A Global Kinase and Phosphatase Interaction Network in the Budding Yeast Reveals Novel Effectors of the Target of Rapamycin (TOR) Pathway

by

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Abstract

In the budding yeast *Saccharomyces cerevisiae*, the evolutionarily conserved Target of Rapamycin (TOR) signaling network regulates cell growth in accordance with nutrient and stress conditions. In this work, I present evidence that the TOR complex 1 (TORC1)-interacting proteins Nnk1, Fmp48, Mks1, and Sch9 link TOR to various facets of nitrogen metabolism and mitochondrial function. The Nnk1 kinase controlled nitrogen catabolite repression-sensitive gene expression via Ure2 and Gln3, and physically interacted with the NAD^+-linked glutamate dehydrogenase Gdh2 that catalyzes deamination of glutamate to α-ketoglutarate and ammonia. In turn, Gdh2 modulated rapamycin sensitivity, was phosphorylated in Nnk1 immune complexes *in vitro*, and was relocalized to a discrete cytoplasmic focus in response to *NNK1* overexpression or respiratory growth. The Fmp48 kinase regulated respiratory function and mitochondrial morphology, while Mks1 linked TORC1 to the mitochondria-to-nucleus retrograde signaling pathway. The Sch9 kinase appeared to act as both an upstream regulator and downstream sensor of mitochondrial function. Loss of Sch9 conferred a respiratory growth defect, a defect in mitochondrial DNA transmission, lower mitochondrial membrane potential, and decreased levels of reactive oxygen species. Conversely, loss of mitochondrial DNA caused loss of Sch9

enrichment at the vacuolar membrane, loss of Sch9 phospho-isoforms, and small cell size suggestive of reduced Sch9 activity. Sch9 also exhibited dynamic relocalization in response to stress, including enrichment at mitochondria under conditions that have previously been shown to induce apoptosis in yeast. Taken together, this work reveals intimate connections between TORC1, nitrogen metabolism, and mitochondrial function, and has implications for the role of TOR in regulating aging, cancer, and other human diseases.

Acknowledgments

Doing a PhD changes you. I'm certainly not the same guy who first started graduate school on a crisp autumn morning back in September of 2002. In fact, if you believe the oft-cited claim (for which, ironically, I can't find a reference) that every atom in one's body is replaced over a period of 7 years, I'm literally not the same guy who started this journey back in 2002. Chalk it up to the cumulative effect of many things over the years: the late nights spent hunched over lab equipment; the afternoons spent reading through stacks of papers; the sudden flashes of inspiration jotted down on a napkin; the weekends where life was squeezed into 1-hour windows coinciding with the incubation times of a Western blot; the roller-coaster ride of exciting and then perplexing data; the cat-and-mouse game of manuscript submission and revision; the growing sense of comfort with ambiguity; the gradual conceptual shift from being a consumer of knowledge to a generator of it; and (near the end) the creeping existential angst of being a "student" into one's thirties.

Let me take this opportunity to salute the fellow travelers with whom I shared a moment (or two) over the years: Debbie Blake, Traci Lee, Joy Nishikawa, Ginny Chen, Jaspal Singh, Jan Wildenhain, Michaela Spitzer, John Rohde, Paul Jorgensen, Ashton Breitkreutz, Lorrie Boucher, Rob Woolstencroft, Marc Angeli, Danielle Dewar, Lionel Pintard, Giora Ben-Ari, Dave Bellows, Sonam Dolma, Nick Jarvik, Yang-Chieh Chou, Pamela Kanellis, Fred Sweeney, Mike Downey, Mike Cook, Marcia Roy, Brett Larsen, Catherine Lebel, Jean Tang, Zhen Lin, and Qingquan Liu. You made sure that graduate school was always interesting, even during those times when my data was not. I'm also grateful to my supervisory committee members, Dr. Tim Hughes and Dr. Brenda Andrews, for their guidance over the years, and to NSERC and the Terry Fox Foundation for taking a chance on me with their scholarships.

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Finally, I owe a great debt of gratitude to my wife, Jennifer Baker, for her love and support. Only a fellow PhD student could have understood and cheerfully accepted the personal sacrifices we both made to see this thing through. Jen, you deserve a medal. (It may take me the next few decades to pay off my household chores "tab".)

Yes, doing a PhD changes you – but it's time well spent.

"After sleeping through a hundred million centuries we have finally opened our eyes on a sumptuous planet, sparkling with color, bountiful with life. Within decades we must close our eyes again. Isn't it a noble, an enlightened way of spending our brief time in the sun, to work at understanding the universe and how we have come to wake up in it?"

– Richard Dawkins

Table of Contents

List of Figures

List of Tables

List of Abbreviations

1-NM-PP1	1-naphthylmethyl-PP1
ADP	adenosine 5’-diphosphate
AMP	adenosine 5’-monophosphate
AMPK	AMP-activated protein kinase
ANT	adenine nucleotide translocator
APC	anaphase promoting complex
AP-MS	affinity purification - mass spectrometry
as	analogue sensitive
ATP	adenosine 5’-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
cAMP	cyclic AMP
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CDK	cyclin-dependent kinase
CEN	centromere
CFP	cyan fluorescent protein
ChIP	chromatin immunoprecipitation

CR	caloric restriction
CTP	cytosine 5’-triphosphate
CWI	cell wall integrity
DAPI	4’,6-diamidino-2-phenylindole
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ERC	extrachromosomal rDNA circle
ESR	environmental stress response
FEAR	fourteen early anaphase release
FKBP12	FK506-binding protein of 12 kDa
GAP	GTPase activating protein
GDH	glutamate dehydrogenase
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLS	glutaminase
GO	Gene Ontology
GTP	guanosine 5’-triphosphate

HA	hemaglutinin
HBD	hormone-binding domain
HOG	high osmolarity glycerol
HRP	horseradish peroxidase
HTP	high-throughput
HTP-HC	high-throughput - high-confidence
HU	hydroxyurea
IGF-1	insulin-like growth factor 1
IR	insulin receptor
IRS	insulin receptor substrate
kanR	kanamycin resistance gene
kb	kilobase pair
kd	kinase-dead
KPI	kinase and phosphatase interaction
LC	liquid chromatography
LTP	low-throughput
MAPK	mitogen activated protein kinase
MBF	MCB binding factor
MCB	MluI cell cycle box
MEN	mitotic exit network

MIPS	Munich Information Center for Protein Sequences
MMS	methyl methanesulphonate
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein particle
MS	mass spectrometry
MSX	methionine sulfoximine
NAD^+	nicotinamide adenine dinucleotide
natR	nourseothricin resistance gene
NCR	nitrogen catabolite repression
NES	nuclear export signal
NLS	nuclear localisation signal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAS	pre-autophagosomal structure
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDK	phosphoinositide-dependent kinase
PI3K	phosphoinositide 3'-kinase
PIKK	phosphoinositide-kinase-related kinase
PKA	protein kinase A

PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PHD	prolyl hydroxylase
PP2A	protein phosphatase 2A
PVDF	poly(vinylidene fluoride)
rDNA	ribosomal DNA
RENT	regulator of nucleolar silencing and telophase exit
RFB	replication fork barrier
RiBi	ribosome biogenesis
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	ribosomal protein
rRNA	ribosomal RNA
RRPE	ribosomal RNA processing element
RT	room temperature
RTG	retrograde
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
S6K	ribosomal protein S6 kinase

SAINT	Significance Analysis of Interactome
SBF	SCB binding factor
SC	synthetic complete
SCB	Swi4/6 cell cycle box
SCF	Skp1-Cdc53/CUL1-F-box
SDS	sodium dodecyl sulphate
SGA	synthetic genetic array
SGD	Saccharomyces Genome Database
TBS	Tris-buffered saline
TBS-T	TBS with Tween-20
TCA	trichloroacetic acid
TE	Tris-EDTA
TOR	target of rapamycin
TORC1	TOR complex 1
TORC2	TOR complex 2
tRNA	transfer RNA
ts	temperature sensitive
TTC	triphenyltetrazolium chloride
UAS	upstream activating sequence
UCP	uncoupling protein

uORF	upstream ORF
WT	wild type
XY	rich medium supplemented with tryptophan and adenine
YFP	yellow fluorescent protein
YNB	yeast nitrogen base

Chapter 1 – Introduction: Nutrient sensing and growth control by the Target of Rapamycin (TOR) signaling network

1.1 The Target of Rapamycin (TOR) signaling network

1.1.1 Nutrient sensing and growth control in the budding yeast

All cells must coordinate their growth and metabolism with external environmental conditions, and the budding yeast *Saccharomyces cerevisiae* – one of biology's powerful model organisms – has made seminal contributions to our understanding of the underlying mechanisms. The yeast cell has evolved a series of interlocking signaling networks that allow it to sense and respond appropriately to nutrients (Bahn, et al., 2007; Schneper, et al., 2004; Schuller, 2003; Smets, et al., 2010; Wilson and Roach, 2002; Zaman, et al., 2008). Decades of work have refined our knowledge of the cellular machinery involved, which includes: the general amino acid control (GAAC) pathway (Hinnebusch, 2005), the Pho85 cyclin-dependent kinase (Huang, et al., 2007; Mouillon and Persson, 2006), the Snf1 glucose repression pathway (Hedbacker and Carlson, 2008; Zhang, et al., 2010), the Ras/protein kinase A (PKA) pathway (Gancedo, 2008; Santangelo, 2006), the Rgt hexose transporter pathway (Johnston and Kim, 2005), the nitrogen catabolite repression (NCR) pathway (Cooper, 2002; Wong, et al., 2008), the mitochondrial retrograde response pathway (Liu and Butow, 2006), the SPS amino acid sensing pathway (Ljungdahl, 2009), and the Target of Rapamycin (TOR) pathway (De Virgilio and Loewith, 2006a).

The evolutionarily conserved TOR network is a major part of this signal transduction apparatus. TOR acts a master integrator of a variety of inputs to coordinate cell growth with nutrient status and stress conditions.

1.1.2 History and discovery of TOR

Rapamycin was first identified 35 years ago as a compound produced by the soil bacterium *Streptomyces hygroscopicus* (Sehgal, et al., 1975; Vezina, et al., 1975). Subsequent characterization revealed that rapamycin had powerful antifungal, antiproliferative, and immunosuppressive activities (Sehgal, 2003), and roused interest in the drug's mechanism of action. A genetic screen in yeast for spontaneous rapamycin-resistant mutants (Heitman, et al., 1991) identified 3 genes – *FPR1*, *TOR1* and *TOR2* – that became the founding members of what has come to be known as the TOR signaling network. *FPR1* was found to encode the peptidylprolyl isomerase FKBP12 (FK506-binding protein of 12 kDa), which – in an unusual

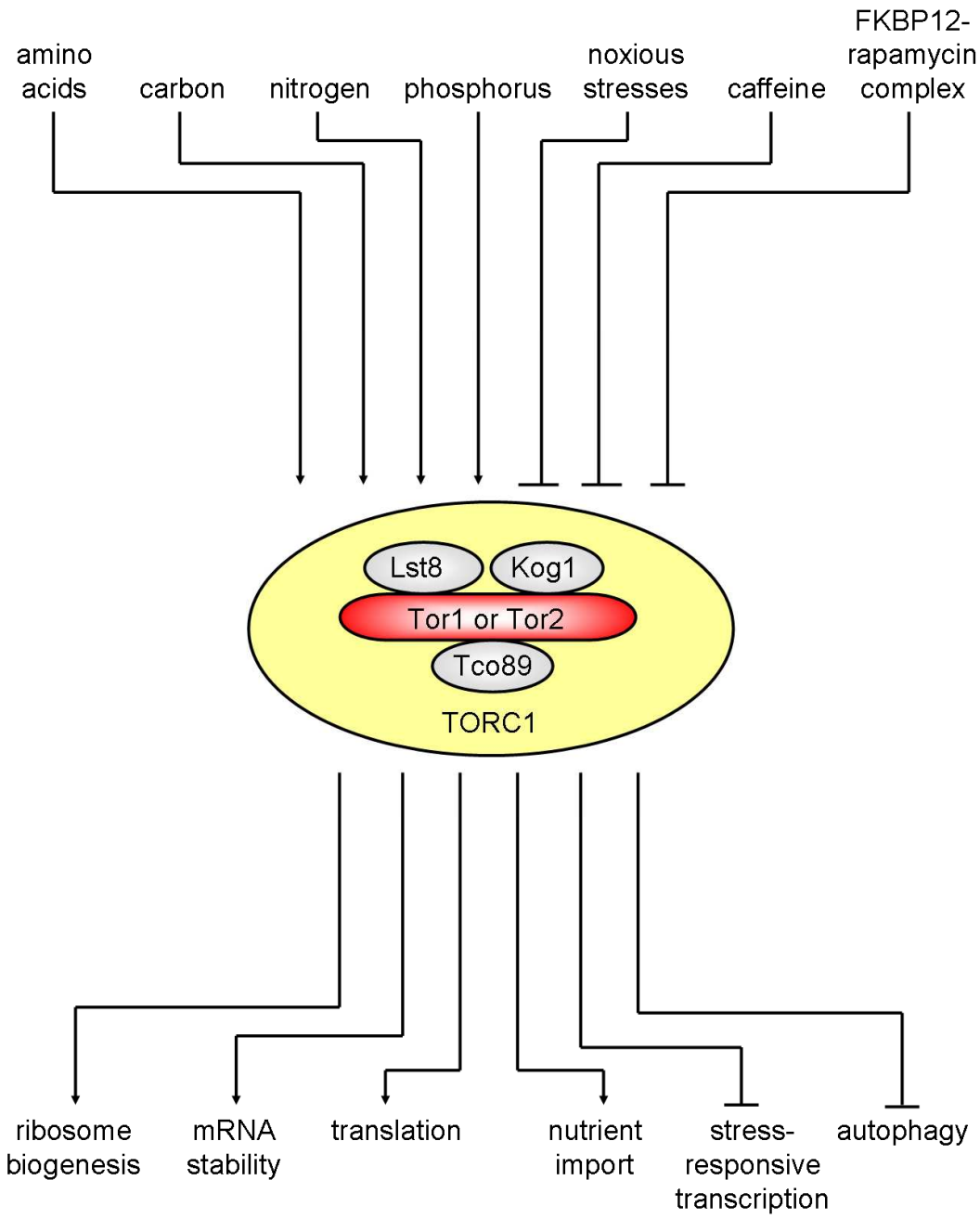
mechanism – bound rapamycin to form a complex that then inhibited the proteins encoded by the *TOR* genes (Heitman, et al., 1991; Koltin, et al., 1991). The true growth-inhibitory targets of rapamycin, encoded by *TOR1* and *TOR2*, were found to be pair of homologous proteins resembling phosphatidylinositol (PI) kinases, and further work showed that the rapamycin-resistant *TOR1* and *TOR2* alleles recovered were dominant, gain-of-function, point mutations that disrupted binding of the FKBP12-rapamycin complex (Cafferkey, et al., 1993; Helliwell, et al., 1994; Kunz, et al., 1993; Stan, et al., 1994). Genetic studies indicated that *TOR1* and *TOR2* shared a rapamycin-sensitive common function that was required for G1 progression, and that *TOR2* carried out another rapamycin-insensitive unique function (Helliwell, et al., 1998a; Helliwell, et al., 1994; Kunz, et al., 1993; Zheng, et al., 1995). In parallel, mammalian TOR (mTOR) was isolated based on its binding to FKBP-rapamycin (Brown, et al., 1994; Chiu, et al., 1994; Sabatini, et al., 1994; Sabers, et al., 1995).

