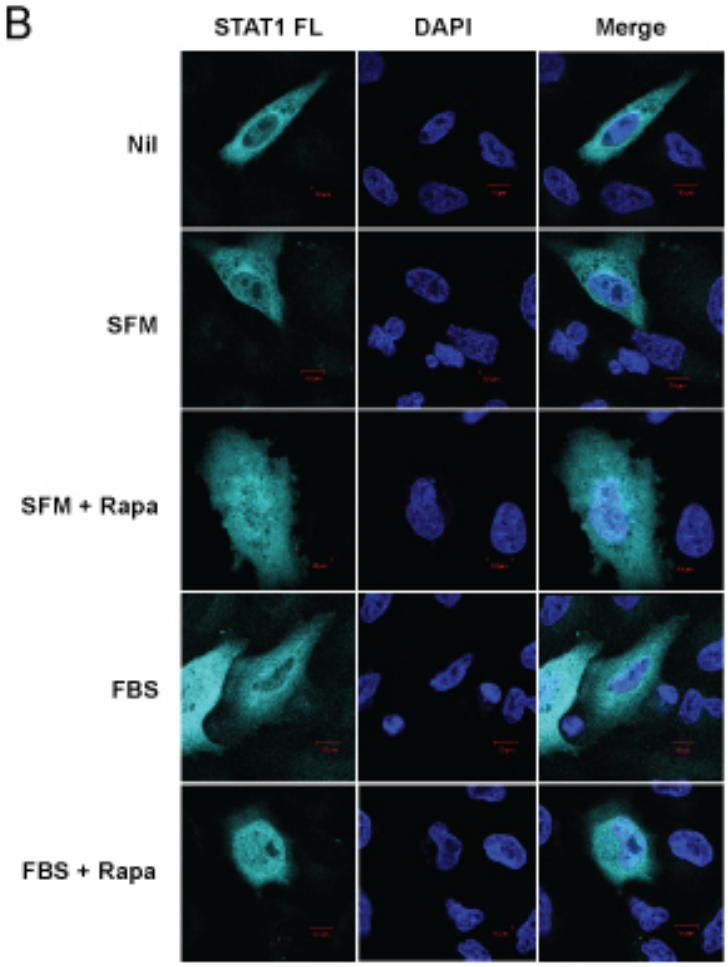
Time 0h 1h

Figure 1B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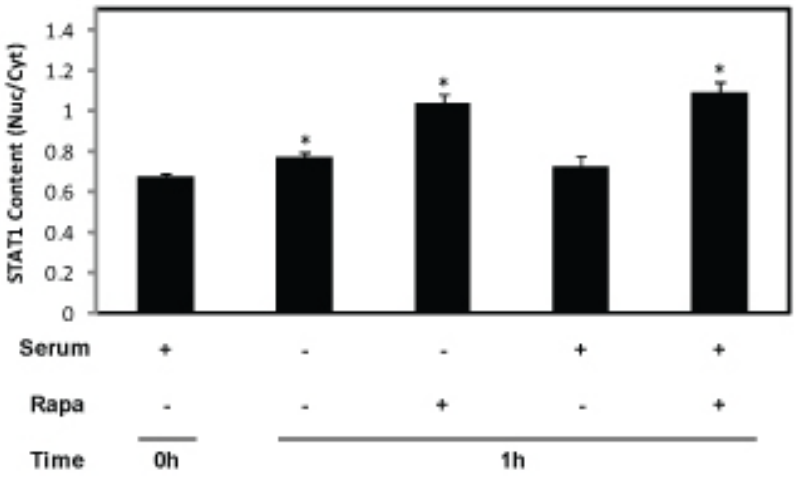
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0.4
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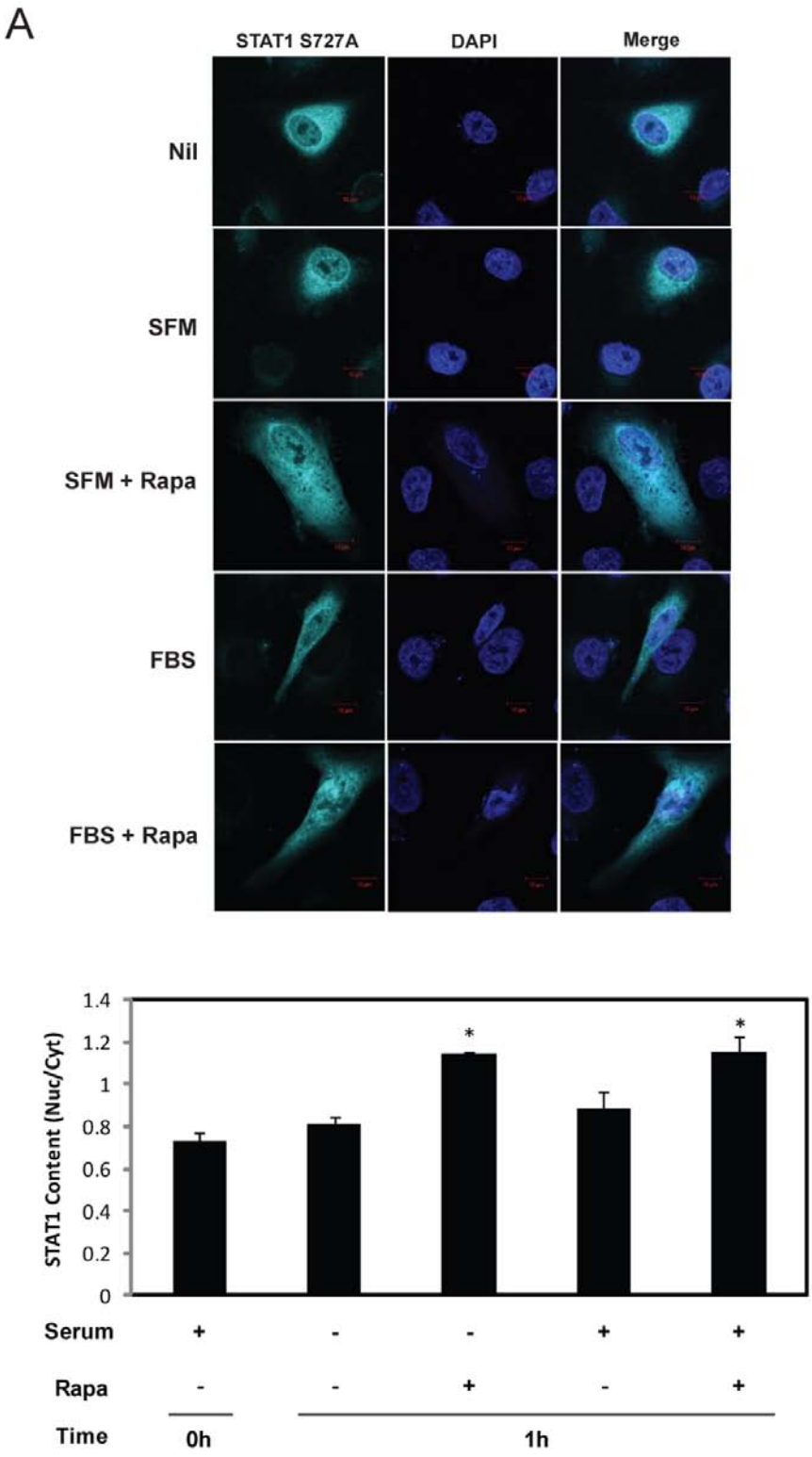
Time 0h 1h