1.1.3 TOR complexes

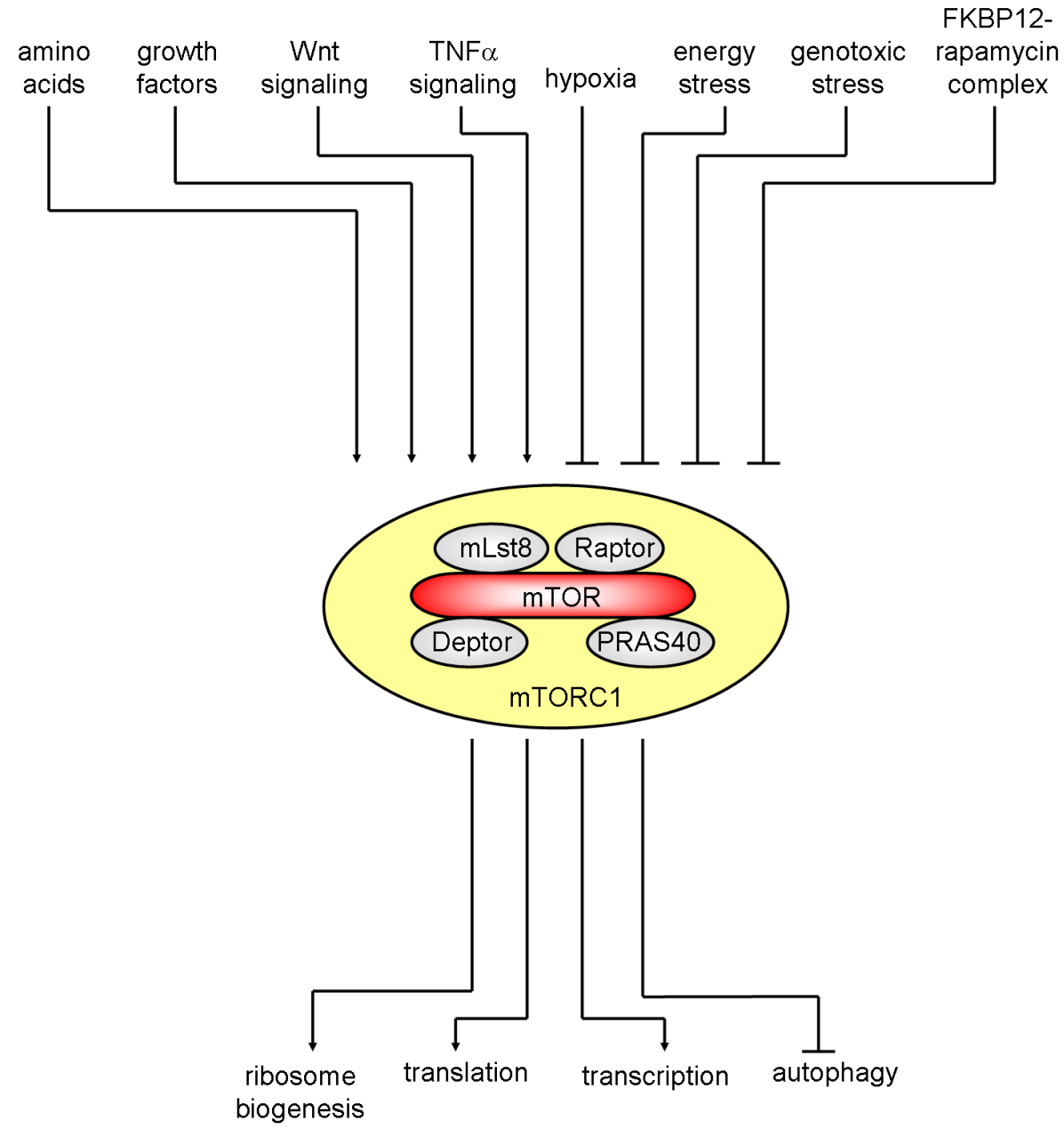
The TOR proteins belong to a class of Ser/Thr protein kinases known as the phosphatidylinositol kinase-related kinases (PIKKs) (Keith and Schreiber, 1995) and form the heart of two evolutionary conserved multi-protein complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 and TORC2 are large complexes that are dimeric or multimeric *in vivo* (Takahara, et al., 2006; Wang, et al., 2006; Wullschleger, et al., 2005; Yip, et al., 2010; Zhang, et al., 2006). TORC1 contains Lst8, Kog1, Tco89, and either Tor1 or Tor2 (Figure 1-1A) (Chen and Kaiser, 2003; Loewith, et al., 2002; Reinke, et al., 2004; Wedaman, et al., 2003). TORC2 contains Lst8, Avo1, Avo2, Avo3, Bit2, Bit61, and Tor2, but not Tor1 (Chen and Kaiser, 2003; Fadri, et al., 2005; Loewith, et al., 2002; Reinke, et al., 2004; Wedaman, et al., 2003). The two TOR complexes are conserved in a wide range of species from yeast to humans (Soulard, et al., 2009), although virtually all eukaryotes other than yeast have only one TOR gene (Wullschleger, et al., 2006). Indeed, in retrospect, the initial characterization of TOR in *S. cerevisiae* was quite fortuitous – the genetic identification in yeast of the rapamycin-sensitive, TOR1/2-shared function and rapamycin-insensitive, TOR2-unique function foreshadowed the later biochemical identification of the two distinct TOR complexes.

Figure 1-1. Composition, major upstream inputs, and major downstream outputs of Target of Rapamycin Complex 1 (TORC1). (A) TORC1 in budding yeast. **(B)** mTORC1 in mammalian cells. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively.

A


amino acids
carbon
nitrogen
phosphorus
noxious stresses
caffeine
FKBP12-rapamycin complex
Lst8
Kog1
Tor1 or Tor2
Tco89
TORC1
ribosome biogenesis
mRNA stability
translation
nutrient import
stress-responsive transcription
autophagy

B


amino acids
growth factors
Wnt signaling
TNFα signaling
hypoxia
energy stress
genotoxic stress
FKBP12-rapamycin complex
mLst8
Raptor
mTOR
Deptor
PRAS40
mTORC1
ribosome biogenesis
translation
transcription
autophagy

In mammalian cells, mTORC1 contains mTOR, Raptor (corresponds to yeast Kog1), mLst8/GβL (corresponds to yeast Lst8), Deptor, and PRAS40 (Figure 1-1B) (Hara, et al., 2002; Kim, et al., 2002; Kim, et al., 2003; Peterson, et al., 2009; Sancak, et al., 2007; Vander Haar, et al., 2007). Raptor appears to modulate TORC1 substrate specificity by recruiting substrates with a TOR signaling (TOS) motif (Beugnet, et al., 2003b; Lee, et al., 2008; Nojima, et al., 2003; Schalm and Blenis, 2002; Schalm, et al., 2003). mTORC2 contains mTOR, mLst8/GβL, Rictor (corresponds to yeast Avo3), Deptor, Protor/PRR5 (corresponds to yeast Bit61), and mSIN1 (corresponds to yeast Avo1) (Frias, et al., 2006; Jacinto, et al., 2006; Jacinto, et al., 2004; Pearce, et al., 2007; Sarbassov, et al., 2004; Thedieck, et al., 2007; Woo, et al., 2007; Yang, et al., 2006). In addition, mammalian Tel2 appears to bind mTOR and regulate its stability (Takai, et al., 2007).

When incorporated into TORC2, Tor2 cannot be bound by the FKBP12-rapamycin complex, thus explaining TORC2's insensitivity to rapamycin (Loewith, et al., 2002). In mammalian cells, mTOR is similarly insensitive to rapamycin when incorporated within mTORC2 (Jacinto, et al., 2004; Sarbassov, et al., 2004), although prolonged rapamycin treatment has been shown to inhibit *de novo* assembly of the complex (Sarbassov, et al., 2006). The specific molecular mechanism by which rapamycin inhibits TORC1 signaling – whether by disruption of the complex, interference with the TOR-Kog1 interaction, disruption of interactions with substrates, or inhibition of TOR kinase activity – has been a matter of debate and remains unclear (Jacinto, et al., 2004; Kim, et al., 2002; Loewith, et al., 2002; McMahon, et al., 2005; Oshiro, et al., 2004; Soliman, et al., 2010; Yan, et al., 2006; Yip, et al., 2010). Interestingly, a new generation of ATP-competitive, active-site TOR inhibitors has shown that rapamycin only inhibits TORC1 signaling towards a subset of its targets, demonstrating that TORC1 has rapamycin-resistant functions (Choo, et al., 2008; Feldman, et al., 2009; Garcia-Martinez, et al., 2009; Thoreen, et al., 2009; Yu, et al., 2009).

Localization studies using a range of experimental techniques have shown that yeast TOR complexes associate with intracellular membranes (Aronova, et al., 2007; Cardenas and Heitman, 1995; Chen and Kaiser, 2003; Kunz, et al., 2000; Reinke, et al., 2004; Wedaman, et al., 2003). In particular, recent studies using GFP-tagged TOR complex components have shown that

TORC1 is enriched at the vacuolar membrane while TORC2 is enriched in punctate foci at the plasma membrane (Araki, et al., 2005; Berchtold and Walther, 2009; Binda, et al., 2009; Sturgill, et al., 2008; Urban, et al., 2007). mTOR has been found in a variety of locations, including mitochondria (Desai, et al., 2002), the endoplasmic reticulum and Golgi apparatus (Drenan, et al., 2004; Withers, et al., 1997), and – most recently – the lysosomal membrane (Sancak, et al., 2010). A pool of TORC1 also appears to enter the nucleus in both yeast and mammalian cells (Bachmann, et al., 2006; Kim and Chen, 2000; Li, et al., 2006a; Tsang, et al., 2010; Zhang, et al., 2002).

In general terms, TORC1 is thought to direct temporal control of cell growth by promoting anabolic processes and inhibiting catabolic processes, while TORC2 is thought to direct spatial control of cell growth by regulating cell polarity (Bhaskar and Hay, 2007; De Virgilio and Loewith, 2006b; Dunlop and Tee, 2009; Laplante and Sabatini, 2009; Polak and Hall, 2009; Rohde, et al., 2008; Sengupta, et al., 2010; Soulard, et al., 2009; Wullschleger, et al., 2006; Yang and Guan, 2007). Compared to TORC1, relatively little is known about TORC2 signaling, although recent years have witnessed significant advances in our understanding (Alessi, et al., 2009; Cybulski and Hall, 2009). In the remainder of this chapter, I concentrate on the upstream regulation and downstream readouts of TORC1, focusing on *S. cerevisiae* and discussing higher eukaryotes where appropriate.

1.1.4 Upstream inputs regulating TORC1

TORC1 is responsive to a range of upstream signals. Yeast TORC1 is activated by nutrients and amino acids, and inhibited by a variety of noxious stresses (Figure 1-1A). In mammalian cells, mTORC1 has been shown to be regulated by amino acids, growth factors and hormones, energy/ATP levels, oxygen levels, and genotoxic stress (Figure 1-1B).

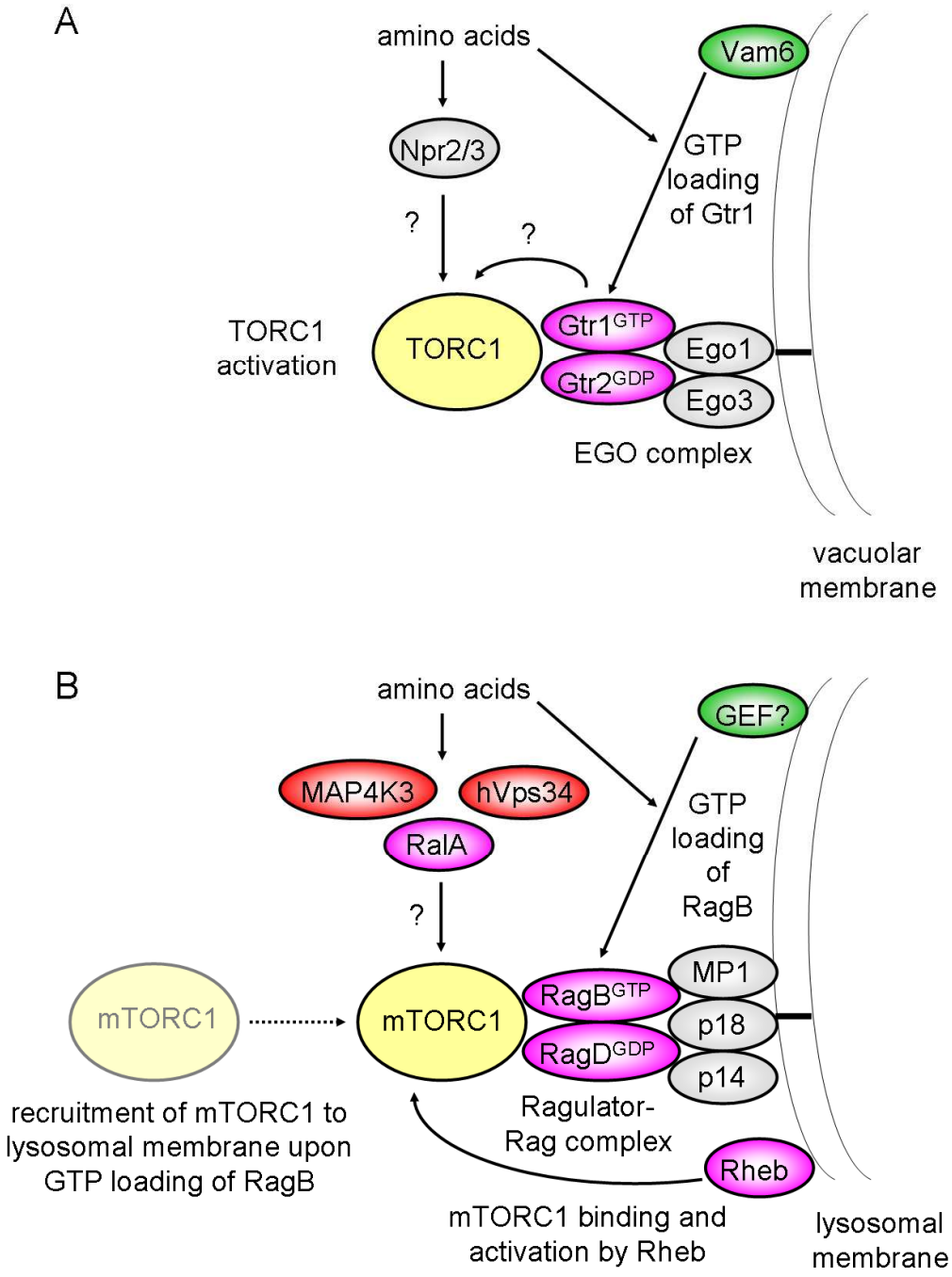
1.1.4.1 Amino acids

Yeast and metazoan TORC1 activity is responsive to the presence of amino acids, particularly leucine (Avruch, et al., 2009; Binda, et al., 2009). How amino acids are sensed by TORC1 has been a longstanding question, and recent work has shed further light on this process in both yeast and higher eukaryotes. In yeast, the vacuolar-membrane-localized EGO complex – composed of Ego1, Ego3, Gtr1, and Gtr2 – has recently been shown to link amino acid signaling to TORC1 (Figure 1-2A) (Binda, et al., 2009). Components of this complex had previously been

implicated in cell cycle re-entry after rapamycin-induced growth arrest (Dubouloz, et al., 2005), trafficking of amino acid permeases (Gao and Kaiser, 2006), and as the target of a small molecule that suppresses the effects of rapamycin (Huang, et al., 2004). The EGO complex, when Gtr1 is in the GTP-bound state and Gtr2 in the GDP-bound state, was found to bind and activate TORC1 via an unknown mechanism involving the TORC1 component Tco89 (Binda, et al., 2009). GTP loading of Gtr1 in response to amino acids was in turn shown to be mediated by the guanine nucleotide exchange factor (GEF) Vam6 (Binda, et al., 2009). How the presence of amino acids is sensed and communicated to Vam6 is unknown.

In mammalian cells, an analogous system appears to operate involving RagA or RagB (the orthologs of yeast Gtr1) and RagC or RagD (the orthologs of yeast Gtr2) (Figure 1-2B) (Kim, et al., 2008; Sancak, et al., 2008). Most recently, the lysosomal-membrane-localized Ragulator-Rag complex (composed of MP1/MAPKSP1, p14/ROBLD3, p18/c11orf59, RagB, and RagD) has been shown to link amino acid signals to mTORC1 (Sancak, et al., 2010). GTP loading of RagB to form a $RagB^{GTP}$-$RagD^{GDP}$ heterodimer triggered binding of mTORC1 via its component Raptor, thus recruiting mTORC1 to the lysosomal membrane and placing it in close proximity to its known upstream activator, the small GTPase Rheb (Sancak, et al., 2010). Interestingly, MP1 and p14 form a complex that appears to be the structural counterpart of yeast Ego3, despite low homology at the amino acid level (Kogan, et al., 2010). However, unlike in yeast – where all TORC1 and EGO complex components appear to be constitutively localized to the vacuolar membrane (Binda, et al., 2009) – the key regulatory step in mammalian cells appears to be the translocation of mTORC1 to the lysosomal membrane. Indeed, mechanisms of TORC1 activation seem to be at least partially divergent between yeast and mammalian cells: the yeast ortholog of the GTPase Rheb (encoded by *RHB1*) does not appear to function in TORC1 signaling; yeast does not carry orthologs of the TSC1/2 proteins (see below); and mammals do not appear to carry an ortholog of the TORC1 component Tco89 (De Virgilio and Loewith, 2006a; Li and Guan, 2009; Soulard, et al., 2009; Wullschleger, et al., 2006). How the presence of amino acids is sensed and communicated to the Ragulator-Rag complex is unknown. Interestingly, a recent report has suggested that the anti-diabetic drug metformin – widely believed to inhibit mTORC1 via activation of the AMP-activated protein kinase (AMPK) (see below) – may actually exert its effects via the Rag GTPases (Kalender, et al., 2010).

Figure 1-2. Upstream activation of TORC1 by amino acids. (A) TORC1 in budding yeast. **(B)** mTORC1 in mammalian cells. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. GTPases are shown in purple, GEFs in green, kinases in red. TORC1, target of rapamycin complex 1; GEF, guanine nucleotide exchange factor.


A
amino acids
Vam6
Npr2/3
GTP
loading
of Gtr1
?
?
TORC1
activation
TORC1
Gtr1GTP
Gtr2GDP
Ego1
Ego3
EGO complex
vacuolar
membrane
B
amino acids
GEF?
MAP4K3
hVps34
RalA
GTP
loading
of
RagB
?
mTORC1
mTORC1
RagBGTP
RagDGDP
MP1
p18
p14
recruitment of mTORC1 to
lysosomal membrane upon
GTP loading of RagB
Ragulator-
Rag complex
Rheb
mTORC1 binding and
activation by Rheb
lysosomal
membrane

Other factors have also been implicated in communicating amino acid status to TORC1. These include, in yeast, the Npr2/3 complex (Neklesa and Davis, 2009), and in higher eukaryotes, the mitogen activated protein kinase (MAPK) homolog MAP4K3 (Bryk, et al., 2010; Findlay, et al., 2007; Yan, et al., 2010), the class III phosphatidylinositol 3-kinase (PI3K) hVps34 (Byfield, et al., 2005; Gulati, et al., 2008; Nobukuni, et al., 2005), the FKBP-family protein FKBP38 (Bai, et al., 2007), and the GTPase RalA (Maehama, et al., 2008). Some of the latter mechanisms have been contested or shown to be species-specific (Juhasz, et al., 2008; Maehama, et al., 2008; Uhlenbrock, et al., 2009; Wang, et al., 2008b), and their relationship to the conserved Gtr/Rag cassette is unclear. One recent study detected a physical interaction between MAP4K3 and the Rag proteins (Bryk, et al., 2010), while another study did not (Yan, et al., 2010).