Figure 1: Regulation of STAT1 cellular localization by mTOR

A549 cells were A. endogenously immunostained against STAT1, or B. transiently transfected with fluorescence-tagged STAT1. Cells were incubated in serum-free media (SFM), 10% serum media in the presence of rapamycin (50ng/mL) for 60 minutes, or IFNγ for 30 minutes followed by detection by fluorescence confocal microscopy. Slides were mounted using media containing the nuclear marker DAPI (blue). STAT1 localization is indicated in cyan. Summarized data (mean nuclear to cytoplasmic density ratio $\pm$ SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 2A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DAPI
Merge
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SFM
SFM + Rapa
FBS
FBS + Rapa
STAT1 Content (Nuc/Cyt)
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1
0.8
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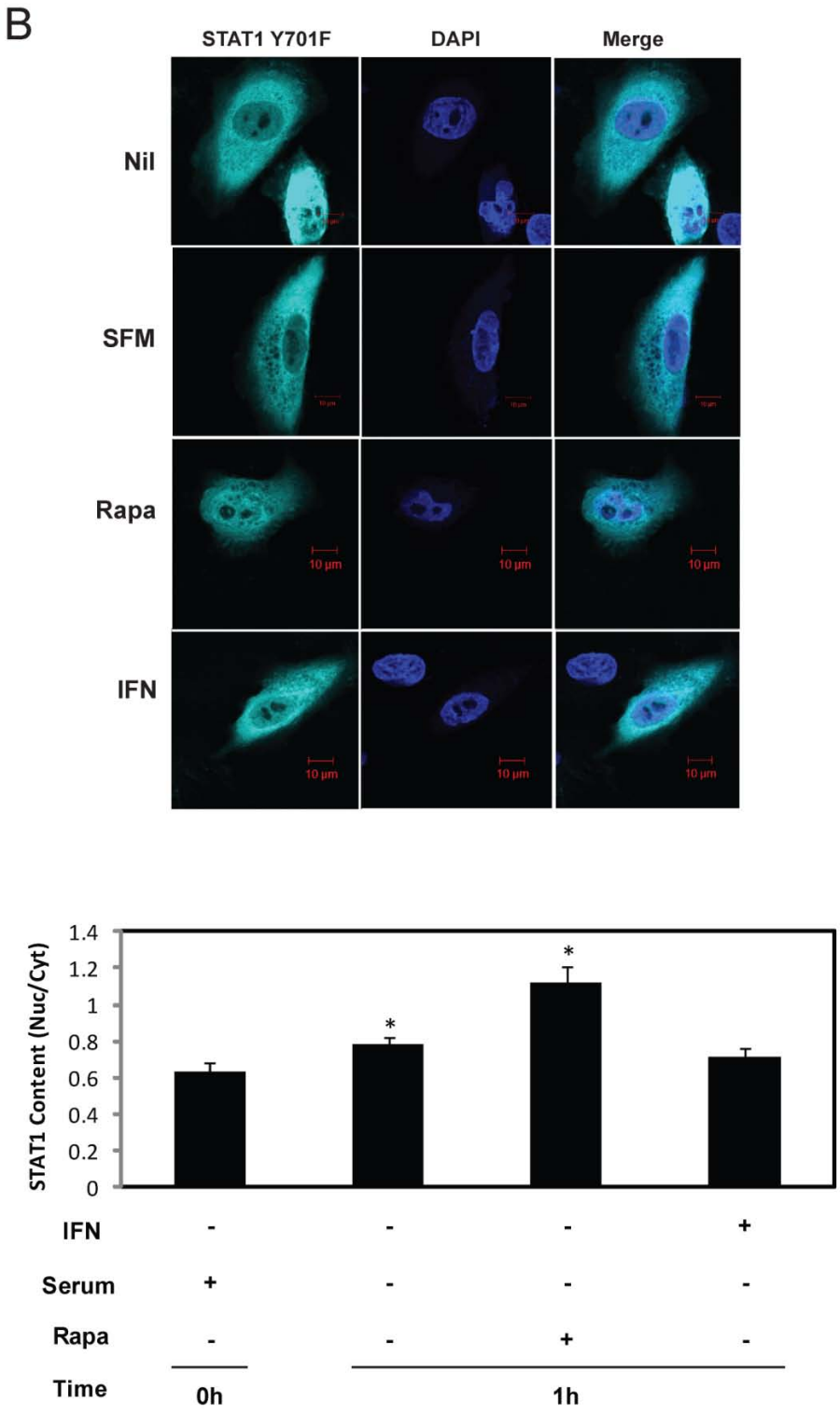
Serum + - - + +
Rapa - - + - +
Time 0h 1h

Figure 2B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0.4
0.2
0
*
*
IFN - - - +
Serum + - - -
Rapa - - + -
Time 0h 1h

Figure 2C

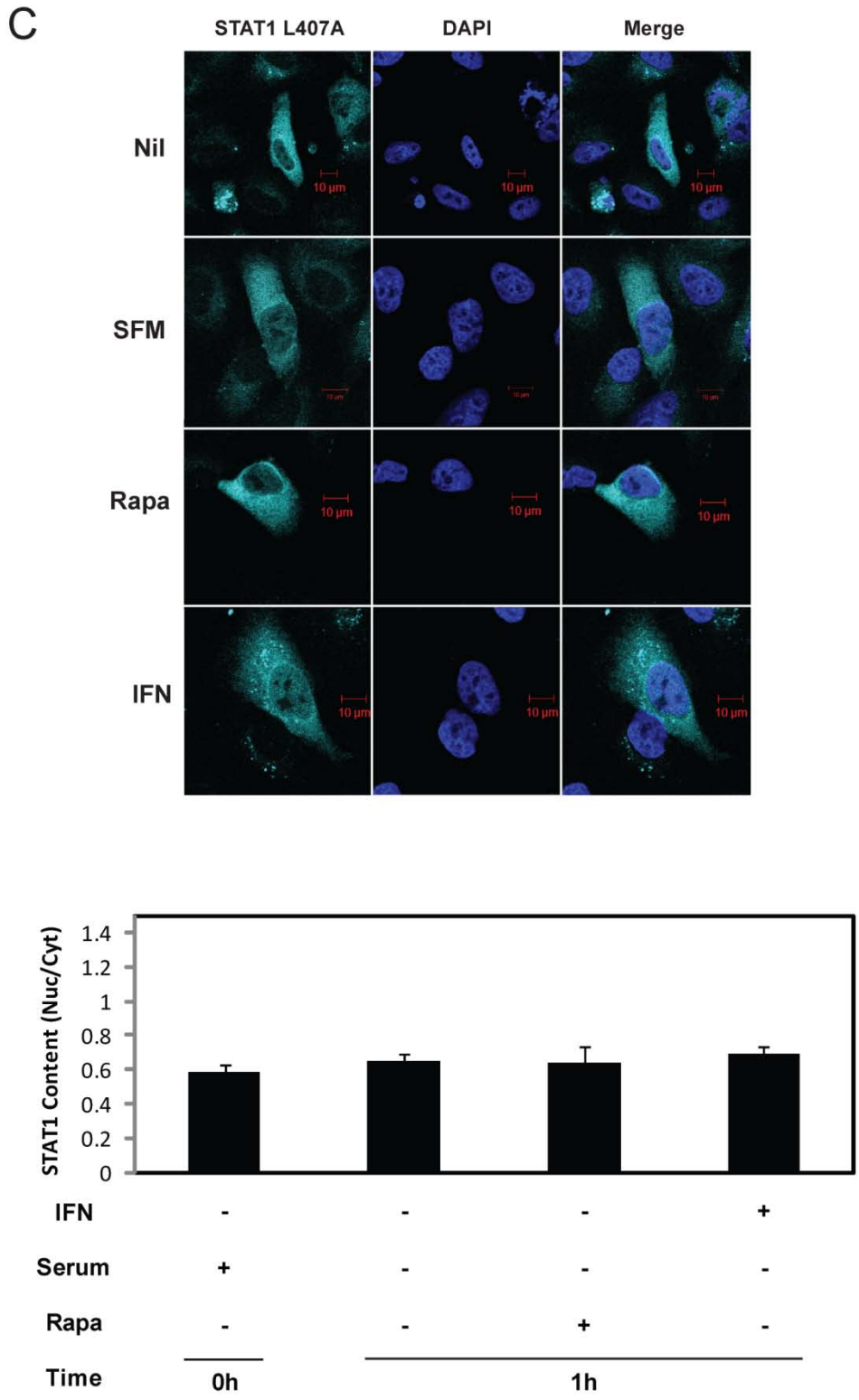

C
STAT1 L407A
DAPI
Merge
Nil
SFM
Rapa
IFN
10 µm
STAT1 Content (Nuc/Cyt)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IFN - - - +
Serum + - - -
Rapa - - + -
Time 0h 1h

Figure 2: Effect of STAT1 mutation on mTOR-dependent STAT1 cellular localization

A549 cells were A. transiently transfected with fluorescence-tagged STAT1 S727A, B. transiently transfected with fluorescence-tagged STAT1 Y701F, or C. transiently transfected with fluorescence-tagged STAT1 L470A. Cells were incubated in 10% serum media in the presence of rapamycin (50ng/mL) for 60 minutes before detection by fluorescence confocal microscopy. Slides were mounted using media containing the nuclear marker DAPI (blue). STAT1 localization is indicated in cyan. Summarized data (mean nuclear to cytoplasmic density ratio $\pm$ SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 3A