In yeast, TORC1 activity has also been shown to be lower in cells growing in less preferred sources of carbon (raffinose, ethanol, glycerol) or nitrogen ($NH4^+$, proline) (Urban, et al., 2007), but the molecular mechanisms by which carbon and nitrogen quality are sensed and communicated to TORC1 are unknown.

1.1.4.2 Growth factors

In addition to sensing amino acids, as in yeast, metazoan TORC1 is also regulated by growth factor and hormone signaling, which act as a proxy for the nutrient status of the entire organism (Polak and Hall, 2009). In mammalian cells, many upstream signals regulate mTORC1 activity by converging on a core cassette composed of the small GTPase Rheb – which directly binds and activates mTORC1 (Long, et al., 2005a) – and the upstream heterodimeric TSC1/TSC2 complex, which inhibits Rheb via TSC2's GTPase activator protein (GAP) activity (Figure 1-3) (Huang and Manning, 2008). Growth factors like insulin and insulin-like growth factor 1 (IGF-1) bind their receptor, causing recruitment of insulin receptor substrate 1 (IRS-1), activation of phosphoinositide 3-kinase (PI3K), plasma membrane recruitment of protein kinase B (PKB)/Akt, and subsequent Akt activation by phosphoinositide-dependent kinase 1 (PDK1) and mTORC2 (Bhaskar and Hay, 2007; Manning and Cantley, 2007). Akt (and other kinases activated downstream of growth factor signaling) impinge on TORC1 signaling by phosphorylating TSC2 (Li, et al., 2003b; Ma, et al., 2005; Potter, et al., 2002; Rolfe, et al., 2005;

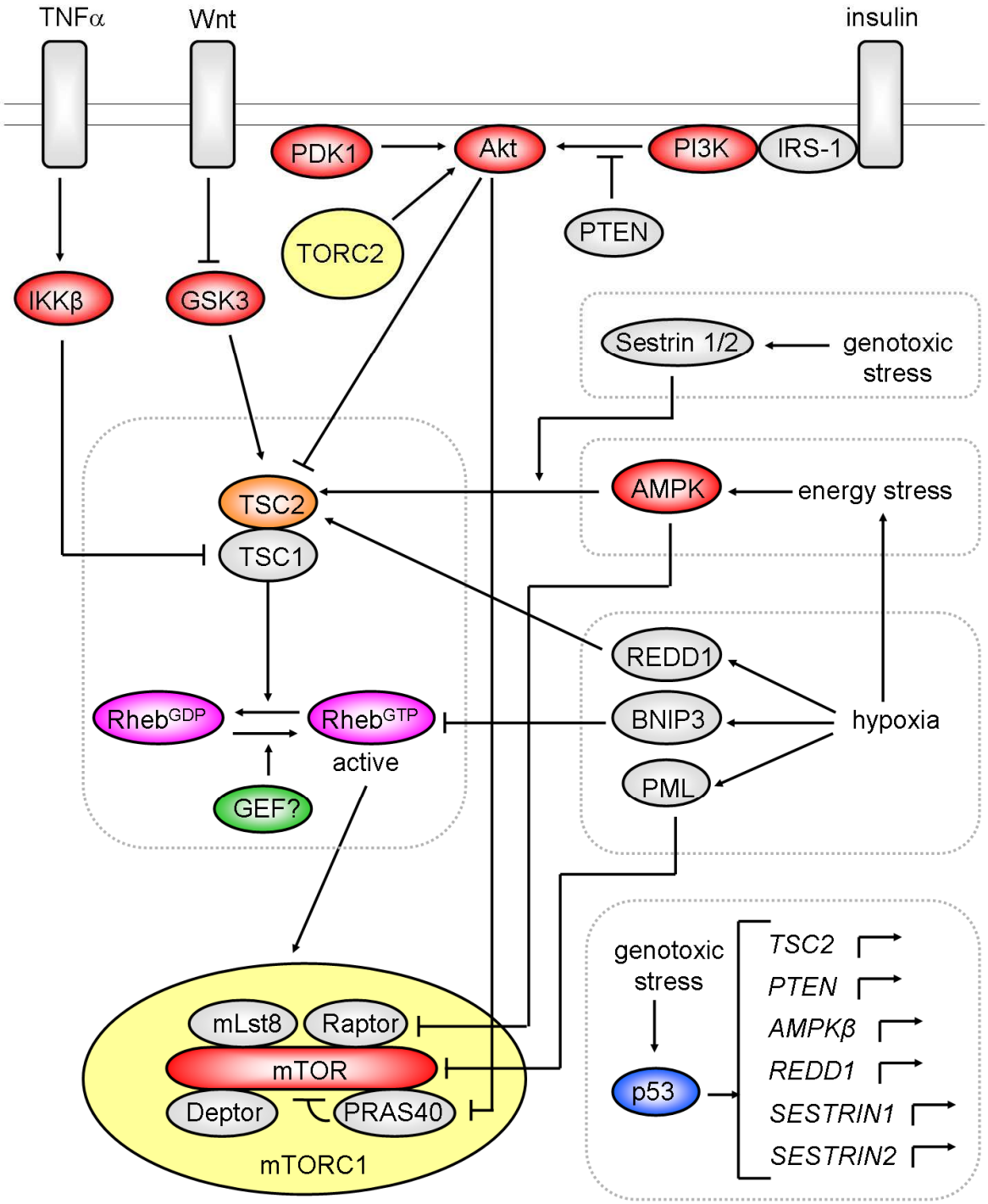
Roux, et al., 2004; Tee, et al., 2003a), thus inhibiting its ability to convert Rheb-GTP to Rheb-GDP (Cai, et al., 2006; Inoki, et al., 2003a; Tee, et al., 2003b). Wnt signaling also targets TSC2 (Inoki, et al., 2006), while TNFα signaling targets TSC1 (Lee, et al., 2007b). In addition, recent studies have uncovered another, TSC2-independent pathway in which Akt phosphorylates and antagonizes the mTORC1 component PRAS40, which also acts as an mTORC1 inhibitor (Oshiro, et al., 2007; Sancak, et al., 2007; Thedieck, et al., 2007; Vander Haar, et al., 2007; Wang, et al., 2008a; Wang, et al., 2007).

1.1.4.3 Noxious stresses

In yeast, TORC1 activity has been found to be inhibited by a range of stress conditions, including: high NaCl concentrations, H_2O_2, arsenic, mercury, nickel, wortmannin, caffeine, upshift to high temperature, mechanical stress from centrifugation, and starvation for carbon, nitrogen, or phosphorus (Hosiner, et al., 2009; Kuranda, et al., 2006; Reinke, et al., 2006; Urban, et al., 2007; Wanke, et al., 2008). The molecular mechanisms by which these stresses are sensed and communicated to TORC1 are unknown. In mammalian cells, many stresses inhibit mTORC1 via the TSC1/TSC2 complex (see below). However, since yeast does not contain orthologs of the TSC1 and TSC2 proteins, the relevant mechanisms are likely different from those in metazoans.

A series of studies characterizing the Golgi Ca^{2+}/Mn^{2+} ATPase Pmr1 have also shown that Mn^{2+} in the Golgi inhibits TORC1 (Devasahayam, et al., 2007; Devasahayam, et al., 2006). This result, whose significance is unclear, joins the growing number of enigmatic connections between TORC1 and components of the membrane trafficking and secretory pathway machinery (Aronova, et al., 2007; Brown, et al., 2010; Flinn, et al., 2010; Lempiainen, et al., 2009; Puria, et al., 2008; Scott, et al., 2009b; Singh and Tyers, 2009; Zurita-Martinez, et al., 2007). The mechanisms linking noxious stresses to TORC1 have been more extensively characterized in metazoans (see below) (Sengupta, et al., 2010).

Figure 1-3. Regulation of mTORC1 in mammalian cells by growth factors and noxious stresses. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. GTPases are shown in purple, GAPs in orange, GEFs in green, kinases in red, transcription factors in blue.


TNFα
Wnt
insulin
PDK1
Akt
PI3K
IRS-1
PTEN
TORC2
IKKβ
GSK3
Sestrin 1/2
genotoxic stress
AMPK
energy stress
TSC2
TSC1
REDD1
RhebGDP
RhebGTP
active
BNIP3
hypoxia
PML
GEF?
mLst8
Raptor
mTOR
Deptor
PRAS40
mTORC1
genotoxic stress
p53
TSC2
PTEN
AMPKβ
REDD1
SESTRIN1
SESTRIN2

1.1.4.4 Energy/ATP levels

In mammalian cells, low energy levels inhibit mTORC1 via the AMP-activated protein kinase (AMPK) (Figure 1-3). A low level of ATP, and thus a high AMP:ATP ratio, triggers activation of AMPK (Hardie, 2007). AMPK inhibits mTORC1 activity by phosphorylating TSC2 on sites that – in contrast to those phosphorylated by Akt – positively regulate TSC2 function (Corradetti, et al., 2004; Inoki, et al., 2003b; Shaw, et al., 2004). Recent studies have also uncovered another, TSC2-independent pathway in which AMPK phosphorylates and inhibits the TORC1 component Raptor (Gwinn, et al., 2008). In addition to AMPK's function upstream of mTORC1, recent reports have also placed AMPK downstream of TORC1 (Aguilar, et al., 2007; Selman, et al., 2009) – similar to the situation in yeast, where Snf1 is downstream of yeast TORC1 (Orlova, et al., 2006).

1.1.4.5 Oxygen levels

In mammalian cells, hypoxia inhibits TORC1 via several mechanisms (Figure 1-3) (Wouters and Koritzinsky, 2008). First, the hypoxia-induced protein REDD1 has been shown to activate TSC2 by releasing it from negative regulation by 14-3-3 proteins (Brugarolas, et al., 2004; DeYoung, et al., 2008; Reiling and Hafen, 2004; Sofer, et al., 2005). Second, the inhibition of respiration that accompanies hypoxia has been found to cause reduced ATP levels, AMPK activation, and inhibition of mTORC1 by AMPK as described above (Liu, et al., 2006). Third, the hypoxia-induced protein BNIP3 has been shown to bind and inhibit Rheb (Li, et al., 2007). Finally, the PML tumour suppressor protein has been reported to bind mTOR and inhibit its interaction with Rheb (Bernardi, et al., 2006).

1.1.4.6 Genotoxic stress

In mammalian cells, inhibition of mTORC1 appears to be an important aspect of the response to DNA damage, and occurs as a result of several points of cross-talk between the p53 and TOR pathways (Figure 1-3) (Feng and Levine, 2010; Levine, et al., 2006). p53 has been shown to transcriptionally upregulate multiple upstream negative regulators of mTORC1, including TSC2, REDD1, the β1 subunit of AMPK, and the lipid phosphatase PTEN (Ellisen, et al., 2002; Feng, et al., 2007; Feng, et al., 2005; Stambolic, et al., 2001). In addition, recent

studies have also found that p53 transcriptionally upregulates the Sestrin 1 and Sestrin 2 proteins, which inhibit mTORC1 by physically interacting with TSC1, TSC2, and AMPK, thereby enhancing phosphorylation and activation of TSC2 by AMPK (Budanov and Karin, 2008). Paradoxically, S6K1 has also been shown to be activated by DNA damage and to stimulate p53 via negative regulation of the p53 inhibitor Mdm2 (Lai, et al., 2010).

1.1.5 Downstream outputs controlled by TORC1

TOR signaling controls cell growth in accordance with environmental conditions. In general terms, TORC1 positively regulates growth and anabolism, and negatively regulates stress responses and catabolism.

1.1.5.1 Major TORC1 effector branches

The two main effector branches downstream of yeast TORC1 are mediated by (i) Tap42-associated phosphatase complexes, and (ii) the AGC kinase Sch9 (Figure 1-4). Tap42 and Sch9 are thought to be direct substrates of TORC1 (Jiang and Broach, 1999; Urban, et al., 2007). A recent phospho-proteomic study succeeded in assigning a large number of rapamycin-sensitive phosphorylation events between the Tap42 and Sch9 effector branches (Huber, et al., 2009).

1.1.5.1.1 Tap42-associated phosphatases

The yeast genome encodes a number of protein phosphatase 2A (PP2A) and PP2A-like phosphatase catalytic subunits, each of which can bind various regulatory and scaffolding subunits to form an array of potential phosphatase complexes (Jiang, 2006). Tap42 is a protein that associates with the PP2A catalytic subunits Pph21 and Pph22, and the PP2A-like phosphatases Sit4, Pph3 and Ppg1 (Di Como and Arndt, 1996; Jacinto, et al., 2001; Jiang and Broach, 1999; Wang, et al., 2003). Tap42-phosphatase complexes may also contain the Rrd1 or Rrd2 peptidylprolyl isomerases (Douville, et al., 2006; Jordens, et al., 2006; Rempola, et al., 2000; Van Hoof, et al., 2005; Zheng and Jiang, 2005) or the SAP regulatory proteins (Sap155, Sap185, Sap190) (Rohde, et al., 2004). Tap42 binds a subset (5-10%) of the total phosphatase pool (Di Como and Arndt, 1996) and subjects it to regulation by TORC1. In response to nutrient starvation or TORC1 inhibition by rapamycin, Tap42 is dephosphorylated and its interaction with phosphatases is disrupted (Di Como and Arndt, 1996; Jiang and Broach, 1999; Wang, et al., 2003). Tap42 may be regulated by TORC1 via the inhibitor Tip41, whose dephosphorylation and

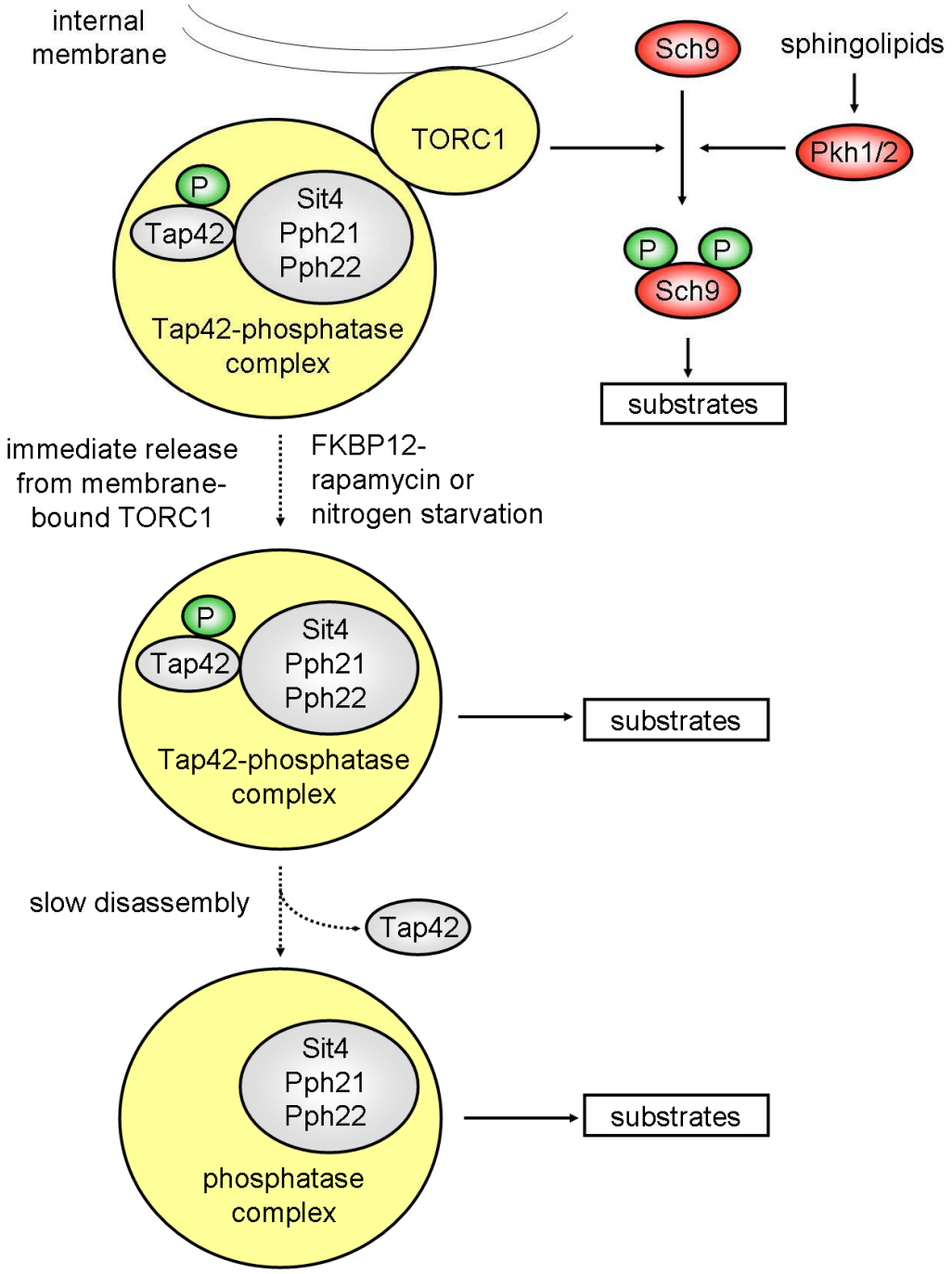
binding to Tap42 can be induced by rapamycin (Jacinto, et al., 2001). Although initially thought to be a negative regulator of phosphatase activity, evidence suggests that Tap42 acts positively toward at least some substrates (Duvel, et al., 2003; Wang, et al., 2003; Yan, et al., 2006), and thus may actually be a regulator of phosphatase substrate specificity. A recent study found that Tap42-phosphatase complexes were sequestered at membranes via physical binding to membrane-associated TORC1, and were released upon rapamycin treatment (Yan, et al., 2006). Time-course data revealed that subsequent dissociation of Tap42 from phosphatases occurred more slowly, as Tap42 was gradually dephosphorylated (Yan, et al., 2006). TORC1's Tap42-phosphatase effector branch has been shown to regulate many downstream targets, including: Npr1 (Schmidt, et al., 1998), Gln3 (Beck and Hall, 1999), Msn2 (Santhanam, et al., 2004), Gcn2 (Cherkasova and Hinnebusch, 2003), and Rtg1/3 (Duvel, et al., 2003) (see below).