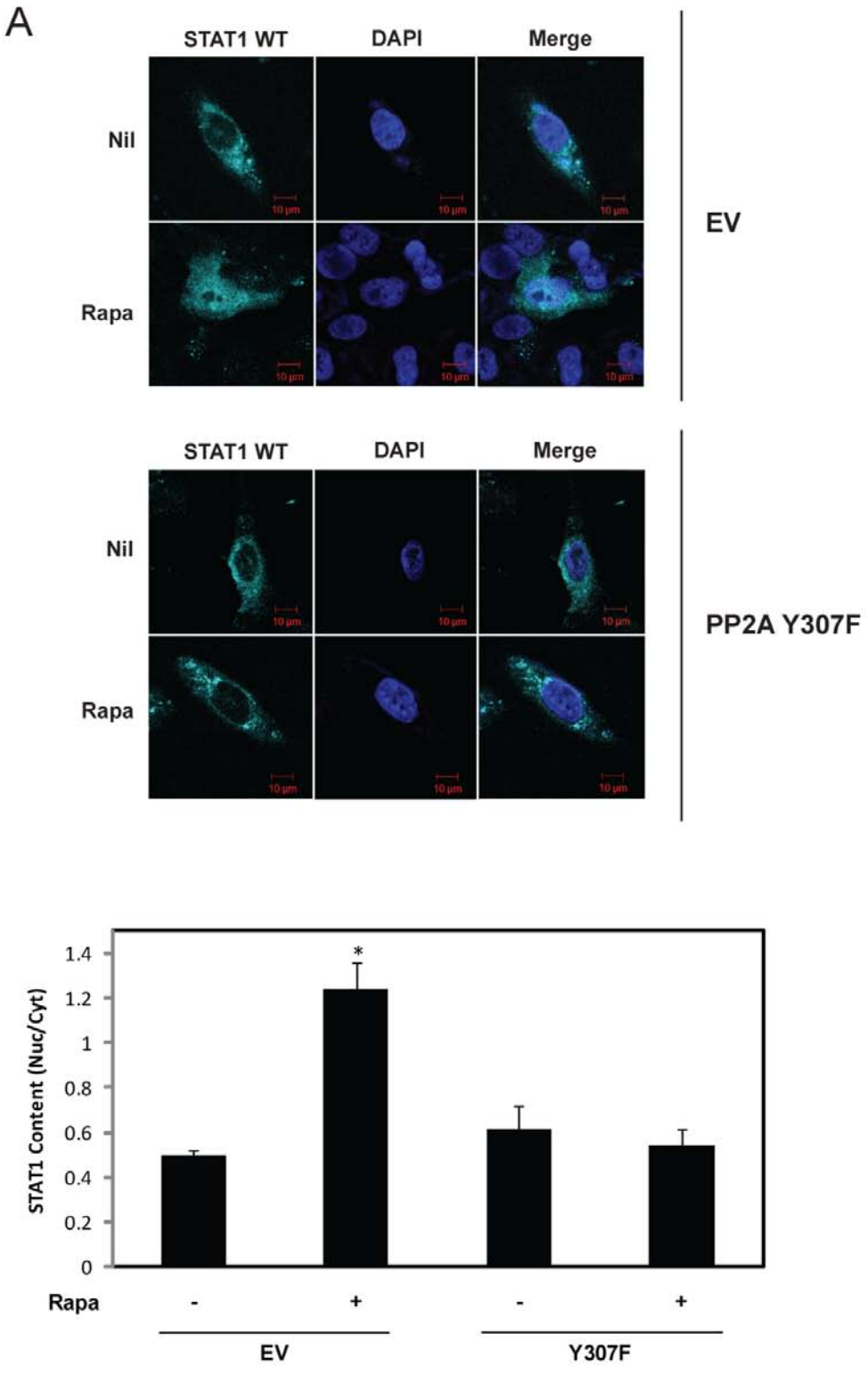
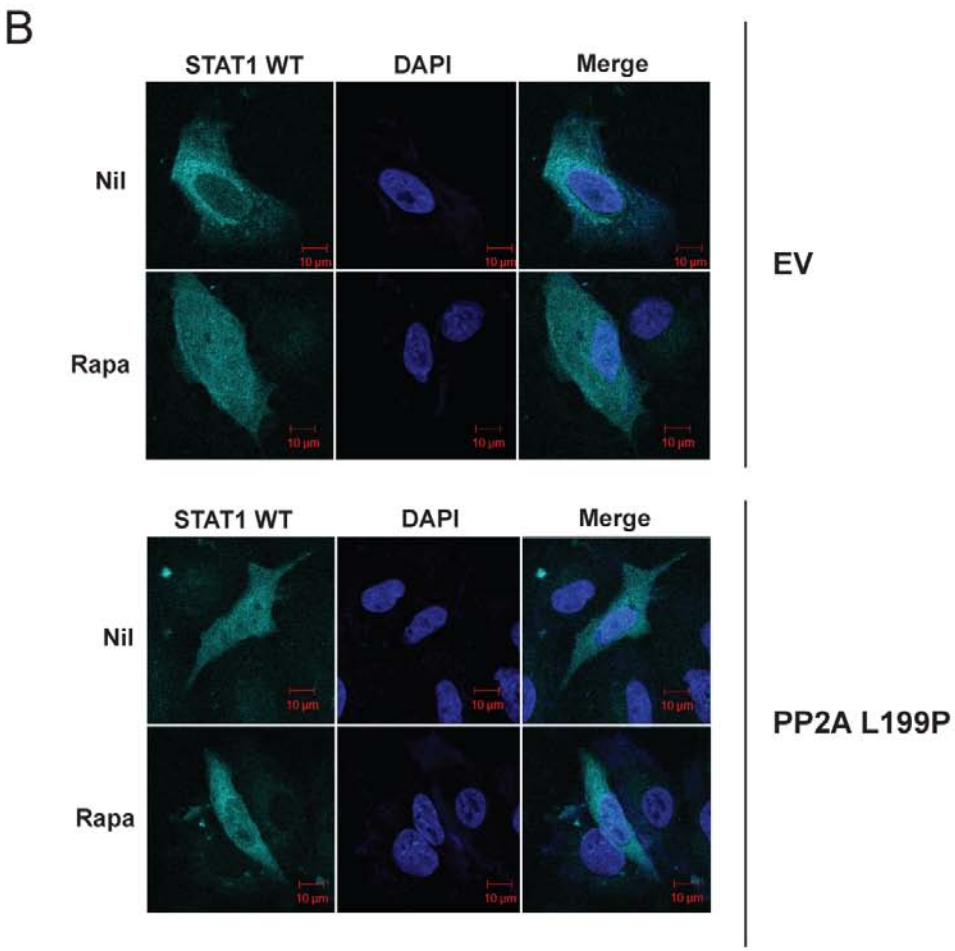

A
STAT1 WT
DAPI
Merge
Nil
Rapa
EV
STAT1 WT
DAPI
Merge
Nil
Rapa
PP2A Y307F
STAT1 Content (Nuc/Cyt)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Rapa
-
+
-
+
EV
Y307F

Figure 3B


B
STAT1 WT
DAPI
Merge
Nil
Rapa
EV
STAT1 WT
DAPI
Merge
Nil
Rapa
PP2A L199P

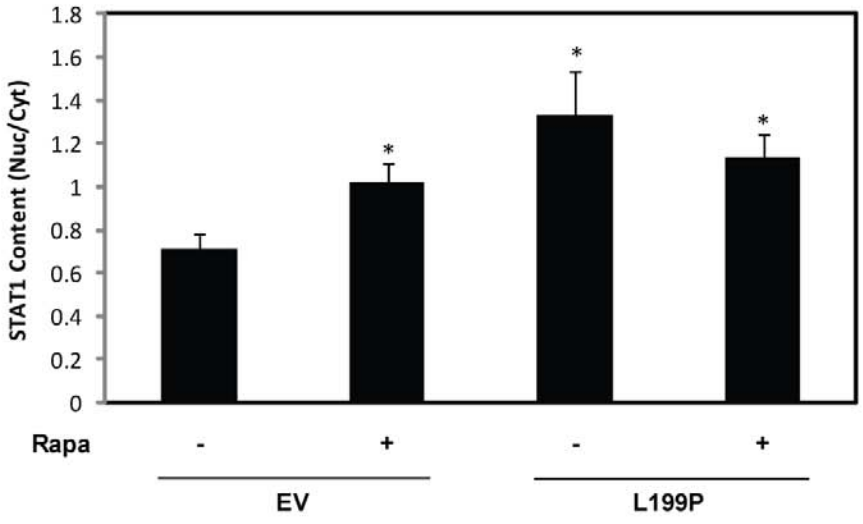
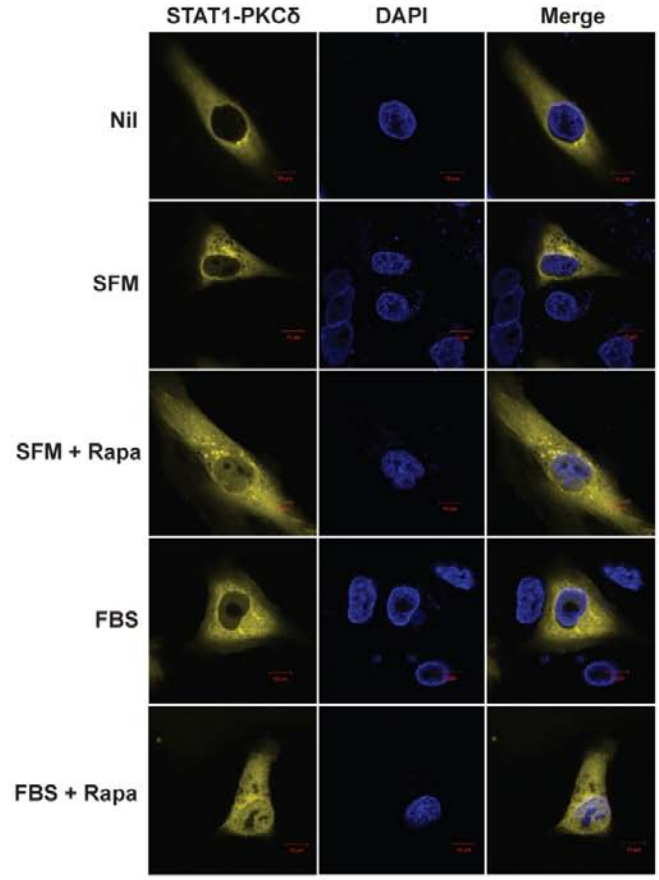

STAT1 Content (Nuc/Cyt)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Rapa
-
+
-
+
EV
L199P

Figure 3: PP2A-dependent regulation of STAT1 localization by mTOR

A549 cells were A. transiently co-transfected with fluorescence-tagged STAT1 and PP2Ac Y307F, B. transiently co-transfected with fluorescence-tagged STAT1 and L199P, along with empty vector (EV) controls. Cells were incubated in 10% serum media in the presence of rapamycin (50ng/mL) for 60 minutes before detection by fluorescence confocal microscopy. Slides were mounted using media containing the nuclear marker DAPI (blue). STAT1 localization is indicated in cyan. Summarized data (mean nuclear to cytoplasmic density ratio $\pm$ SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 4


STAT1-PKCδ
DAPI
Merge
Nil
SFM
SFM + Rapa
FBS
FBS + Rapa

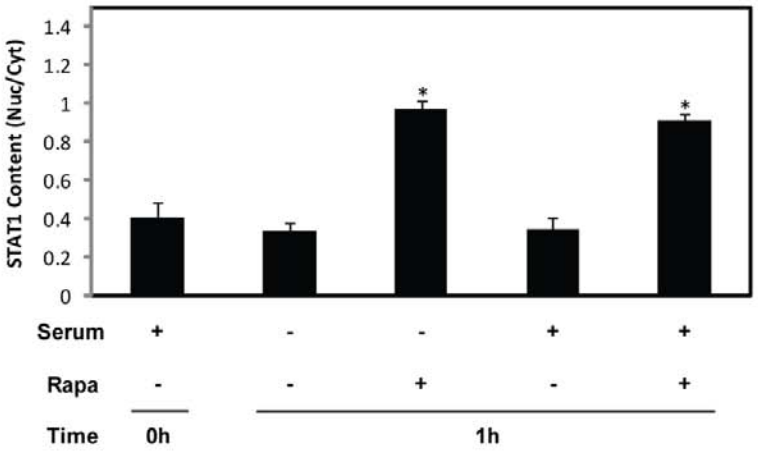

STAT1 Content (Nuc/Cyt)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Serum + - - + +
Rapa - - + - +
Time 0h 1h

Figure 4: mTOR-dependent localization of STAT1-containing heterodimer

A549 cells were transiently co-transfected with STAT1 and PKCδ constructs tagged with complementary fragments of the YFP gene. Cells were incubated in 10% serum media in the presence of rapamycin (50ng/mL) for 60 minutes before detection by fluorescence confocal microscopy. Slides were mounted using media containing the nuclear marker DAPI (blue). Co-localization of STAT and PKCδ is indicated in yellow. Summarized data (mean nuclear to cytoplasmic density ratio $\pm$ SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 5A

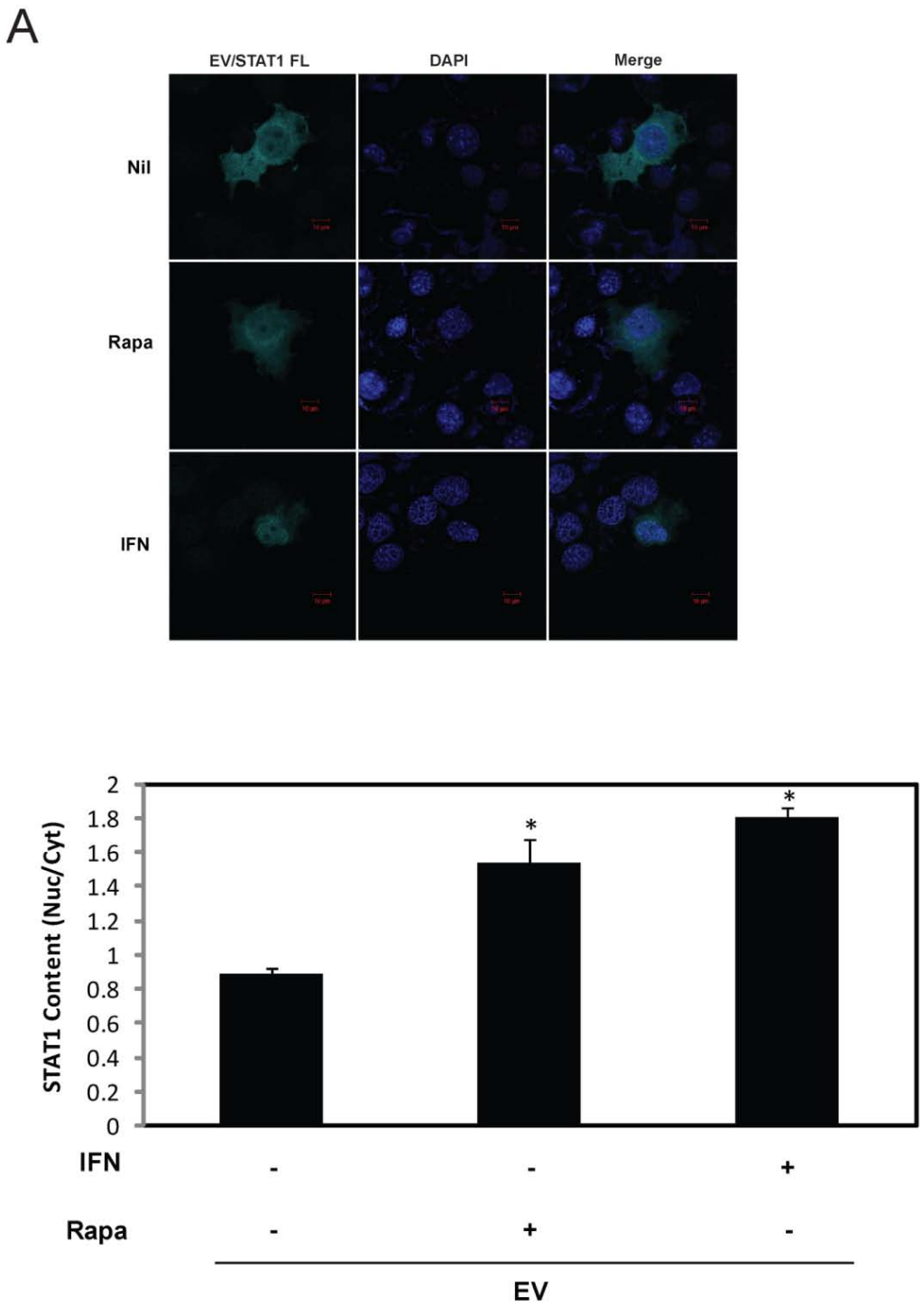

A
EV/STAT1 FL
DAPI
Merge
Nil
Rapa
IFN
STAT1 Content (Nuc/Cyt)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
IFN
-
-
+
Rapa
-
+
-
EV

Figure 5B

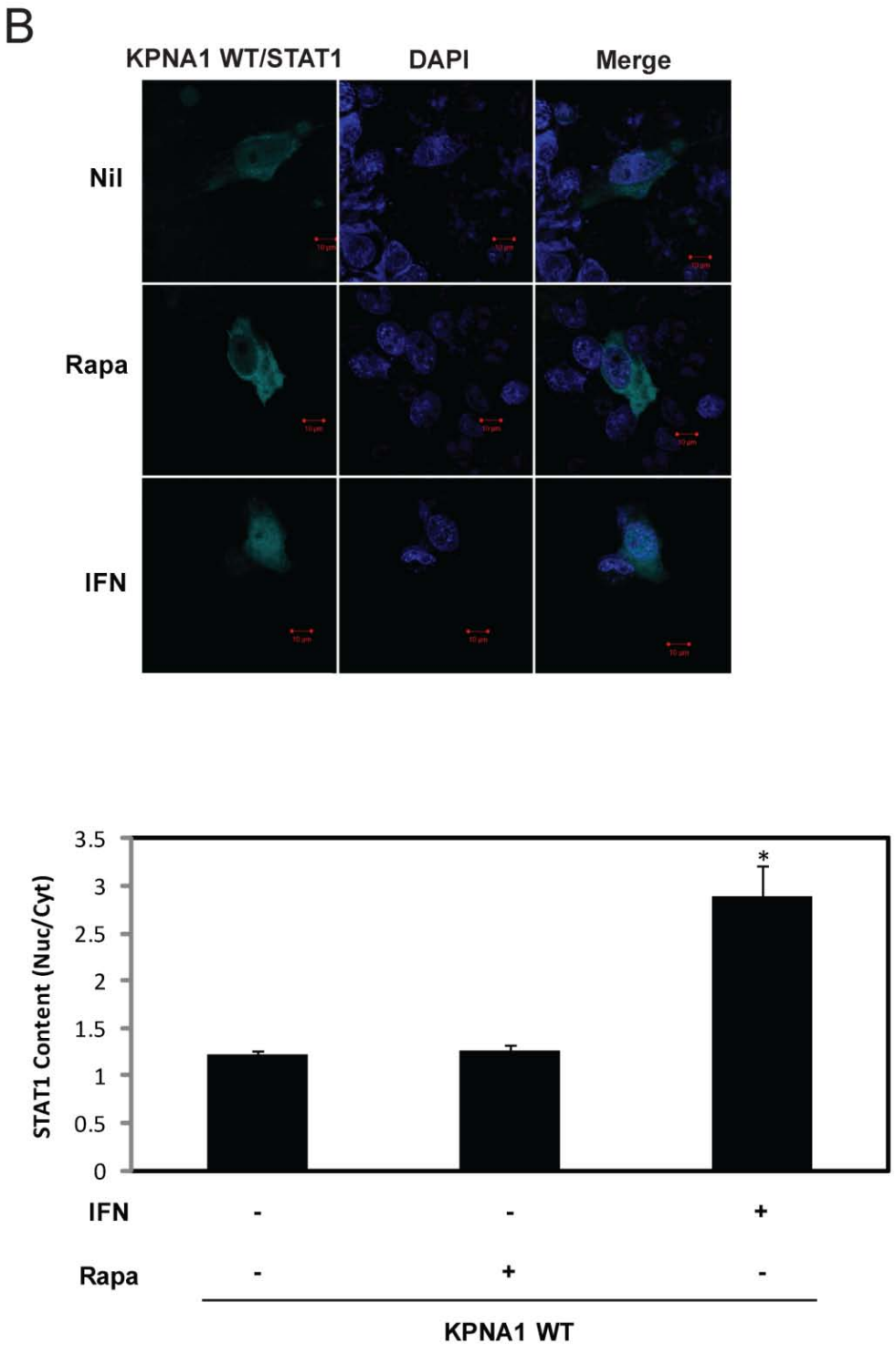

B
KPNA1 WT/STAT1
DAPI
Merge
Nil
Rapa
IFN
STAT1 Content (Nuc/Cyt)
3.5
3
2.5
2
1.5
1
0.5
0
*
IFN
-
-
+
Rapa
-
+
-
KPNA1 WT