1.1.5.1.2 The Sch9 kinase

Sch9 is an AGC-family kinase that has been shown to be activated via phosphorylation by TORC1 at 6 Ser/Thr residues in its C-terminal region (Urban, et al., 2007), consistent with regulation of AGC kinases being an evolutionarily conserved function of TOR (Jacinto and Lorberg, 2008). Although once thought to be the yeast ortholog of mammalian protein kinase B (PKB)/Akt (Sobko, 2006), Sch9 phosphorylation by TORC1 suggests that it is in fact the yeast ortholog of ribosomal protein S6 kinase (S6K) (Powers, 2007). TORC1's Sch9 effector branch has been found to regulate many downstream processes, including: entry into G0 phase via phosphorylation of the kinase Rim15 (Wanke, et al., 2008); translation initiation via eIF2α phosphorylation (Urban, et al., 2007); and transcription of polymerase I-, II- and III-dependent genes required for ribosome biogenesis (Huber, et al., 2009; Jorgensen, et al., 2004; Lee, et al., 2009; Urban, et al., 2007; Wei and Zheng, 2009) (see below).

It should be noted that Sch9 also appears to have TORC1-independent functions. Sch9 has been shown to receive input from sphingolipid signaling and the yeast PDK1-like kinases Pkh1/2 (Liu, et al., 2005; Roelants, et al., 2004; Urban, et al., 2007), and to function antagonistically to TORC1 with respect to some transcriptional responses (Smets, et al., 2008). Also, in contrast to the downregulation of TORC1 activity in response to many stresses, during osmotic stress Sch9 has been shown to positively regulate osmostress genes at the level of transcription elongation (Pascual-Ahuir and Proft, 2007).

Figure 1-4. Two major effector pathways downstream of TORC1 in yeast are controlled by Tap42-associated phosphatases and the AGC kinase Sch9. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. Dashed arrows represent relocalization or the passage of time, as indicated. See legend to Figure 1-3 for colour scheme.


internal
membrane
TORC1
Sch9
sphingolipids
Pkh1/2
P
Tap42
Sit4
Pph21
Pph22
Tap42-phosphatase
complex
P
P
Sch9
substrates
immediate release
from membrane-
bound TORC1
FKBP12-
rapamycin or
nitrogen starvation
P
Tap42
Sit4
Pph21
Pph22
Tap42-phosphatase
complex
substrates
slow disassembly
Tap42
Sit4
Pph21
Pph22
phosphatase
complex
substrates

1.1.5.2 Ribosome biogenesis

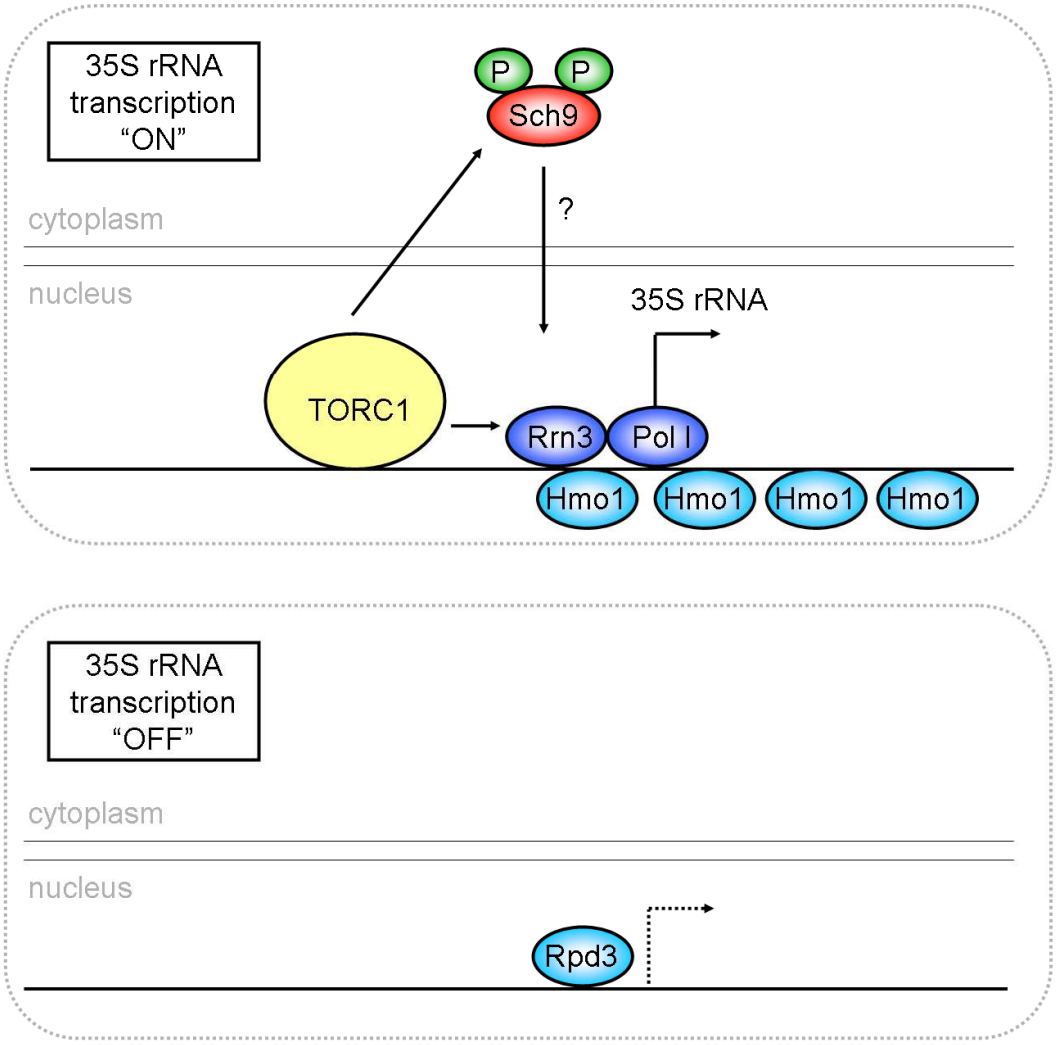
Producing yeast ribosomes is a complex process that requires all three polymerases and represents up to 95% of total transcription during exponential growth – as a result, it is carefully coordinated with nutrient status (Warner, 1999). The process of ribosome biogenesis involves transcription of the 25S, 18S, 5.8S, and 5S rRNAs, 137 ribosomal protein (RP) genes, and over 200 co-regulated ribosome biogenesis (RiBi) genes encoding trans-acting factors required for ribosome assembly and translation (rRNA modification factors, tRNA synthetases, translation factors, RNA polymerase I and III subunits, etc) (Henras, et al., 2008; Kressler, et al., 2010). TORC1 inhibition by rapamycin has been shown to rapidly decrease expression of rRNA, tRNA, RP, and RiBi genes (Cardenas, et al., 1999; Hardwick, et al., 1999; Powers and Walter, 1999; Shamji, et al., 2000; Zaragoza, et al., 1998). Recent work has revealed many of the molecular mechanisms by which TORC1 regulates ribosome synthesis (Figure 1-5) (Lempiainen and Shore, 2009; Mayer and Grummt, 2006; Xiao and Grove, 2009).

1.1.5.2.1 RNA polymerase I-transcribed 35S rRNA

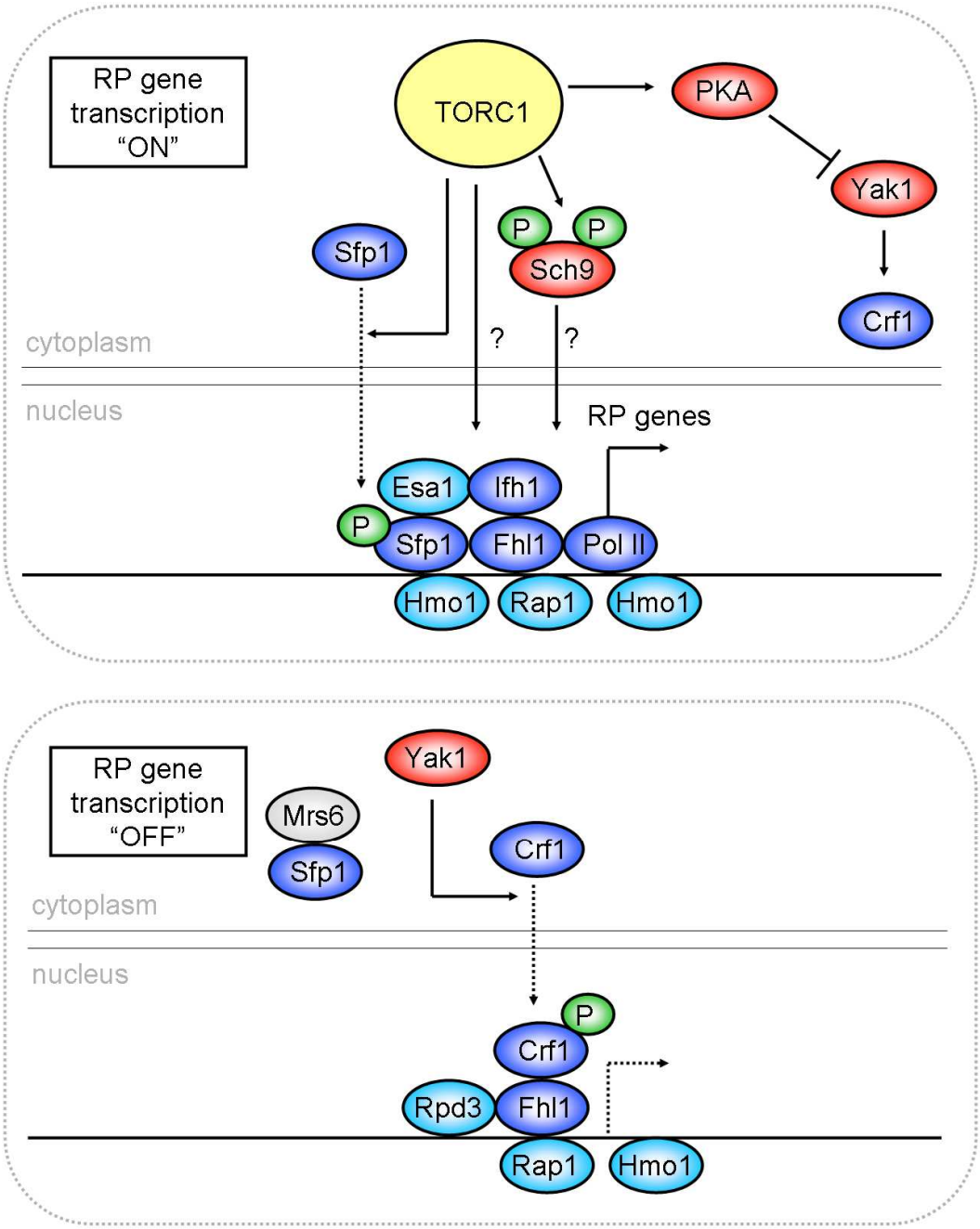
The 35S pre-rRNA, which is later processed into the mature 25S, 18S, and 5.8S rRNAs, is transcribed by RNA polymerase I (Figure 1-5A). TORC1 has been proposed to regulate this process via the polymerase I initiation factor Rrn3 (Claypool, et al., 2004), although subsequent evidence has suggested that this may not be the primary mechanism (Huber, et al., 2009; Philippi, et al., 2010). A recent study has shown that the TORC1 effector Sch9 regulates recruitment of polymerase I to the rDNA, 35S pre-rRNA transcription, and 35S pre-rRNA processing (Huber, et al., 2009). In addition, TORC1 has been found to physically interact directly with the rDNA promoter (Li, et al., 2006a) and to regulate binding of the HMG box protein Hmo1 (Berger, et al., 2007), which is bound all along actively transcribed rDNA (Kasahara, et al., 2007; Merz, et al., 2008). Repression of polymerase I transcription upon TORC1 inhibition has been associated with recruitment of the RSC chromatin-remodeling complex (Damelin, et al., 2002) and the histone deacetylase Rpd3 (Humphrey, et al., 2004; Tsang, et al., 2003), although the latter claim has been disputed (Oakes, et al., 2006). Interestingly, a recent report presented evidence that polymerase I transcription, via an unknown mechanism, can regulate RP gene transcription by polymerase II and 5S rRNA transcription by polymerase III (Laferte, et al., 2006).

Figure 1-5. Regulation of ribosome biogenesis by TORC1 in yeast. (A) 35S rRNA transcription by RNA polymerase I. **(B)** Ribosomal protein (RP) gene transcription by RNA polymerase II. **(C)** Ribosome biogenesis (RiBi) gene transcription by RNA polymerase II. **(D)** 5S rRNA transcription by RNA polymerase III. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. See legend to Figure 1-3 for colour scheme.

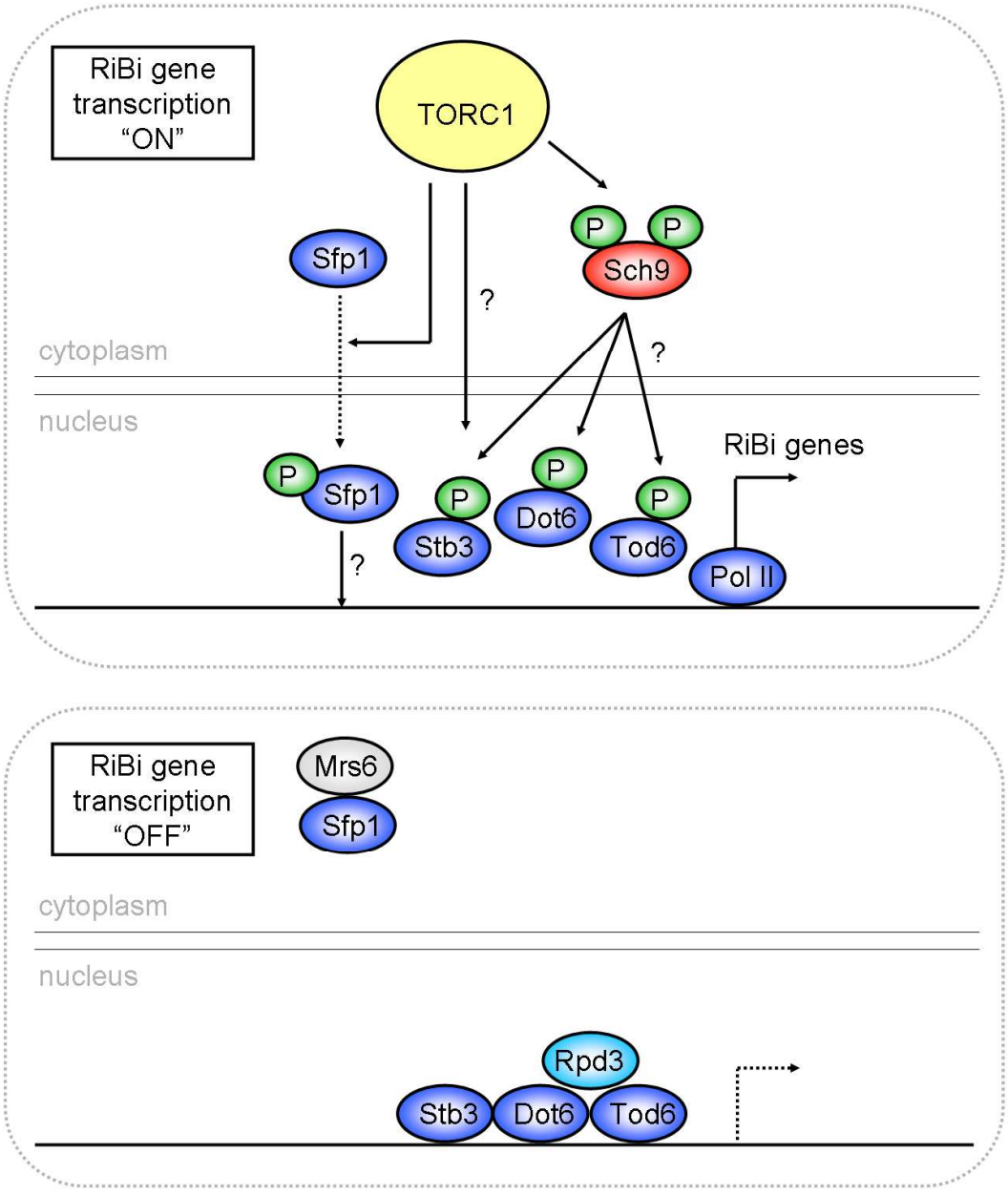
A


35S rRNA transcription "ON"
P
P
Sch9
?
cytoplasm
nucleus
35S rRNA
TORC1
Rrn3
Pol I
Hmo1
Hmo1
Hmo1
Hmo1
35S rRNA transcription "OFF"
cytoplasm
nucleus
Rpd3

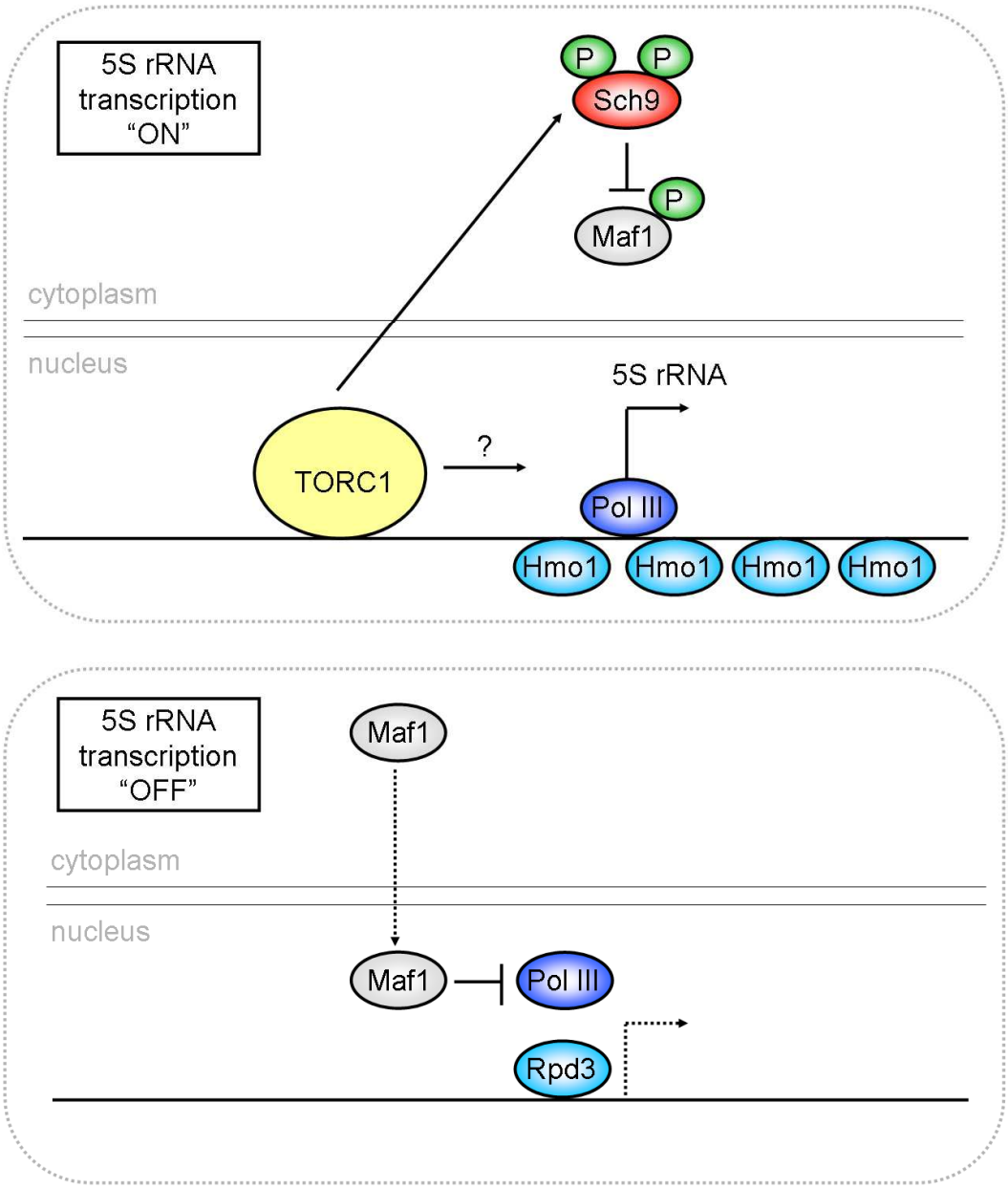
B


RP gene transcription "ON"
TORC1
PKA
Yak1
Sfp1
P
P
Sch9
Crf1
?
?
cytoplasm
nucleus
RP genes
Esa1
Ifh1
P
Sfp1
Fhl1
Pol II
Hmo1
Rap1
Hmo1
RP gene transcription "OFF"
Yak1
Mrs6
Sfp1
Crf1
cytoplasm
nucleus
P
Crf1
Rpd3
Fhl1
Rap1
Hmo1

C


RiBi gene transcription "ON"
TORC1
Sfp1
P
P
Sch9
?
?
cytoplasm
nucleus
RiBi genes
P
Sfp1
P
Stb3
P
Dot6
P
Tod6
Pol II
?
RiBi gene transcription "OFF"
Mrs6
Sfp1
cytoplasm
nucleus
Rpd3
Stb3
Dot6
Tod6

D


5S rRNA transcription "ON"
P
P
Sch9
P
Maf1
cytoplasm
nucleus
5S rRNA
TORC1
?
Pol III
Hmo1
Hmo1
Hmo1
Hmo1
5S rRNA transcription "OFF"
Maf1
cytoplasm
nucleus
Maf1
Pol III
Rpd3

Analogous mechanisms appear to operate in metazoans. In mammalian cells, association of the coactivator TIF-1A (the analog of yeast Rrn3) with polymerase I has been found to be regulated by mTORC1 phosphorylation (Mayer, et al., 2004) and TIF-1A is also regulated by TOR in flies (Grewal, et al., 2007). In addition, the mTORC1 effector S6K (the analog of Sch9) has been reported to be required for phosphorylation of the activation domain of UBF1 (Hannan, et al., 2003). mTOR has also been found to associate with the rDNA in mammalian cells (Tsang, et al., 2010).

1.1.5.2.2 RNA polymerase II-transcribed ribosomal protein (RP) genes

The yeast cell's 78 ribosomal proteins, most of which are present in the genome in duplicate copies, are encoded by 137 ribosomal protein (RP) genes that are transcribed by RNA polymerase II (Figure 1-5B). In recent years, a number of factors have been found at RP genes, including Rap1, Abf1, Fhl1, Ifh1, Crf1, Sfp1, and Hmo1 (Hall, et al., 2006; Jorgensen, et al., 2004; Kasahara, et al., 2007; Lee, et al., 2002; Marion, et al., 2004; Martin, et al., 2004; Rudra, et al., 2005; Schawalder, et al., 2004; Wade, et al., 2004; Zhao, et al., 2006). Expression of RP genes has been associated with the recruitment of the Esa1 histone acetylase (Reid, et al., 2000; Rohde and Cardenas, 2003), while RP gene repression has been associated with the presence of the Rpd3 histone deacetylase (Humphrey, et al., 2004; Rohde and Cardenas, 2003).

TOR is known to impinge upon RP genes in several ways. First, TORC1 has been shown to regulate the phosphorylation state and nucleocytoplasmic localization of the co-repressor Crf1, via an indirect mechanism involving PKA and the Yak1 kinase (Martin, et al., 2004). Under favourable growth conditions, TORC1 activity was found to maintain Crf1 in the cytoplasm. Upon TORC1 inhibition, Crf1 underwent phosphorylation by Yak1, accumulated in the nucleus, and competed with the co-activator Ifh1 for binding to Fhl1, which is itself constitutively bound at RP gene promoters (Martin, et al., 2004). However, the function of Crf1 has since been reported to be strain-specific (Zhao, et al., 2006), and RP gene transcription has been reported to be unaffected by the absence of Ifh1 and Fhl1 binding to RP genes (Hall, et al., 2006; Zhao, et al., 2006). These results indicate the existence of alternate mechanisms for controlling RP gene transcription, perhaps involving Sch9 (Jorgensen, et al., 2004; Urban, et al., 2007). Interestingly, the finding that Ifhl1 can be observed in a complex with casein kinase 2 (CK2) and the rRNA

processing factors Utp22 and Rrp7 (Rudra, et al., 2007) suggests a potential regulatory connection between RP gene transcription and transcription of 35S rDNA, which is tightly coupled to rRNA processing (Schneider, et al., 2007). Second, TOR signaling may also regulate RP promoters via Hmo1 (Berger, et al., 2007). Hmo1 is required for proper TORC1-dependent regulation of RP genes, and is released from some RP gene promoters upon rapamycin treatment (Berger, et al., 2007). Third, TORC1 activity has been found to regulate the nucleocytoplasmic localization of Sfp1, a positive regulator of RP gene transcription (Jorgensen, et al., 2004; Marion, et al., 2004). Recent studies have further shown that Sfp1 is bound and phosphorylated by TORC1, and that TORC1 inhibition causes Sfp1 dephosphorylation and release from RP promoters (Lempiainen, et al., 2009). Sfp1 nuclear localization has been reported to be regulated by the vesicular-trafficking-related Rab escort protein Mrs6, although studies have reached differing conclusions as to whether the Sfp1-Mrs6 interaction is regulated by TORC1 activity (Lempiainen, et al., 2009; Singh and Tyers, 2009).

1.1.5.2.3 RNA polymerase II-transcribed ribosome biogenesis (RiBi) genes

The ribosome biogenesis (RiBi) genes encode ~200 transcriptionally co-regulated factors involved in ribosome assembly and translation that are transcribed by RNA polymerase II (Figure 1-5C) (Jorgensen, et al., 2004; Wade, et al., 2001). Recent studies have uncovered the long sought-after DNA-binding proteins that bind to the RRPE and PAC elements in the upstream region of RiBi genes – Stb3 was found to bind the RRPE element (Liko, et al., 2007), while Tod6 and Dot6 were found to bind the PAC element (Badis, et al., 2008; Freckleton, et al., 2009; Zhu, et al., 2009). Consistent with evidence that these factors are phosphorylated in an Sch9-dependent manner (Huber, et al., 2009), further characterization of Stb3, Tod6, and Dot6 suggested that they act as transcriptional repressors that are antagonized by TORC1 signaling (Liko, et al., 2010; Lippman and Broach, 2009). Transcription of RiBi genes has also been found to be activated by the TORC1-regulated transcription factor Sfp1 – however, since Sfp1 can be cross-linked to RP genes but not RiBi genes, the mechanism by which Sfp1 exerts this effect is unclear (Cipollina, et al., 2008a; Cipollina, et al., 2008b; Fingerman, et al., 2003; Jorgensen, et al., 2004; Marion, et al., 2004).

1.1.5.2.4 RNA polymerase III-transcribed 5S rRNA

The 5S rRNA and tRNA genes are transcribed by RNA polymerase III (Figure 1-5D). In yeast, TORC1 has been shown to antagonize the polymerase III repressor Maf1 (Willis and Moir, 2007) at least in part via regulation of Maf1 nuclear localization by Sch9 (Huber, et al., 2009; Lee, et al., 2009; Wei and Zheng, 2009). Maf1 has also been reported to be downstream of mTOR in human cells (Kantidakis, et al., 2010; Michels, et al., 2010; Shor, et al., 2010). In addition, TOR signaling may directly impinge on other components at polymerase III-transcribed genes, as TORC1 has been found to physically associate directly with the 5S rDNA in yeast (Wei, et al., 2009b) and mammalian cells (Kantidakis, et al., 2010).

1.1.5.2.5 Ribosome assembly

During the process of ribosome biogenesis, many trans-acting factors transiently associate with nascent pre-ribosomes as they sequentially travel through the nucleolus, nucleus, and cytoplasm (Henras, et al., 2008; Tschochner and Hurt, 2003). Short-term rapamycin treatment has been reported to cause many ribosome assembly factors to accumulate in the nucleolus or nucleus (Honma, et al., 2006; Shin, et al., 2009; Vanrobays, et al., 2008), suggesting that nucleocytoplasmic translocation of pre-ribosomal particles may be regulated by TORC1.

1.1.5.3 mRNA stability

TORC1 has long been known to control mRNA degradation (Albig and Decker, 2001), but the mechanisms have remained unclear. Interestingly, a recent study has shown that the Igo1 and Igo2 proteins are direct targets of the kinase Rim15, which is repressed downstream of TORC1 (Talarek, et al., 2010). TORC1-regulated Igo1/2 was found to control the degradation of mRNAs via the 5'-3' mRNA decay pathway (Talarek, et al., 2010).

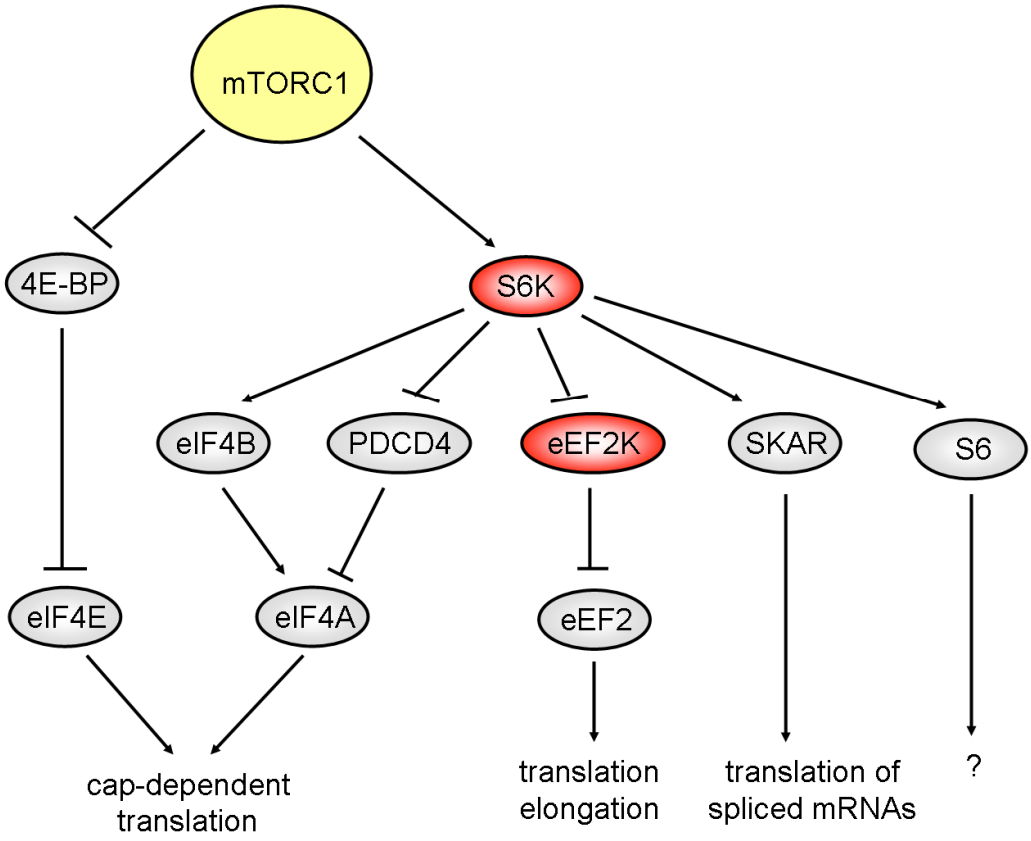
1.1.5.4 Translation

Regulation of translation was one of the first readouts found to be downstream of TOR signaling (Barbet, et al., 1996). TOR was hypothesized to regulate translation initiation via the translation initiation factor 4E (eIF4E) (the 5'-mRNA cap-binding protein, encoded by *CDC33* in yeast) (Barbet, et al., 1996). Since then, TORC1 been shown to control translation via several mechanisms.

First, TORC1 has been found to regulate the eukaryotic translation initiation factor 2α (eIF2α; encoded by *SUI2* in yeast) via the eIF2α kinase Gcn2 (Cherkasova and Hinnebusch, 2003; Kubota, et al., 2003). TORC1 activity, via inhibition of Tap42-associated phosphatases, was shown to maintain Gcn2 phosphorylation at Ser 577. This, in turn, antagonizes Gcn2's inhibitory kinase activity towards eIF2α and thus sustains high rates of global translation initiation (Hinnebusch, 2005). Interestingly, TORC1 has also been shown to regulate eIF2α phosphorylation via the second TOR effector branch downstream of Sch9 (Urban, et al., 2007). Sch9 can also phosphorylate ribosomal protein S6 (Urban, et al., 2007), but the functional significance of this modification in yeast is unclear (Johnson and Warner, 1987). Second, TORC1 inhibition has been shown to result in degradation of eukaryotic translation initiation factor 4G (eIF4G) (Berset, et al., 1998). Loss of Eap1 – the yeast analog of the metazoan eIF4E-binding proteins (4E-BPs) – has been found to confer rapamycin resistance (Cosentino, et al., 2000), although it is unclear if Eap1 functions downstream of yeast TORC1 as 4E-BPs do in higher eukaryotes (Matsuo, et al., 2005).

The connections between TORC1 and translation have been more extensively characterized in metazoans (Figure 1-6). In mammalian cells, mTORC1 has been found to regulate translation via two major substrates: eIF4E-binding proteins (4E-BPs) and S6 kinases (S6Ks) (Ma and Blenis, 2009). TORC1 phosphorylation of 4E-BP prevents 4E-BP from binding to eIF4E, thus allowing eIF4E to promote cap-dependent translation of mRNAs with complex secondary structures in their 5' untranslated regions (5' UTRs) – such mRNAs tend to encode proteins that promote growth and proliferation (Ma and Blenis, 2009). The TORC1 effector S6K has been shown to control translation via several downstream proteins, including eukaryotic elongation factor 2 kinase (eEF2K), eukaryotic translation initiation factor 4B (eIF4B), programmed cell death 4 (PDCD4), S6K1 aly/REF-like target (SKAR), and ribosomal protein S6.

Figure 1-6. Regulation of translation by mTORC1 in mammalian cells. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. See legend to Figure 1-3 for colour scheme.