Figure 5C

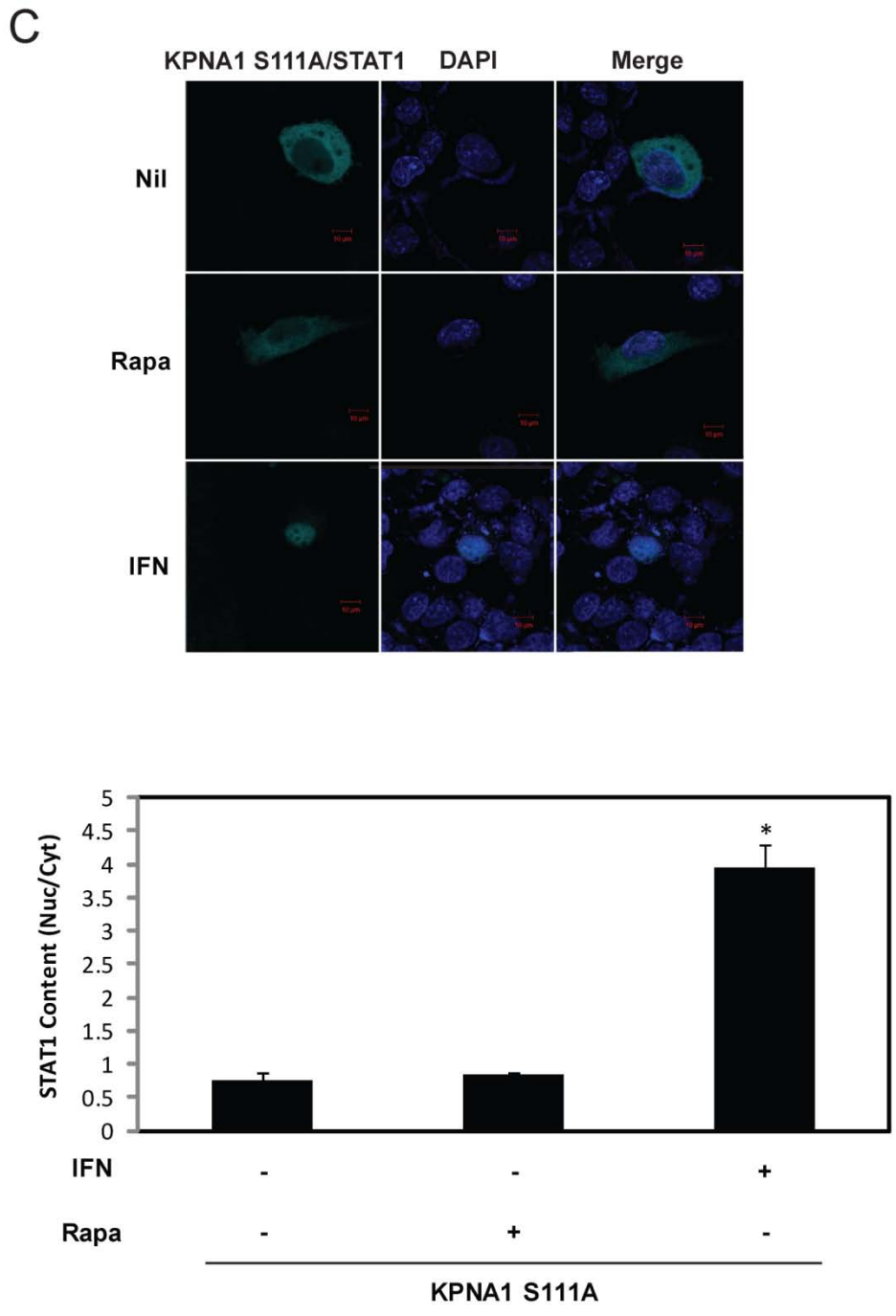

C
KPNA1 S111A/STAT1
DAPI
Merge
Nil
Rapa
IFN
STAT1 Content (Nuc/Cyt)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
IFN
-
-
+
Rapa
-
+
-
KPNA1 S111A

Figure 5: Importin α5 site-dependent regulation of STAT1 nuclear localization

COS7 cells were transiently transfected with ECFP-STAT1 and A. empty vector, B. KPNA1 WT, or C. KPNA1 S111A. Cells were incubated in the presence or absence of rapamycin (50ng/mL) for 1 hour in 10% serum media, or IFNγ (100U/mL) for 30 minutes before visualization by fluorescence confocal microscopy. Slides were mounted using media containing the nuclear marker DAPI (blue). STAT1 localization is indicated in yellow. Summarized data (mean nuclear to cytoplasmic density ratio, $\pm$ SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 6A

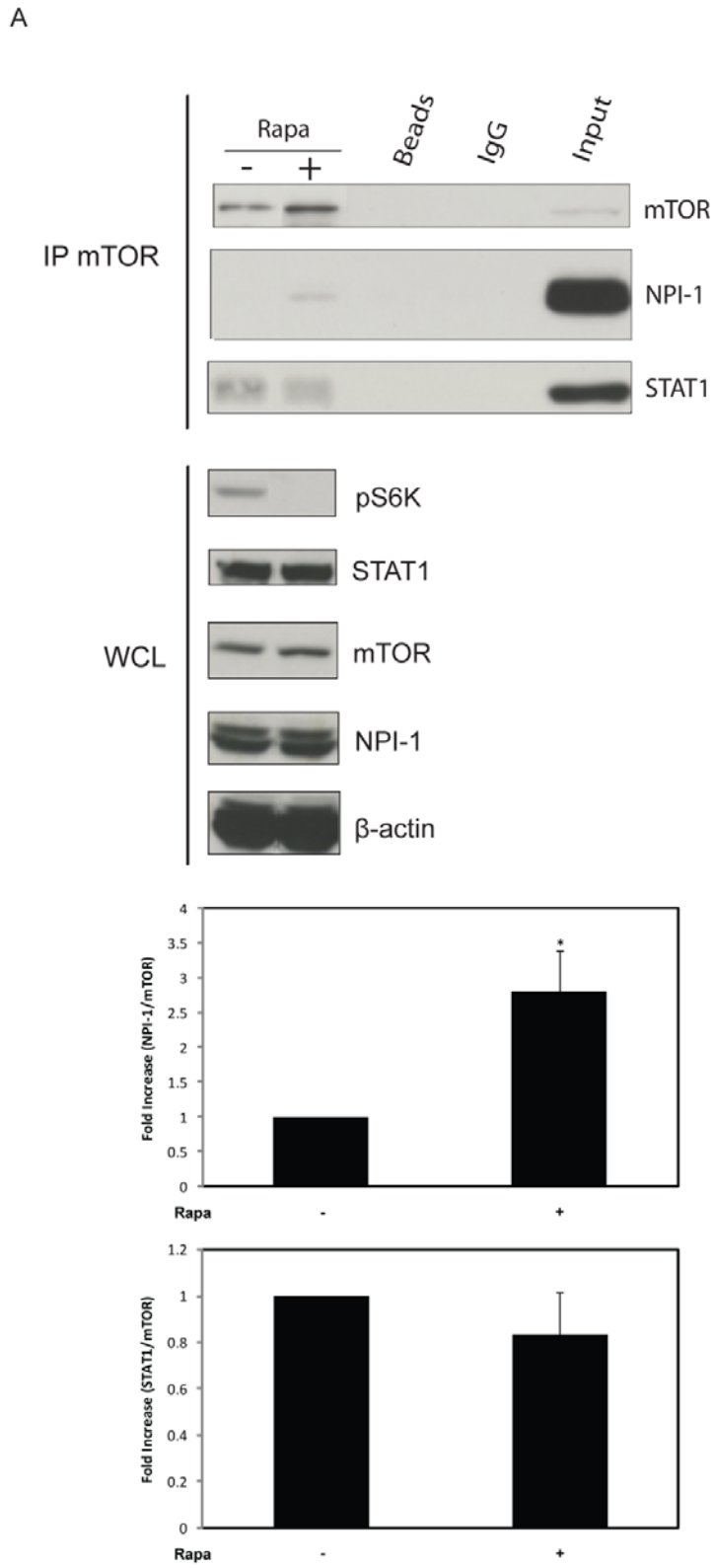

A
Rapa
-
+
Beads
IgG
Input
IP mTOR
mTOR
NPI-1
STAT1
WCL
pS6K
STAT1
mTOR
NPI-1
β-actin
Fold Increase (NPI-1/mTOR)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Rapa
-
+
Fold Increase (STAT1/mTOR)
1.2
1
0.8
0.6
0.4
0.2
0
Rapa
-
+

Figure 6B

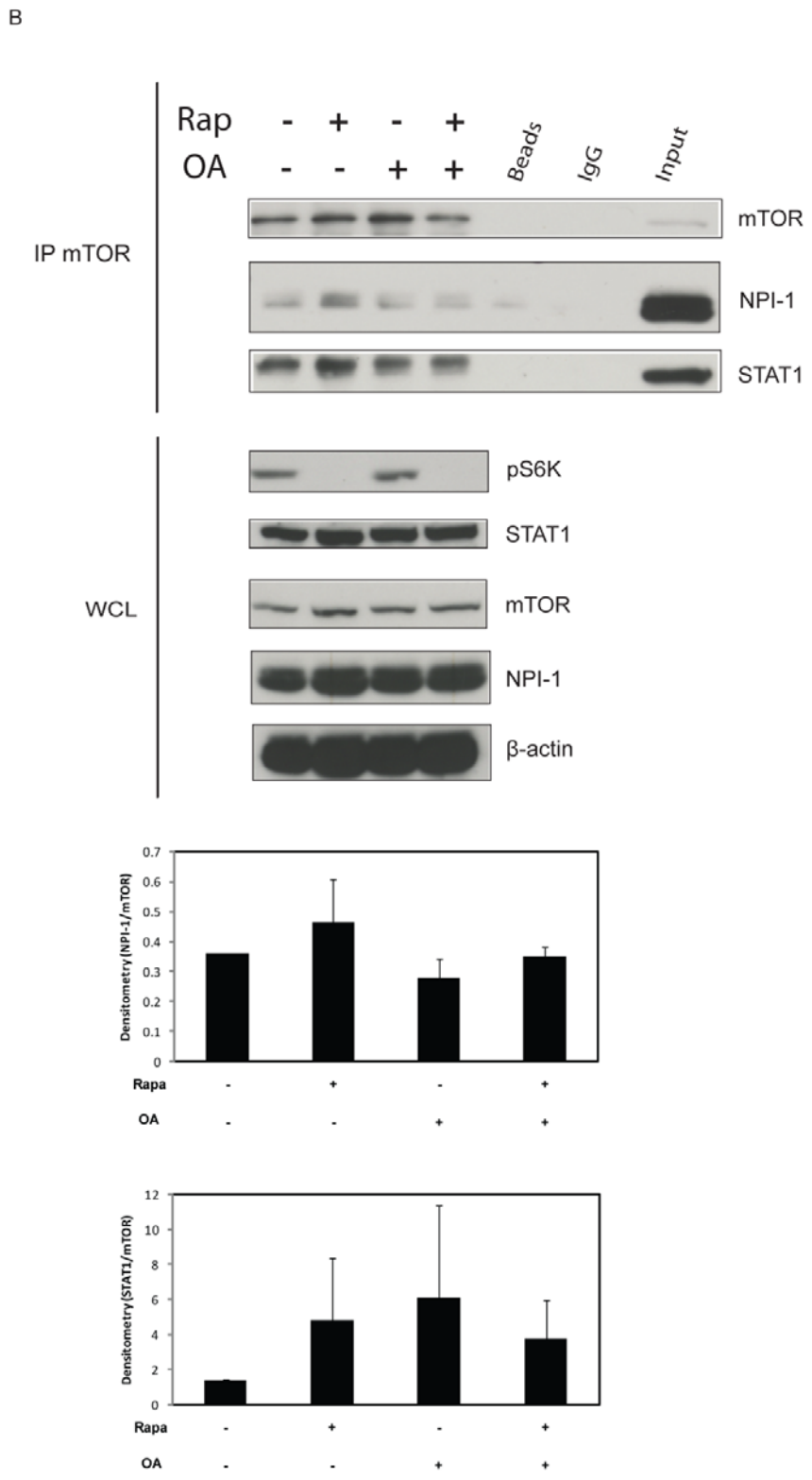
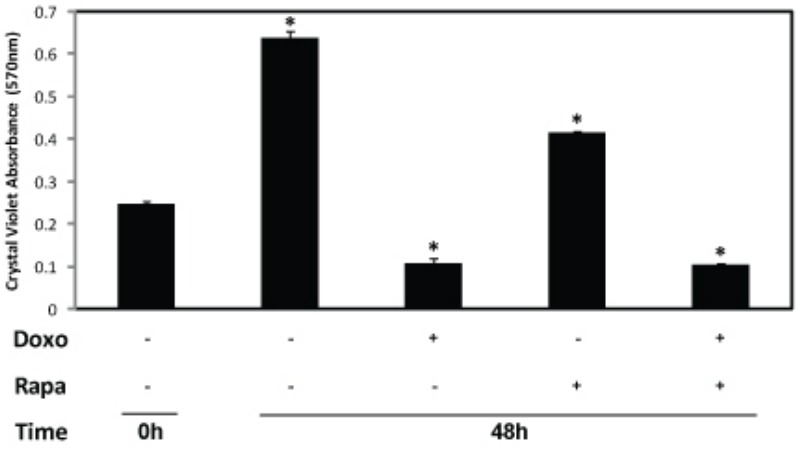

B
Rap - + - +
OA - - + +
Beads
IgG
Input
IP mTOR
mTOR
NPI-1
STAT1
WCL
pS6K
STAT1
mTOR
NPI-1
β-actin
Densitometry (NPI-1/mTOR)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Rapa - + - +
OA - - + +
Densitometry (STAT1/mTOR)
12
10
8
6
4
2
0
Rapa - + - +
OA - - + +

Figure 6: The effect of mTOR-inhibition on mTOR-importin α5 association

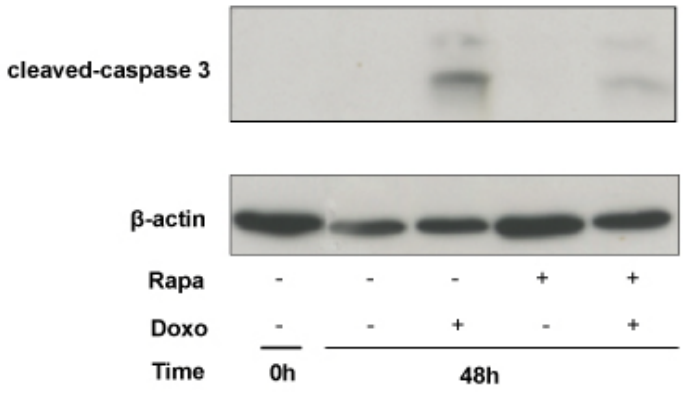
HEK 293T cells were A. incubated in 10% serum media in the presence or absence of rapamycin (50ng/mL) for 30 minutes, B. incubated in the presence or absence of the phosphatase inhibitor PP2A (50nM) for 1 hour before incubation in 10% serum media with rapamycin (50ng/mL) for 30 minutes. Proteins in whole cell lysates, 2mg, were immunoprecipitated with 5ug of anti-mTOR antibody. Proteins immunoprecipitated from lysates were separated by SDS-PAGE and detected by Western blot analysis. Data shown are representative of 3 independent experiments (densitometry measurements, $\pm$ SEM). *$p < 0.05$ vs. control.

Figure 7

A


Crystal Violet Absorbance (570nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
Doxo - - + - +
Rapa - - - + +
Time 0h 48h

B


cleaved-caspase 3
β-actin
Rapa - - - + +
Doxo - - + - +
Time 0h 48h

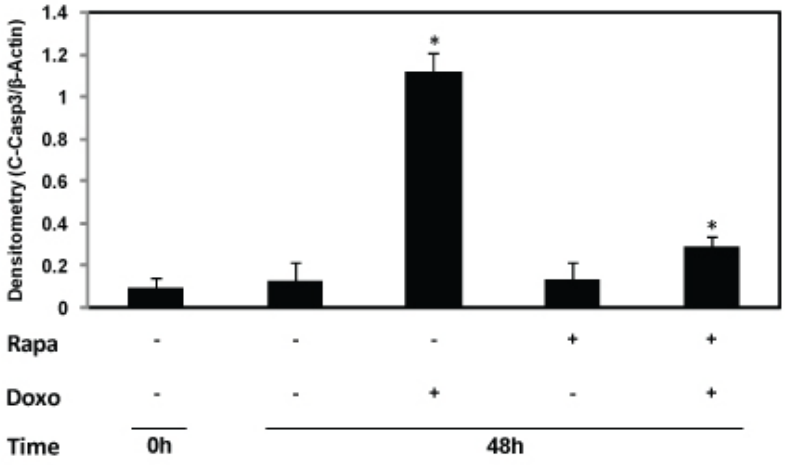

Densitometry (C-Casp3/β-Actin)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Rapa - - - + +
Doxo - - + - +
Time 0h 48h