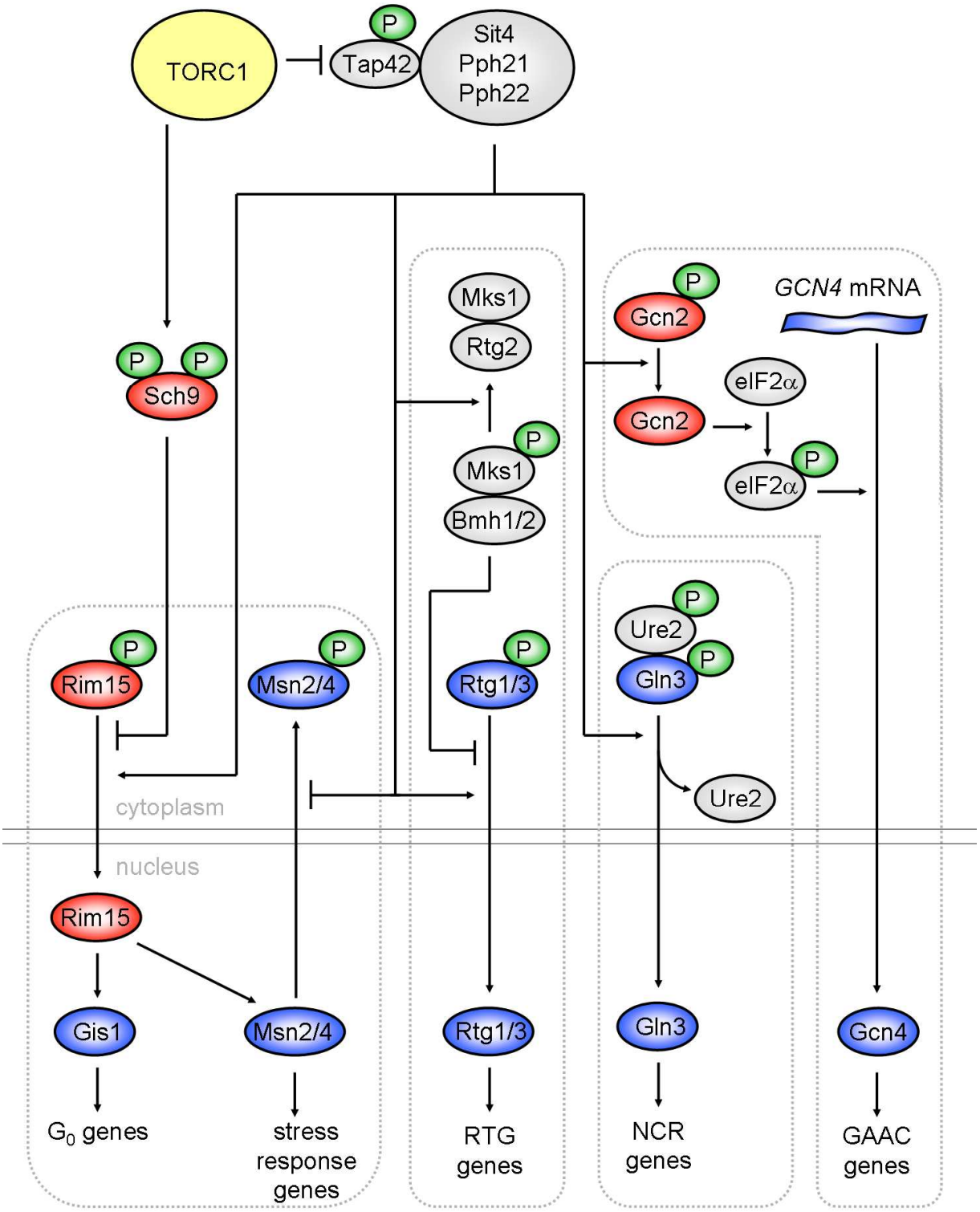

mTORC1
4E-BP
S6K
eIF4B
PDCD4
eEF2K
SKAR
S6
eIF4E
eIF4A
eEF2
cap-dependent
translation
translation
elongation
translation of
spliced mRNAs
?

First, S6K phosphorylation of eEF2K prevents eEF2K from phosphorylating and inhibiting eukaryotic elongation factor 2 (eEF2), thus allowing eEF2 to promote translation elongation (Wang, et al., 2001). Second, S6K phosphorylation of eIF4B enhances its ability to activate eIF-4A, a helicase that facilitates translation initiation of mRNAs with secondary structure in their 5' UTR (Raught, et al., 2004; Shahbazian, et al., 2006). Third, S6K phosphorylation of PDCD4 earmarks PDCD4 for degradation and prevents it from inhibiting the helicase activity of eIF-4A (Dorrello, et al., 2006). Fourth, S6K phosphorylation of (and interaction with) SKAR recruits S6K to the exon junction complex (EJC) on newly synthesized mRNAs, thereby enabling S6K to phosphorylate additional targets to increase the translation efficiency of spliced mRNAs (Ma, et al., 2008; Richardson, et al., 2004). Finally, S6K phosphorylates the substrate for which it was named – ribosomal protein S6, a component of the 40S ribosomal subunit. For many years it was hypothesized that S6 phosphorylation by S6K promoted the translation of a class of mRNAs with a 5' terminal oligopyrimidine tract (5' TOP mRNAs), which encode components of the translation machinery (Meyuhas, 2008). However, recent studies using cells lacking S6K (Pende, et al., 2004) or expressing a non-phosphorylatable version of S6 (Ruvinsky, et al., 2005) have clearly falsified this model. Thus, as in yeast (Johnson and Warner, 1987), the functional significance of S6 phosphorylation in mammalian cells is unclear. The mechanism by which TOR regulates the translation of 5' TOP mRNAs remains unknown, but has been suggested to involve a TOR complex distinct from the canonical mTORC1 and mTORC2 (Patursky-Polischuk, et al., 2009).

1.1.5.5 Transcription

TORC1 regulates a large swath of the transcriptome by controlling the activity of downstream transcription factors, often by regulating their nucleocytoplasmic localization. Transcriptional profiling experiments have shown that TORC1 antagonizes stress-responsive transcription (Figure 1-7) (Cardenas, et al., 1999; Chen and Powers, 2006; Hardwick, et al., 1999; Komeili, et al., 2000; Shamji, et al., 2000).

Figure 1-7. Regulation of stress-responsive transcription by TORC1 in yeast. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. See legend to Figure 1-3 for colour scheme. GAAC, general amino acid control; NCR, Nitrogen catabolite repression; RTG, retrograde.


TORC1
P
Tap42
Sit4
Pph21
Pph22
P
P
Sch9
Mks1
Rtg2
P
Mks1
Bmh1/2
P
Gcn2
GCN4 mRNA
Gcn2
eIF2α
P
eIF2α
P
Ure2
P
Gln3
Ure2
P
Rim15
P
Msn2/4
P
Rtg1/3
cytoplasm
nucleus
Rim15
Gis1
Msn2/4
Rtg1/3
Gln3
Gcn4
G0 genes
stress response genes
RTG genes
NCR genes
GAAC genes

1.1.5.5.1 General amino acid control (GAAC)

The general amino acid control (GAAC) pathway is activated under stress and poor nutrient conditions – its underlying rationale is to conserve amino acids by decreasing rates of protein synthesis, while simultaneously inducing amino acid biosynthesis genes (Figure 1-7) (Hinnebusch, 2005). As noted above, inhibition of TORC1 has been shown to lead to loss of Gcn2 phosphorylation at Ser 577 (Cherkasova and Hinnebusch, 2003; Kubota, et al., 2003). This enhances Gcn2's kinase activity (which is also activated by uncharged tRNAs, a proxy for amino acid scarcity). Subsequent phosphorylation of eIF2α by Gcn2 leads to decreased rates of global translation initiation, but – paradoxically – derepression of the mRNA encoding the transcription factor Gcn4. The latter occurs via a fascinating mechanism involving four short upstream open reading frames (uORFs) in the *GCN4* mRNA leader (Hinnebusch, 2005). Gcn4 then activates the transcription of more than 30 amino acid biosynthesis genes from 12 different pathways.

1.1.5.5.2 Nitrogen catabolite repression (NCR)

Nitrogen catabolite repression (NCR)-sensitive transcription is activated in the presence of non-preferred nitrogen sources (proline, allantoin) – its underlying rationale is to induce genes required for the import and catabolism of these compounds (Figure 1-7) (Cooper, 2002; Hofman-Bang, 1999; Wong, et al., 2008). The NCR system comprises four DNA-binding GATA factors: two transcriptional activators (Gln3 and Gat1), and two transcriptional repressors (Dal80 and Gzf3). TORC1 inhibition by rapamycin has been shown to cause Gln3 dephosphorylation, Gln3 dissociation from its cytoplasmic binding partner Ure2, Gln3 nuclear entry, and the induction of NCR-sensitive transcription (Beck and Hall, 1999; Carvalho, et al., 2001). TORC1 activity has also been found to regulate phosphorylation of Gln3's cytoplasmic repressor protein Ure2 (Cardenas, et al., 1999; Hardwick, et al., 1999).

However, since these seminal observations, the picture has revealed itself to be much more complex and nuanced: Gln3 phosphorylation status does not always correlate with its nuclear localization (Tate and Cooper, 2007; Tate, et al., 2009; Tate, et al., 2005); Gln3 and Gat1 are regulated differently (Crespo, et al., 2002; Georis, et al., 2008; Kulkarni, et al., 2006; Kuruvilla, et al., 2001; Tate, et al., 2010); and outputs of the system depend on multiple aspects

of strain genetic background (Georis, et al., 2009; Tate, et al., 2006) and the particular nitrogen source used (Godard, et al., 2007). In particular, there is now a growing body of evidence suggesting that rapamycin treatment and nitrogen limitation are not equivalent interventions, and elicit NCR-sensitive transcription via partially distinct mechanisms (Cox, et al., 2004a; Cox, et al., 2002; Cox, et al., 2004b; Magasanik, 2005; Puria and Cardenas, 2008; Puria, et al., 2008; Tate and Cooper, 2003; Tate, et al., 2002).

1.1.5.5.3 Mitochondrial retrograde (RTG) response

The mitochondria-to-nucleus retrograde (RTG) pathway is activated in response to loss of mitochondrial function – its underlying rationale is to induce genes required for the production of α-ketoglutarate (critical for the synthesis of glutamate, glutamine, and other amino acids), which would otherwise be depleted in the absence of a functional tricarboxylic acid (TCA) cycle in respiration-deficient cells (Figure 1-7) (Butow and Avadhani, 2004; Liu and Butow, 2006). The core RTG pathway comprises the heterodimeric transcription factor Rtg1/Rtg3, the upstream positive regulator Rtg2, and the upstream negative regulator Mks1. TORC1 inhibition by rapamycin has been shown to cause Mks1 dephosphorylation, Mks1 dissociation from the 14-3-3 proteins Bmh1/2, Mks1 interaction with Rtg2, and nuclear entry of Rtg1/Rtg3 (Dilova, et al., 2004; Dilova, et al., 2002; Komeili, et al., 2000; Liao and Butow, 1993; Liu, et al., 2003; Sekito, et al., 2002; Tate, et al., 2002). The relationship between RTG pathway activation by rapamycin and activation by mitochondrial dysfunction is complex not completely understood (Giannattasio, et al., 2005). Interestingly, Lst8 – a known component of TORC1 and TORC2 – has been shown to negatively regulate the RTG pathway at two points, one upstream and one downstream of Rtg2 (Liu, et al., 2001).

1.1.5.5.4 Stress response

Nutrient starvation and a wide range of noxious stresses activate several transcription factors in yeast, including Msn2 and Msn4 (which regulate STRE element-controlled genes), and Gis1 (which regulates PDS element-controlled genes). These factors are regulated in part by TORC1 and drive the expression of a large suite of genes encoding molecular chaperones, antioxidant defense enzymes, and other factors involved in the response to stress and starvation (Figure 1-7) (Cameroni, et al., 2004; Gasch, 2007; Gasch and Werner-Washburne, 2002). TORC1 antagonizes the nuclear localization of Msn2/4 (Beck and Hall, 1999; Gorner, et al.,

2002) by means of both the Tap42 and Sch9 effector branches (Santhanam, et al., 2004; Urban, et al., 2007). Interestingly, TORC1 also regulates Msn2/4 and Gis1 in part by antagonizing the nuclear localization of their upstream activator, the kinase Rim15 (Pedruzzi, et al., 2003; Swinnen, et al., 2006; Wanke, et al., 2008; Wanke, et al., 2005).

1.1.5.5.5 Metabolism

In yeast, TORC1 activates transcription of glycolytic genes and represses transcription of respiratory genes (Hardwick, et al., 1999; Lavoie and Whiteway, 2008; Shamji, et al., 2000), consistent with it also repressing mitochondrial function at the posttranscriptional level (Bonawitz, et al., 2007; Lavoie and Whiteway, 2008; Pan and Shadel, 2009). In contrast, in mammalian cells, mTOR activates transcription of respiratory genes via the transcription factor YY1 and cofactor PCG1α (Cunningham, et al., 2007), and positively regulates mitochondrial function (Bentzinger, et al., 2008; Chen, et al., 2008; Ramanathan and Schreiber, 2009; Schieke, et al., 2006). In mammalian cells, mTOR has also been shown to transcriptionally regulate many other metabolic pathways, including glycolysis, the pentose phosphate pathway, and lipid biosynthesis, via the transcription factors SREBP-1, HIF1α, and PPARγ (Brugarolas, et al., 2003; Duvel, et al., 2010; Hudson, et al., 2002; Kim and Chen, 2004; Li, et al., 2010; Peng, et al., 2002; Porstmann, et al., 2008; Zhong, et al., 2000).

1.1.5.6 Nutrient import

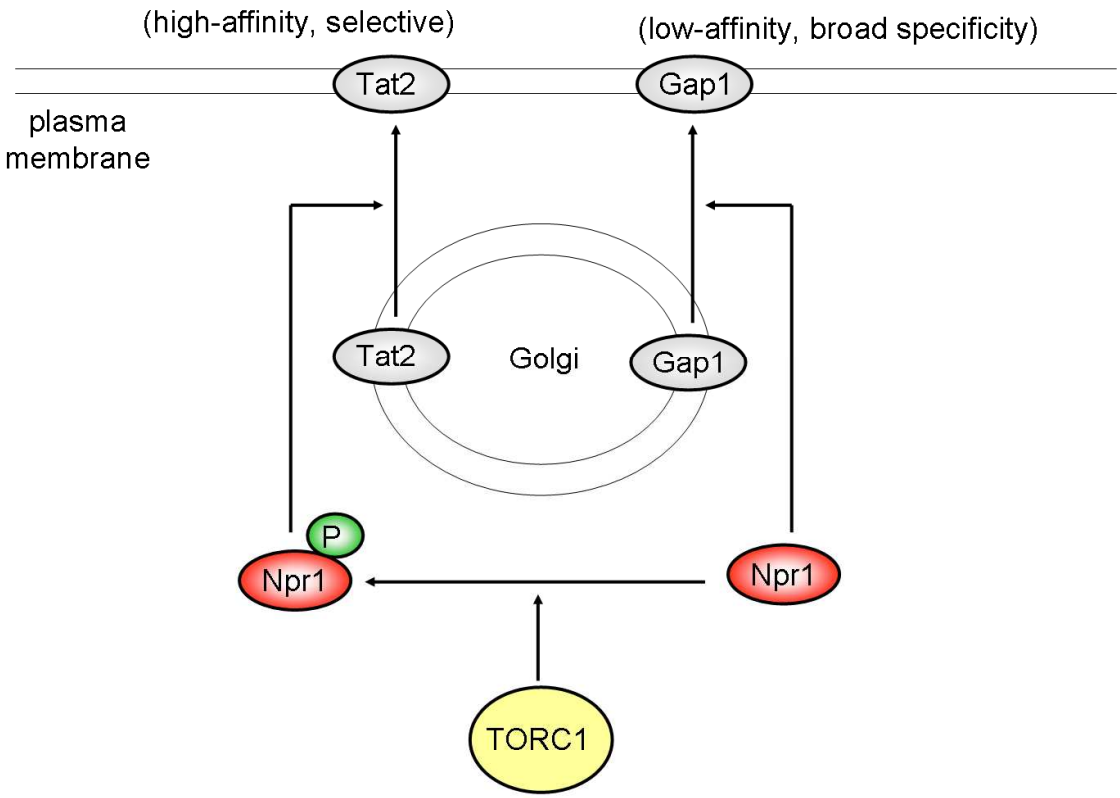
Yeast cells display a clear preference for consumption of certain “preferred” carbon and nitrogen sources, which they will selectively use before starting to consume “non-preferred” sources. In general terms, high-affinity, selective permeases for preferred nutrients are active under favourable nutrient conditions, while low-affinity, non-selective permeases for non-preferred nutrients are active under poor nutrient conditions (Magasanik and Kaiser, 2002). TORC1 is thought to impinge on this system by regulating the expression, activity, and intracellular sorting of nutrient permeases (Figure 1-8). First, by antagonizing NCR-sensitive transcription (see above), TORC1 negatively regulates the expression of low-affinity, non-selective permeases like the general amino-acid permease (encoded by *GAP1*) and the ammonia permease (encoded by *MEP2*) (Magasanik and Kaiser, 2002). Second, TORC1 has been shown to positively regulate the plasma membrane targeting of high-affinity, selective permeases like the tryptophan permease (encoded by *TAT2*) by promoting inhibitory phosphorylation of the

Npr1 kinase (Beck, et al., 1999; Jacinto, et al., 2001; Schmelzle, et al., 2004; Schmidt, et al., 1998). Npr1 has also been implicated in intracellular trafficking of the general amino-acid permease Gap1 (De Craene, et al., 2001; Soetens, et al., 2001), but the mechanisms by which Npr1 controls permease sorting are unclear.

1.1.5.7 Autophagy

Autophagy is a process in which cellular material is enclosed in double-membrane vesicles (autophagosomes), delivered to the vacuole, and degraded to release its macromolecules for recycling (Cebollero and Reggiori, 2009; He and Klionsky, 2009; Nakatogawa, et al., 2009). One of the earliest steps in autophagy is formation of the pre-autophagosomal structure (PAS) – the site of autophagosome nucleation – by a protein complex containing the kinase Atg1 and its binding partner Atg13 (Stephan and Herman, 2006; Xie and Klionsky, 2007). TORC1, in concert with PKA, has been found to antagonize autophagy via inhibitory phosphorylation of Atg1 and Atg13, thus preventing formation of the Atg1-Atg13 complex (Kamada, et al., 2000; Kamada, et al., 2010; Noda and Ohsumi, 1998; Schmelzle, et al., 2004; Stephan, et al., 2009; Yorimitsu, et al., 2007). TORC1 may also antagonize autophagy by repressing the transcription of autophagy genes by Gln3 (Chan, et al., 2001) and Gcn4 (Natarajan, et al., 2001). Paradoxically, Tap42-associated phosphatases – which typically act in the opposite direction to TORC1-dependent kinase activity – have been found, like TORC1, to negatively regulate autophagy (Yorimitsu, et al., 2009). Consistent with overall conservation of the autophagy machinery between yeast and higher eukaryotes (Diaz-Troya, et al., 2008; Jung, et al., 2010; Neufeld, 2010; Yang and Klionsky, 2010), recent work has revealed that mTORC1 also controls autophagy in mammalian cells via a multi-protein assembly analogous to the yeast Atg1 complex (Chan, 2009; Ganley, et al., 2009; Hosokawa, et al., 2009; Jung, et al., 2009).

Figure 1-8. Regulation of amino acid permeases by TORC1 in yeast. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. See legend to Figure 1-3 for colour scheme.


(high-affinity, selective)
(low-affinity, broad specificity)
Tat2
Gap1
plasma
membrane
Tat2
Golgi
Gap1
P
Npr1
Npr1
TORC1

1.1.6 Feedback loops

TORC1 appears to be subject to a variety of regulatory loops, both positive and negative. Negative feedback loops would be expected to dampen large changes in TORC1 activity, while positive feedback loops would be expected to amplify small initial changes in TORC1 activity.

Inhibiting protein synthesis with cycloheximide has been shown to increase TORC1 activity in yeast (Urban, et al., 2007) and mammalian cells (Price, et al., 1989), likely due to an increase in intracellular amino acid pools under conditions where translation is blocked (Beugnet, et al., 2003a; Binda, et al., 2009). TORC1 activity was also increased by deletion of either of its substrates Sch9 or Sfp1 (Lempiainen, et al., 2009), indicating the existence of negative feedback on TORC1 activity by one or more processes downstream of these effectors. In contrast, a positive feedback loop appears to be mediated by the Tip41 protein, which binds and inhibits the TORC1 effector Tap42 (Jacinto, et al., 2001). TORC1 inhibition has been shown to lead to Tip41 dephosphorylation and increased association with Tap42, thus reinforcing the decrease in TORC1 signaling (Jacinto, et al., 2001).

In metazoans, TORC1 is also subject to positive and negative feedback loops (Efeyan and Sabatini, 2010). The most well-characterized negative feedback loop impinging on TOR is the downregulation of PI3K/Akt signaling in response to increased mTORC1 activity. Several mechanisms have been proposed to contribute to this effect (Harrington, et al., 2004; Shah and Hunter, 2006; Tremblay, et al., 2007; Tzatsos, 2009), but most involve negative regulation of IRS-1, which bridges PI3K and the insulin receptor (Harrington, et al., 2005; Manning, 2004). In addition, mTORC1 appears to be the target of negative feedback from autophagy – inhibition of mTORC1 induces autophagy, leading to the release of amino acids, which in turn positively regulate mTORC1 (Yu, et al., 2010). Chronic activation of mTORC1 triggers production of the Sestrin 1 and Sestrin 2 proteins, which in turn negatively regulate mTORC1 in yet another negative feedback loop (Lee, et al., 2010).

On the other hand, mTORC1 appears to be subject to positive feedback loops involving Deptor, PRAS40, and Atg1. Deptor and PRAS40 appear to be both components and substrates of TORC1, and have been proposed to act as negative regulators of mTORC1 that are inhibited by

mTORC1-mediated phosphorylation (Fonseca, et al., 2008; Fonseca, et al., 2007; Oshiro, et al., 2007; Peterson, et al., 2009; Sancak, et al., 2007; Thedieck, et al., 2007; Vander Haar, et al., 2007; Wang, et al., 2008a; Wang, et al., 2007). Similarly, the autophagy-inducing kinase Atg1 is negatively regulated by TORC1 and also acts to inhibit TORC1 signaling (Lee, et al., 2007c; Scott, et al., 2007).

1.2 TORC1 control of the cell cycle

The most obvious effect of TORC1 on the yeast cell cycle is illustrated by the well-established ability of rapamycin to rapidly induce cell cycle arrest in G1/G0. The G1 arrest caused by rapamycin is likely due to inhibition of translation initiation and the consequent loss of unstable G1 cyclins required for the G1/S transition (Barbet, et al., 1996; Danaie, et al., 1999; Moore, 1988; Polymenis and Schmidt, 1997; Popolo, et al., 1982). Other work has implicated TORC1 in the mechanisms that coordinate cell growth and cell division (Jorgensen, et al., 2002; Jorgensen, et al., 2004). Yeast cells must grow to a minimum cell size before initiating the G1/S transition, and this size threshold is known to be modified according to nutrient conditions – the threshold is set high in favourable nutrient conditions, and set low in poor nutrient conditions (Johnston, et al., 1979). Recent studies have shown that loss of either Sfp1 or Sch9 results in a striking decrease in the cell size threshold for division, similar to that observed in poor nutrient conditions (Jorgensen, et al., 2002; Jorgensen, et al., 2004). This finding suggests that a signal or process downstream of these TORC1 effectors – potentially having to do with ribosome biogenesis – communicates nutrient status to the G1/S cell cycle machinery (Jorgensen, et al., 2004). In contrast to the rapamycin-induced G1 arrest noted above, the relevant signal is unlikely to be reduced translation because partial blockade of translation with cycloheximide *increases* (rather than decreases) the size threshold (Jorgensen, et al., 2004).

TORC1 also impinges on the cell cycle in other ways. A recent study has found that TORC1 positively regulates the G2/M transition via the Polo-like kinase Cdc5 (Nakashima, et al., 2008). In addition, in diploid yeast, TORC1 activity has been shown to influence the developmental switch to sporulation by antagonizing nuclear localization of the transcription factor Ime1, a master regulator of meiosis (Colomina, et al., 2003). In metazoans, loss of mTORC1 signaling has been shown to enforce G1 arrest, at least in part, by activation of the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ (Kawamata, et al., 1998; Luo, et al., 1996) and

repression of G1 cyclins at the mRNA and protein level (Averous, et al., 2008; Decker, et al., 2003; Hashemolhosseini, et al., 1998; Hleb, et al., 2004).

1.3 TORC1 control of lifespan

The mechanisms that regulate aging and age-related disease have been subjected to intense study in recent years, leading to many significant advances in what has become a fast-moving field (Greer and Brunet, 2008; Houtkooper, et al., 2010; Kenyon, 2010; Kirkwood, 2008; Partridge, 2010; Sahin and Depinho, 2010; Vijg and Campisi, 2008). In particular, TOR signaling has emerged as a evolutionary conserved regulator of lifespan (Hands, et al., 2009; Kapahi, et al., 2010; Stanfel, et al., 2009), and yeast has emerged as a powerful model system in which to study the underlying regulatory mechanisms (Kaeberlein, 2010; Kaeberlein, et al., 2007; Piper, 2006). Caloric restriction (CR) is an intervention that reduces an organism's nutrient intake without causing malnutrition, and is the only condition that has been shown to extend lifespan in both yeast, flies, worms, rodents (Fontana, et al., 2010; Mair and Dillin, 2008), and – most recently – primates (Colman, et al., 2009). The mechanisms by which CR extends lifespan have been highly contentious (Baur, et al., 2010; Chen and Guarente, 2007; Kaeberlein and Powers, 2007; Kennedy, et al., 2007; Sinclair, 2005; Steinkraus, et al., 2008a). Nevertheless, on balance, genetic epistasis tests in multiple organisms suggest that CR regulates lifespan, at least in part, via TOR signaling (Kapahi, et al., 2010; Stanfel, et al., 2009). Indeed, rapamycin treatment has recently been shown to increase lifespan in mice (Harrison, et al., 2009). In yeast, two measures of lifespan can be assayed – replicative lifespan and chronological lifespan – and TORC1 activity has been found to regulate both.

1.3.1 Yeast replicative lifespan

Yeast cells undergo asymmetric division, producing a large mother cell and small daughter cell (Hartwell and Unger, 1977). Replicative lifespan is measured by the number of daughter cells that a mother can produce before undergoing permanent cell cycle arrest, and is thought to be a useful model for the aging of proliferating cells in metazoans (Steinkraus, et al., 2008a). The number of yeast cell divisions before senescence is finite because one or more putative aging factors are preferentially inherited by the mother at division, while the age of the daughter is reset to zero (Henderson and Gottschling, 2008). Replicative lifespan is typically

assessed in a painstaking assay in which individual yeast mother cells are grown on solid media and repeatedly separated from their daughters by micro-dissection (Steinkraus, et al., 2008a).

Decreased TORC1 signaling has been shown to increase replicative lifespan, and recent studies have implicated protein synthesis as the key effector pathway downstream of TORC1. Yeast replicative lifespan has been extended by loss of Tor1 (Kaeberlein, et al., 2005b), Sch9 (Kaeberlein, et al., 2005b), and factors involved in translation such as ribosomal proteins, translation initiation factors, and rRNA processing factors (Chiocchetti, et al., 2007; Kaeberlein, et al., 2005b; Steffen, et al., 2008). Loss of TORC1 signaling has also extended the lifespan of worms (Jia, et al., 2004; Vellai, et al., 2003), flies (Kapahi, et al., 2004; Luong, et al., 2006) and mice (Harrison, et al., 2009; Selman, et al., 2009). As in yeast, mRNA translation also seems to be a key determinant of longevity in flies (Bjedov, et al., 2010; Zid, et al., 2009) and worms (Curran and Ruvkun, 2007; Hansen, et al., 2007; Pan, et al., 2007; Syntichaki, et al., 2007; Tohyama, et al., 2008). Thus, the cellular processes regulating replicative lifespan in yeast have proven to be relevant to longevity in higher eukaryotes. Interestingly, differential translation of certain mRNAs could be one mechanism by which translation impacts lifespan – the lifespan increase observed in yeast deleted for ribosomal proteins was linked to increased translation of the transcript encoding the nutrient- and stress-responsive transcription factor Gcn4 (Steffen, et al., 2008).

Alternatively, TORC1 may also regulate replicative lifespan, at least in part, via modulation of extrachromosomal rDNA circles (ERCs), which have been correlated with replicative aging in yeast (Sinclair and Guarente, 1997; Steinkraus, et al., 2008a). Loss of Tor1, rapamycin treatment, and loss of Sch9 have recently been reported to decrease recombination at the rDNA (Medvedik, et al., 2007; Riesen and Morgan, 2009). Indeed, this is consistent with previous reports suggesting that Sch9 can regulate recombination and genomic instability (Defossez, et al., 1999; Lin and Keil, 1991; Madia, et al., 2008; Prusty and Keil, 2004; Scholes, et al., 2001). However, the significance of this observation is unclear, given that ERCs are proposed to be a “private” aging mechanism (Partridge and Gems, 2002) that is specific to yeast (Steinkraus, et al., 2008a), while TORC1’s control of lifespan is conserved across species (Kapahi, et al., 2010; Stanfel, et al., 2009).

1.3.2 Yeast chronological lifespan

Chronological lifespan is measured by the length of time yeast cells can survive in a non-dividing state, and is thought to be a useful model for the aging of non-proliferating cells in metazoans (Fabrizio and Longo, 2003). It is typically assessed by growing yeast cells to saturation in synthetic liquid media and then measuring the viability of the population over time, by checking what fraction of the population can re-enter the cell cycle when moved to fresh media (Fabrizio and Longo, 2003).

Decreased TORC1 signaling has been shown to increase chronological lifespan, and recent studies have implicated stress resistance, autophagy, and a metabolic shift towards respiration as the key effector pathways downstream of TORC1. Recent reports have linked chronological lifespan extension conferred by loss of Tor1 and Sch9 to expression of stress response genes downstream of Rim15, Msn2/4, and Gis1 (Fabrizio, et al., 2003; Fabrizio, et al., 2001; Powers, et al., 2006; Wei, et al., 2008), autophagy (Alvers, et al., 2009b), and increased respiration (Bonawitz, et al., 2007; Lavoie and Whiteway, 2008; Pan and Shadel, 2009).

These mechanisms appear to be conserved across model organisms: increased respiration has been linked to extended longevity in yeast (Lin, et al., 2002; Oliveira, et al., 2008; Smith, et al., 2007), worms (Bishop and Guarente, 2007; Schulz, et al., 2007), and flies (Tong, et al., 2007; Zid, et al., 2009); autophagy has been shown to be a key determinant of lifespan in yeast (Alvers, et al., 2009a; Eisenberg, et al., 2009; Fabrizio, et al., 2010; Matecic, et al., 2010), worms (Eisenberg, et al., 2009; Hansen, et al., 2008; Hars, et al., 2007; Jia and Levine, 2007; Melendez, et al., 2003; Toth, et al., 2008), and flies (Eisenberg, et al., 2009); and stress-responsive transcription factors have been implicated in regulating lifespan in yeast (see above), worms (Bishop and Guarente, 2007; Chen, et al., 2009; Libina, et al., 2003; Lin, et al., 1997; Murphy, et al., 2003; Ogg, et al., 1997; Panowski, et al., 2007; Sheaffer, et al., 2008; Steinkraus, et al., 2008b; Tullet, et al., 2008), and flies (Giannakou, et al., 2004; Hwangbo, et al., 2004). Thus, as with replicative lifespan, the cellular processes regulating chronological lifespan in yeast have proven to be relevant to longevity in higher eukaryotes. Interestingly, this is true despite the fact that the key underlying factor that limits lifespan in the standard yeast chronological aging assay appears to be accumulation of extracellular acetic acid (Burtner, et al., 2009), a mechanism that is unlikely to be directly relevant in metazoans.

1.4 TOR signaling and human disease

mTOR signaling is a therapeutic target for several important human diseases and pathologies (Tsang, et al., 2007). First, mutations in upstream regulators of mTOR are known to cause a number of inherited syndromes characterized by tumour-like growths (hamartomas), including tuberous sclerosis (mutant TSC1 or TSC2), Peutz-Jeghers syndrome (mutant LKB1), Cowden syndrome (mutant PTEN), Bannayan-Riley-Ruvalcaba syndrome (mutant PTEN), Lhermitte-Duclos disease (mutant PTEN), Proteus syndrome (mutant PTEN), von Hippel-Lindau disease (mutant VHL), Neurofibromatosis type 1 (mutant NF1), and Polycystic kidney disease (mutant PKD1) (Inoki, et al., 2005; Rosner, et al., 2008). Second, mTOR activation plays a key role in some types of pathologic tissue hypertrophy, such as cardiac muscle hypertrophy, skeletal muscle hypertrophy, vascular restenosis, and compensatory nephrotic hypertrophy (Lee, et al., 2007a). Third, mTOR has been implicated in immune function, where it is a key regulator of both the innate and adaptive immune responses (Saemann, et al., 2009; Thomson, et al., 2009; Weichhart and Saemann, 2009). Fourth, mTOR has crucial roles in neurological function and neuropathology, by controlling neuronal development and synaptic plasticity (Hoeffer and Klann, 2010; Jaworski and Sheng, 2006), and by regulating apoptosis and the clearance of toxic protein aggregates linked to neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease (Swiech, et al., 2008; Zemke, et al., 2007). Fifth, mTOR signaling is intimately associated with the pathologies of nutrient overload, including insulin resistance, diabetes, obesity, and metabolic disease (Dann, et al., 2007; Inoki, 2008; Um, et al., 2006). Finally, mTOR has long been recognized as a major target for cancer therapeutics (Chiang and Abraham, 2007; Easton and Houghton, 2006; Faivre, et al., 2006; Gibbons, et al., 2009; Guertin and Sabatini, 2005; Guertin and Sabatini, 2007; Meric-Bernstam and Gonzalez-Angulo, 2009; Sabatini, 2006; Yap, et al., 2008). Although single-agent therapy with rapamycin-based mTOR inhibitors has thus far exhibited only modest efficacy, recent developments – such as a better understanding of the regulatory loops in the mTOR signaling network, a new generation of active-site mTOR inhibitors, and combination strategies using multiple inhibitors – mean that the prospect of mTOR-based anti-cancer drugs still holds great promise (Albert, et al., 2010; Guertin and Sabatini, 2009; Lane and Breuleux, 2009; Wang and Sun, 2009).