Figure 7: mTOR regulation of apoptosis and cell death

MDA-MB-231 cells were pre-treated with rapamycin (50ng/mL), followed by treatment with doxorubicin (1μM) in 10% serum media for 48h. Cells were A. stained with crystal violet, or B. lysed in non-denaturing detergent buffer. Proteins in whole cell lysates were separated by SDS-PAGE and detected by Western blot analysis. Crystal violet stained cells were solubilised in 1% SDS followed by measurement of spectrophotometer absorbance at 570nm. Summarized data (densitometry readings and crystal violet absorbances, $\pm$ SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 8

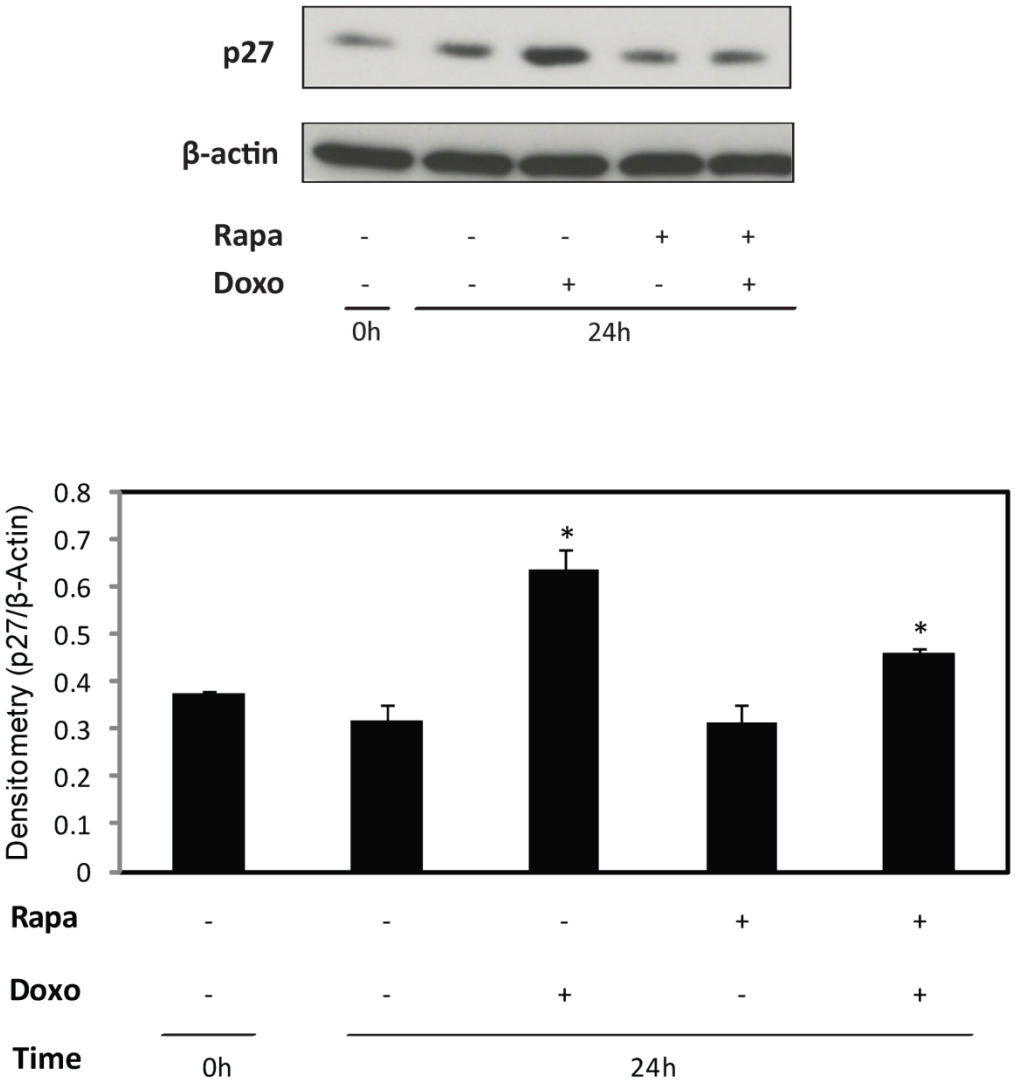

p27
β-actin
Rapa - - - + +
Doxo - - + - +
0h
24h
Densitometry (p27/β-Actin)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Rapa - - - + +
Doxo - - + - +
Time
0h
24h

Figure 8: mTOR regulation of cell proliferation

MDA-MB-231 cells were pre-treated with rapamycin (50ng/mL), followed by treatment with doxorubicin (1μM) in 10% serum media for 24h. Cells were lysed in non-denaturing detergent buffer. Proteins in whole cell lysates were separated by SDS-PAGE and detected by Western blot analysis. Summarized data (densitometry readings, ± SEM) are representative of 3 independent experiments. *$p < 0.05$ vs. control.

Figure 9

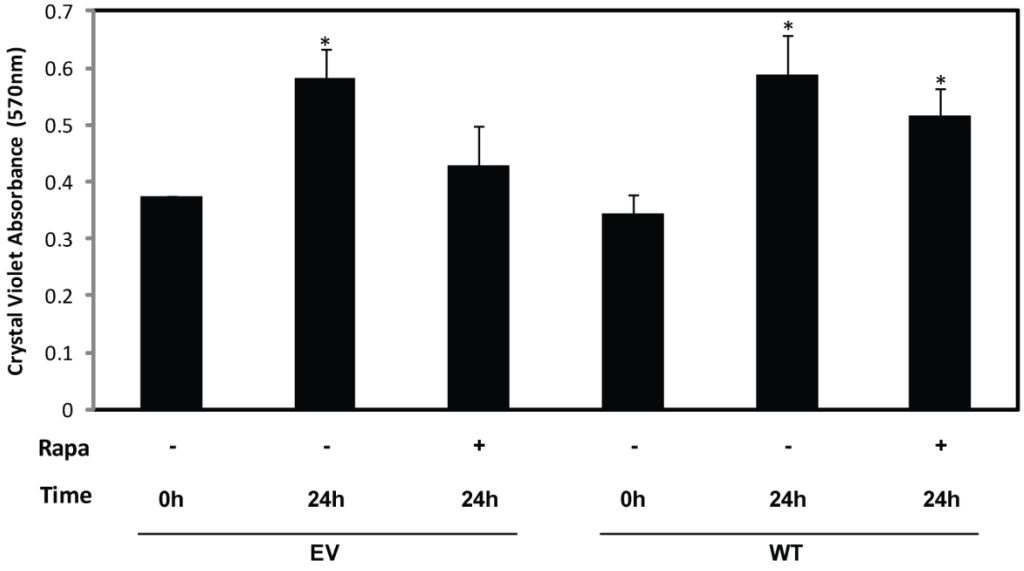

Crystal Violet Absorbance (570nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
Rapa
-
-
+
-
-
+
Time
0h
24h
24h
0h
24h
24h
EV
WT

Figure 9: Effect of importin α5 overexpression on cell proliferation

COS7 cells were transiently transfected with A. EV, or B. KPNA1 WT. Cells were incubated in the presence or absence of rapamycin (50ng/mL) in 10% serum media for 24h. Cells were stained with crystal violet, followed by solubilisation in 1% SDS. Spectrophotometer readings were taken at 570nm. Summarized data (crystal violet absorbances, $\pm$ SEM) are representative of 2 independent experiments. *$p < 0.05$ vs. control.

Discussion

In the absence of cytokine stimulation, STAT1 shuttles constitutively between the cytoplasm and the nucleus[41]. Previous studies of latent STAT1 nucleo-cytoplasmic shuttling have been performed in the context of STAT1 regulation of constitutive gene expression, such as in the case of LMP2[42]. The ability of latent STAT1 to regulate gene expression is indicative that unphosphorylated STAT1 undergoes nuclear import by a mechanism that is distinct to the nuclear import of tyrosine-phosphorylated STAT1. However, the ability of STAT1 to regulate gene expression in its unphosphorylated state is poorly understood. This is due in part to the lack of a canonical nuclear import signal in latent STAT1[41]. Although rapamycin does not induce STAT1 Y701 phosphorylation, it is likely that STAT1 nuclear localization in response to inhibition of mTOR by rapamycin occurs by a separate mechanism.

The objective of this study was to characterize the ability of mTOR to regulate nuclear STAT1 levels, and to understand the mechanisms involved in this process. Here, we showed that inactivation of mTOR, with the inhibitor rapamycin, induces rapid accumulation of STAT1 in the nucleus. We showed that the regulation of STAT1 nuclear content is dependent on specific STAT1 residues. We further demonstrated that inhibition of mTOR induces nuclear accumulation of a STAT1-containing complex that consists of STAT1 and the kinase PKCδ. The idea that tyrosine-phosphorylation independent STAT1 nuclear import still depends on its association with specific nuclear import machinery is consistent with the notion that large proteins cannot pass through the nuclear pore complex alone. We showed that STAT1 S727A only slightly altered the ability of mTOR to regulate STAT1 nuclear levels (figure 2A), but did enhance constitutive STAT1 nuclear levels (figure 1A). This is consistent with previous work that showed that STAT1

S727 mediates the efficient nuclear export of STAT1 proteins from the nucleus, but that it is not completely required for the process[10]. In addition, we showed that STAT1 Y701F blocks the ability of IFNγ to induce STAT1 nuclear accumulation, but that this effect is seen in response to rapamycin (figure 2B). This is consistent with the concept of dimerization-dependent activation of STAT1 in response to Y701 phosphorylation, where dimerization and nuclear import are dependent on the ability of STAT1 to become tyrosine phosphorylated[12]. Rapamycin sensitivity to STAT1 accumulation, despite the Y701 mutation, indicates that homo-dimerization of STAT1 is not required for the constitutive nuclear trafficking of STAT1. In this case, STAT1 dimerization may occur through an alternate hetero-dimerization process, independent of any tyrosine phosphorylation event. Finally, mutation of STAT1 L407 blocked both rapamycin and IFNγ-mediated nuclear import events (figure 2C). Mutation of STAT1 L407 has been shown to block IFNγ-mediated nuclear accumulation, through the masking of a nuclear localization signal that becomes apparent in the homo-dimerization process[12]. However, STAT1 L407 does not correspond to any classical basic NLS, making it puzzling that STAT1 L407A would block rapamycin-induced nuclear accumulation of a STAT1 monomer. It is possible that STAT1 L407A still undergoes a hetero-dimerization process with another protein, which is still able to generate the atypical nuclear localization signal needed for import. This may be elucidated by our finding that STAT1 accumulates in the nucleus in response to rapamycin in association with the kinase PKCδ (figure 4).

We showed here that inhibition of mTOR activity by rapamycin induces physical association between mTOR and importin α5 (figure 5A). This suggests a possible model whereby rapamycin treatment leads to recruitment of importin α5 to an mTOR/STAT1-containing complex, which results in enhanced nuclear trafficking of STAT1. Consistent

with this, the amount of STAT1 associated with mTOR remained constant in mTOR immunoprecipitation experiments. It is possible that mTOR, or another intermediary protein, mediates the interaction of STAT1 with importin α5, and that the translocation of STAT1 to the nucleus in response to rapamycin may be in conjunction with 2 or more proteins. This conjugation may compensate for the lack of an NLS sequence in latent STAT1. However, further studies must be performed to determine the nature of this translocating complex.

We have shown that, similar to yeast, regulation of the cellular localization of the STAT1 transcription factor by mTOR requires the activity of an associated phosphatase (PP2A). We demonstrated that the effects of mTOR on STAT1 localization were mediated by PP2A activity acting downstream of mTOR activity (figure 3). This was further elaborated on through protein-interaction studies that showed that inhibition of mTOR by rapamycin is sufficient to induce association of mTOR with the α karyopherin importin α5, and that inhibition of the associated phosphatase PP2A with okadaic acid blocked the ability of rapamycin to induce an association between the two proteins. Overexpression of constitutively active PP2Ac Y307F of PP2A blocked the ability of rapamycin to cause nuclear accumulation of STAT1 (figure 3A). Conversely, overexpression of a catalytically inactive PP2Ac L199P (figure 3B), caused nuclear accumulation of STAT1, similar to the effect of rapamycin. This is in opposition to previous work that has shown that mTOR inhibits PP2A activity by acting through α4 to inhibit apoptosis[109]. Inactivation of mTOR by stress or under rapamycin conditions, leads to α4 dephosphorylation and activation of PP2Ac function[113]. While the effect of mTOR inhibition on STAT1 trafficking was mimicked by PP2Ac L199P expression, and opposed directly by PP2Ac Y307F expression, in immunoprecipitation experiments

(figure 5B), pre-treatment with okadaic acid blocked the ability of rapamycin to induce mTOR-importin α5 association. It is possible that inhibition of PP2A by okadaic acid, and overexpression of a constitutively active form of PP2A, do not act in the same fashion on the STAT1 nuclear transport system. Overexpression of PP2Ac mutants may not entirely bypass the regulatory effects mediated by α4 expression, whose function was not looked at in this study. Given the ability of PP2A mutants to emulate the effect of rapamycin treatment, it is likely that PP2A acts downstream of mTOR signalling. mTOR may alter the phosphorylation status of PP2A or its regulatory protein α4, enabling PP2A catalytic phosphatase activity. Activated PP2A may affect the phosphorylation status of STAT1, or a STAT1 accessory protein. This may in turn inhibit the ability of STAT1 to physically associate with importin α5, thus regulating STAT1 cellular trafficking. Inhibition of mTOR by rapamycin or during metabolic stress may inhibit PP2A activity, allowing for STAT1 to associate with importin α5. Importin α5 may also be a target for PP2Ac, affecting phosphorylation at the S111 site. Further studies are required to determine the precise mechanism by which PP2A regulates STAT1 localization.

Using *in silico* tools, we showed that the karyopherin importin α5 contains a highly conserved site that is required for importin α-dependent nuclear import. Using this as a guide, we generated importin α5 recombinant mutants to test this hypothesis with imaging studies. We observed that the importin α5 S111A site is required for rapamycin-mediated STAT1 nuclear accumulation. Overexpression of importin α5 S111A blocked the ability of rapamycin to induce nuclear STAT1 accumulation (figure 6C) as compared to controls (figure 6A), while expression of wild-type importin α5 alone induced STAT1 nuclear accumulation (figure 6B). This result indicates that phosphorylation of importin α5 at S111A is necessary for its activity, and is consistent with previous studies

performed in yeast[114]. Importin has been shown to be phosphorylated in yeast, though its phosphorylation has not yet been demonstrated to have any impact on nuclear import of the proteins studied. The role of importin α5 S111 phosphorylation in the regulation of STAT1 nuclear levels remains to be determined.

Given previous studies that showed that doxorubicin is a STAT1-dependent inducer of apoptosis[112], we hypothesized that rapamycin-induced nuclear STAT1 accumulation had the effect of priming the cell for apoptosis, and that this effect would be increased with doxorubicin. In fact, the opposite was observed. Caspase 3-cleavage was decreased in cells treated with rapamycin prior to treatment with doxorubicin (figure 7A). This effect was verified using crystal violet staining to detect viable cells. Samples treated with rapamycin exhibited decreased cell viability as compared to untreated controls, but not increased apoptosis (figure 7B). In fact, rapamycin blocked apoptosis by decreasing doxorubicin-induced caspase-3 cleavage. However, despite the dampened effect of rapamycin on apoptotic cell death, rapamycin did not block doxorubicin-mediated decreases in cell viability as determined by crystal violet readings. Thus, alternative mechanisms are involved in the ability of rapamycin to induce changes in cell viability. It is possible that inhibition of caspase activation shifts the morphology of cell death from the classical aspects of apoptosis to such processes as autophagic cell death[115, 116], or necrosis[117]. Previous studies have shown that in cells whereby caspase-8 is inactivated, cells still underwent non-apoptotic cell death after death stimulation[115]. Such cells exhibit autophagosomes and autolysosomes, markers of autophagy. In addition, chemical inhibition of autophagy by 3-methyl adenine suppressed cell death, implicating a possible role of autophagic processes. STAT1-dependent apoptotic cell death has been well described in the context of IFNγ and STAT1 phosphorylation at Y701. However, recent

evidence has shown that FAS-FASL-induced apoptosis is dependent on S727 phosphorylation and not Y701 phosphorylation[118]. Furthermore, STAT1 has been shown to dimerize in conjunction with other proteins, not just with other STATs. It is plausible that nuclear localization of STAT1 in response to inhibition of mTOR by rapamycin requires a priming event, such as phosphorylation at S727, to promote the expression of pro-apoptotic genes and apoptosis itself.

We further hypothesized that the effect of rapamycin inhibition of cell viability occurs via STAT1-dependent cell cycle arrest. We assessed the ability of rapamycin to increase the expression of the cyclin-dependent kinase inhibitor p27. Rapamycin had no observable effect on p27 expression (figure 8), though this does not necessarily rule out the possibility of mTOR inhibition on affecting cell cycle progression. Similar to the effect of rapamycin on caspase 3-cleavage, rapamycin dampened doxorubicin-induced enhancement of p27 expression. This indicates that mTOR may not regulate the direct expression of p27, but rather the global expression of inhibitory proteins. The notion that inhibition of mTOR would decrease total cellular protein synthesis is consistent with prescribed cellular roles of mTOR in growth and anabolism. Overexpression of wild-type KPNA1 reversed the rapamycin inhibition on cell proliferation (figure 9). Although the mechanism by which overexpression of KPNA1 promotes cellular proliferation is unclear, its ability to reverse the effects of rapamycin on cell proliferation implicate it in the anti-proliferative effects of rapamycin.

The ability of mTOR, a regulator of metabolic activity, to regulate STAT1 has important clinical implications. The anti-proliferative effects of rapamycin have proven to be useful in treating cancer, and this raises important questions linking the nutritional status of patients to the ability to control tumour development and the onset of

malignancy. Combined therapies using the STAT1-dependent pro-apoptotic drug doxorubicin, as well as rapamycin, have proven to be efficient treatments for Akt-dependent clinical drug resistance[119], however the mechanism of action is unknown. In our studies, we have shown that inhibition of mTOR by rapamycin increases STAT1-dependent pro-apoptotic gene expression[44]. However, it is clear that for apoptosis itself to occur, additional events are required. This may involve further exogenous signals that may tilt the cell toward the cell death fate. Further studies are necessary to understand the full extent of mTOR-regulation of STAT1 and the processes associated with it. These may provide further insight into clinical treatments for metabolic and proliferative disorders.

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