1.5 Outlook

Recent years have witnessed a gradual but unmistakable conceptual shift in which the traditional metaphor of "signaling pathways" has given way to an emerging paradigm of "signaling networks". This evolution in thinking has been necessitated by the mounting body of evidence showing that signaling pathways – once thought to be linear and insulated – are in fact highly interconnected (Gibson, 2009). Indeed, there is now a growing recognition that "pathways" often have multiple points of cross-talk or converge on shared downstream targets. For instance, TOR signaling has been found to overlap or interact with many other pathways in yeast, including: the SPS amino-acid-sensing pathway (Shin, et al., 2009), the general amino acid control (GAAC) pathway (Cherkasova and Hinnebusch, 2003; Kubota, et al., 2003; Matsuo, et al., 2005; Rohde, et al., 2004; Sosa, et al., 2003; Staschke, et al., 2010; Valenzuela, et al., 2001), the Snf1 glucose repression pathway (Bertram, et al., 2002; De Wever, et al., 2005; Mayordomo, et al., 2002; Orlova, et al., 2006), the phosphate-sensing Pho80-Pho85 pathway (Wang and Jiang, 2003; Wanke, et al., 2005), the cell wall integrity (CWI) pathway (Ai, et al., 2002; Araki, et al., 2005; Krause and Gray, 2002; Kuranda, et al., 2006; Reinke, et al., 2004; Torres, et al., 2002), and the PKA pathway (Chen and Powers, 2006; Crauwels, et al., 1997; Gorner, et al., 1998; Marion, et al., 2004; Pedruzzi, et al., 2003; Roosen, et al., 2005; Santhanam, et al., 2004; Schmelzle, et al., 2004; Soulard, et al., 2010; Stephan, et al., 2009; Toda, et al., 1988; Yorimitsu, et al., 2007; Zurita-Martinez and Cardenas, 2005).

In reviewing our evolving understanding of TOR signaling over the last decade, a few general observations can be made. First, it is striking to note the degree to which progress in the field has been shaped by the availability of rapamycin. In some ways, the existence of this small-molecule inhibitor has been a double-edged sword. It has greatly facilitated our characterization of the downstream readouts of TORC1, but at the expense of the comfortable assumption – now known to be false (Choo and Blenis, 2009; Dowling, et al., 2010) – that rapamycin inhibits all TORC1 functions equally and completely. Indeed, not only is rapamycin not a perfect inhibitor of TORC1, but it may also have TOR-independent effects in the cell (Limson and Sweder, 2010). Meanwhile, progress in characterizing TORC2 – which is rapamycin-insensitive – has lagged behind. However, the field now seems poised to make rapid progress, armed with a new generation of ATP-competitive, active-site TOR inhibitors capable of blocking both TORC2 and the rapamycin-insensitive functions of TORC1 (Guertin and Sabatini, 2009).

Second, the longstanding absence of a well-characterized readout for TORC1 activity in yeast (analogous to S6K phosphorylation in mammalian cells) has hampered mapping of the upstream signals impinging on TORC1. Here too, the field now seems poised to make further rapid progress with the identification of the kinase Sch9 as a *bona fide* TORC1 substrate and putative ortholog of metazoan S6K (Powers, 2007; Urban, et al., 2007). In addition, the powerful genome-wide approaches available for yeast – for instance, screening the yeast proteome for proteins whose localization (Shin, et al., 2009) or phosphorylation (Huber, et al., 2009) are responsive to TORC1 – hold great promise for expanding our knowledge of the TOR signaling network.

Many future challenges remain. First, how amino acids and other nutrients are sensed at the molecular level and how this information is communicated to TORC1 still represents a major outstanding question. Although important players in this process have recently been identified (Binda, et al., 2009; Kim, et al., 2008; Sancak, et al., 2010; Sancak, et al., 2008), the most proximal signaling events – in which a nutrient or small-molecule metabolite physically interacts with a sensor protein of some type – remain a mystery. Indeed, with some exceptions (Holsbeeks, et al., 2004; Scott, et al., 2009a; Sellick and Reece, 2005), the specific molecular mechanisms by which nutrient and metabolite molecules are sensed by the cell are still generally poorly understood.

Second, we still have an incomplete picture of the substrates and signaling networks that emanate from TORC1. For instance, a strain bearing TOR-independent alleles of both *SCH9* and *TAP42* – the two major known TORC1 effectors – is still sensitive to rapamycin (Huber, et al., 2009), suggesting that TORC1 has additional substrates that have yet to be uncovered. In addition, the mechanisms linking Sch9 and Tap42 to the many downstream readouts of TORC1 have only been partially characterized. Ultimately, further knowledge of the network connectivity and information flow upstream and downstream of TORC1 will enable us to model and further refine of our picture of the TOR signaling (Kuepfer, et al., 2007).

1.6 Rationale

In this work, I sought to expand the TOR signaling network in *Saccharomyces cerevisiae*. Our laboratory's ongoing effort to systematically chart the yeast kinase and phosphatase "interactome" uncovered many new physical interactors of TORC1. In this work, I present

evidence that the novel TORC1 interactors Mks1, Fmp48, and Nnk1 link TOR signaling to various facets of nitrogen metabolism and mitochondrial function. In a separate series of experiments – performed before Sch9 was known to be a direct substrate of TORC1 – I present evidence that Sch9 acts as both an upstream regulator and downstream sensor of mitochondrial function. Taken together, the findings presented herein underscore the central role of TORC1 and its partners as a nexus for nutrient sensing and cell growth in yeast.

Chapter 2 – A global kinase and phosphatase interaction network in the budding yeast reveals novel effectors of the target of rapamycin (TOR) pathway

Work in this chapter was included in the publication:

Breitkreutz, A.*, Choi, H.*, Sharom, J.R.*, Boucher, L.*, Neduva, V.*, Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, X.S., Pawson, T., Gingras, A.-C., Nesvizhskii, A.I., and Tyers, M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* **328**, 1043-1046.

* contributed equally and share first authorship

Contributions:

Some of the experiments shown in this chapter were done in collaboration with Dr. Lorrie Boucher. Dr. Boucher assisted with immune-complex kinase assays (where she helped with cell extract preparation, performed the kinase reactions, and prepared the relevant figures) and expression profiling experiments (where she grew the cells and extracted their RNA). Dr. Boucher also performed the immunoblots shown in this chapter. Cytoscape network diagrams in this chapter were constructed by Dr. Anne-Claude Gingras. The kinase and phosphatase interaction network that provides the foundation for the experiments in this chapter was generated as part of a large-scale collaborative project (see above publication).

2.1 Abstract

The target of rapamycin (TOR) kinases are evolutionarily conserved regulators of cell growth. Our laboratory's systematic charting of kinase and phosphatase interactions in the budding yeast revealed many novel physical interactors of the TOR kinases. Here, I demonstrate that the target of rapamycin complex 1 (TORC1) interactors Mks1, Fmp48, and Nnk1 link TOR signaling to various facets of nitrogen metabolism and mitochondrial function. Prompted by interactions in the KPI network, I also show that deregulation of the Cdc14 phosphatase confers cryptic phenotypes that suggest novel roles for Cdc14 in nutrient, metabolic, and stress signaling. The uncharacterized kinase Nnk1 (Ykl1171w) modulated rapamycin sensitivity, interacted with the nitrogen catabolite repression (NCR) regulator Ure2, impacted Gln3 nuclear localization, and controlled transcription of Gln3 target genes. Nnk1 also interacted with the NAD^+-linked glutamate dehydrogenase Gdh2. In turn, Gdh2 modulated rapamycin sensitivity, was phosphorylated in Nnk1 immune complexes *in vitro*, and was relocalized to a discrete cytoplasmic focus in response to *NNK1* overexpression or respiratory growth. The uncharacterized kinase Fmp48 modulated rapamycin sensitivity, regulated respiratory function and mitochondrial morphology, and Fmp48-associated kinase activity was increased by rapamycin treatment. The retrograde response regulator Mks1 modulated rapamycin sensitivity, regulated respiratory function, and ectopic expression of *MKS1* caused severe growth inhibition with properties suggestive of reduced TORC1 signaling. Taken together, this work reveals intimate connections between TORC1, nitrogen metabolism, and mitochondrial function. These findings are intriguing when viewed in the larger context of emerging evidence from higher eukaryotes that suggests an intimate link between TOR signaling, metabolism, and cancer.

2.2 Results

To chart the budding yeast kinase and phosphatase interaction (KPI) network, our laboratory employed a sensitive method based on rapid magnetic-bead capture of protein complexes, minimal washes, direct on-bead protein digestion, and mass spectrometric identification (Breitkreutz, et al., 2010). Bait proteins were tagged with the HA or FLAG epitope and transiently expressed from the inducible *GAL1* promoter, or tagged with the tandem affinity purification (TAP) tag at their endogenous chromosomal locus. The analysis included 130 protein kinase catalytic subunits, 24 lipid and metabolic kinases, 47 kinase regulatory subunits,

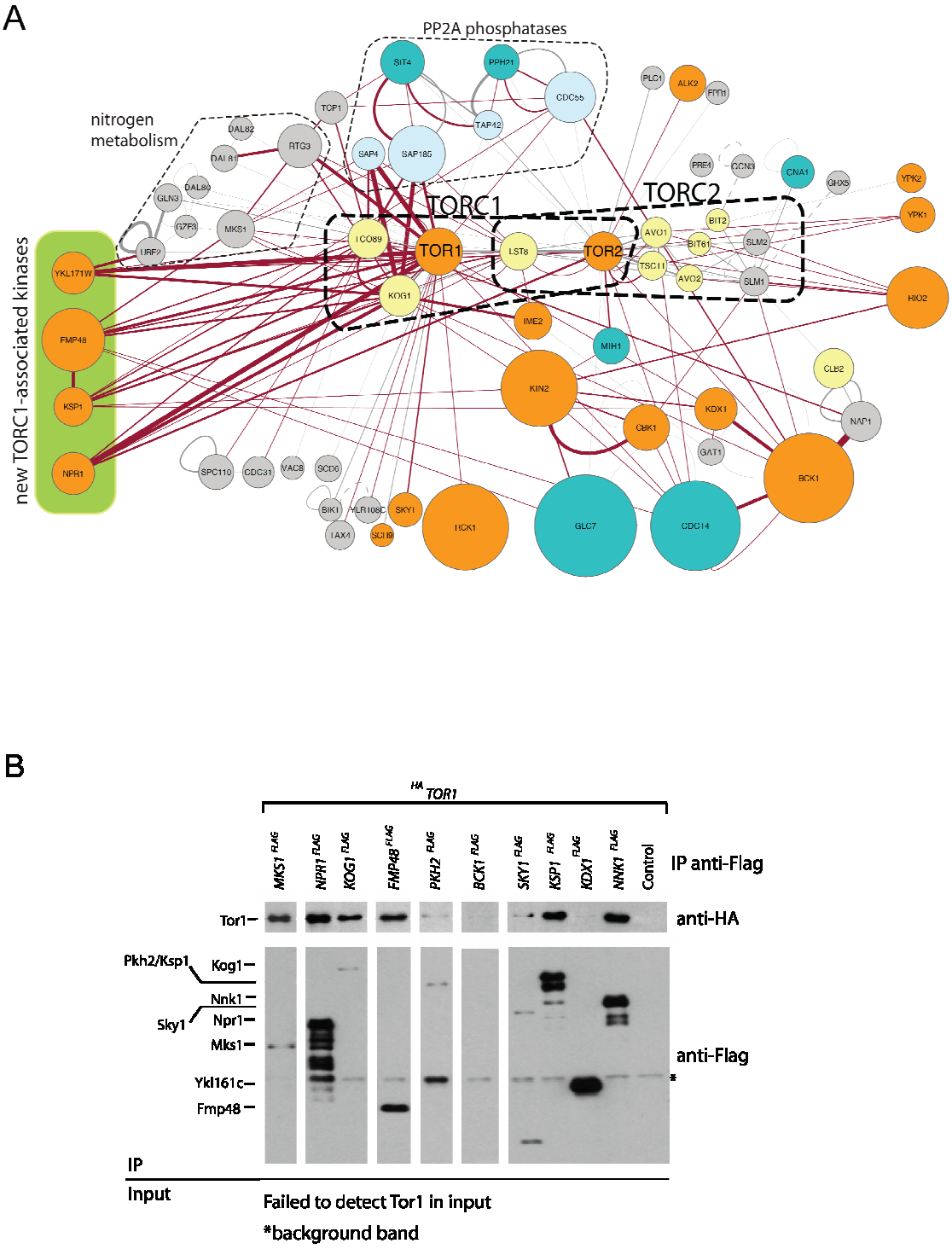
38 protein phosphatases, 32 phosphatase regulatory subunits, and 5 metabolic phosphatases (Breitkreutz, et al., 2010). A dataset of more than 38,000 unfiltered identifications was refined to a final dataset of 1844 interactions between 887 proteins using a statistical model called Significance Analysis of Interactome (SAINT) (Choi, et al., 2011). To investigate the physiological relevance of the interactions in the KPI dataset, I focused on the biological consequences predicted by regions of the network surrounding the target of rapamycin complex 1 (TORC1), and to a lesser extent, the cell-cycle phosphatase Cdc14.

2.2.1 A kinase and phosphatase interaction network reveals novel interactors of the target of rapamycin (TOR) pathway

The TOR kinases are conserved from yeast to human cells and partition into the TORC1 complex (composed of Tor1 or Tor2, Kog1, Lst8 and Tco89) and the TORC2 complex (composed of Tor2, Lst8, Avo1, Avo2, Tsc11/Avo3, Bit2 and Bit61) (De Virgilio and Loewith, 2006a; Wullschleger, et al., 2006). TORC1 controls many aspects of cell growth, including protein synthesis and amino acid metabolism, and is sensitive to the macrolide rapamycin, while TORC2 controls the cytoskeleton in a rapamycin-insensitive manner (De Virgilio and Loewith, 2006a; Wullschleger, et al., 2006). Although TORC1 and TORC2 are not observed in most high-throughput protein-protein interaction datasets (Collins, et al., 2007), Tor1 and Tor2 represent a major hub in the KPI network (Figure 2-1A).

The KPI network recapitulated many known or suspected TOR associations, including those with protein phosphatase 2A (PP2A) and PP2A-like phosphatase complexes (Yan, et al., 2006). Also identified were several proteins that, while not previously shown to physically interact with TORC1, have been reported to be phosphorylated in a rapamycin-sensitive manner: Npr1 (Gander, et al., 2008; Schmidt, et al., 1998), Mks1 (Dilova, et al., 2002; Sekito, et al., 2002), Rtg3 (Sekito, et al., 2002), and most recently Ksp1 and Sky1 (Huber, et al., 2009). The KPI network also identified the known TORC2 effectors Ypk1/2 (Kamada, et al., 2005). Thus, the KPI network appeared to capture *bona fide* components of the TOR signaling network. Affinity capture-immunoblot was used to confirm novel interactions between Tor1 and Mks1, Npr1, Fmp48, Pkh2, Bck1, Sky1, Ksp1, Kdx1, and Nnk1 (Figure 2-1B). I chose to characterize 3 novel TORC1 interactors in further detail: Nnk1, Fmp48, and Mks1.

Figure 2-1. Physical interactors of the target of rapamycin (TOR) pathway. (A) Interaction network for the target of rapamycin complex 1 (TORC1) and target of rapamycin complex 2 (TORC2). Kinases are shown in orange, kinase-associated proteins in yellow, phosphatases in dark blue, phosphatase-associated proteins in light blue, and other proteins in gray. Red connecting lines indicate KPI network interactions, gray lines indicate low-throughput (LTP) interactions, and gray dashed lines indicate high-throughput - high-confidence (HTP-HC) interactions [previously reported in two or more HTP studies; see (Breitkreutz, et al., 2010) for details]. Line thickness is proportional to the evidence for an interaction and node size is proportional to the total number of connections for a node. Novel TORC1-associated kinases are boxed in green. Figure constructed by Dr. Anne-Claude Gingras. **(B)** Validation of Tor1 interactions by affinity capture-immunoblot. A *pep4Δ* strain (yMT2398) carrying a $<^{HA}TOR1>$ plasmid with HA-tagged Tor1 expressed from its endogenous promoter (pMT4436) and the indicated $<pGAL1\text{-}ORF^{FLAG}>$ expression plasmid was subject to anti-FLAG immunoprecipitation and co-precipitating ^{HA}Tor1 detected with anti-HA antibody. Control lane carries no tagged bait protein. Asterisk (*) indicates a background band. Inputs were blotted for ^{HA}Tor1 but none was detectable (data not shown). Experiment performed by Dr. Lorrie Boucher. Panels A and B are from: Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, X.S., Pawson, T., Gingras, A.-C., Nesvizhskii, A.I., and Tyers, M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* **328**, 1043-1046. Reprinted with permission from AAAS.


A
PP2A phosphatases
nitrogen metabolism
new TORC1-associated kinases
TORC1
TORC2
TOR1
TOR2
SIT4
PPH21
CDC55
TAP42
SAP4
SAP185
TCP1
RTG3
DAL82
DAL81
DAL80
GLN3
GZF3
MKS1
IRA2
ICS89
LST8
KOG1
AVO1
BIT2
BIT61
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TSC11
AVO2
SLM1
PLC1
ALK2
FPR1
PRE4
CCN1
CNA1
GHX5
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YPK1
HIO2
YKL171W
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NPR1
IME2
MIH1
KIN2
CBK1
KDX1
NAT1
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NNK1 FLAG
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Tor1
anti-HA
Pkh2/Ksp1
Kog1
Nnk1
Sky1
Npr1
Mks1
Ykl161c
Fmp48
anti-Flag
*
IP
Input
Failed to detect Tor1 in input
*background band

2.2.2 Nnk1 links TORC1 to nitrogen signaling and metabolism

In the KPI network generated by our laboratory, the Nnk1 kinase – encoded by *YKL171W* and subsequently renamed *NNK1* for nitrogen network kinase – associated with all subunits of TORC1 (Figure 2-2A), and the interaction of Nnk1 with Tor1 was confirmed by affinity capture-immunoblot (Figure 2-1B). Consistent with the physical connection to TORC1, overexpression of *NNK1* conferred sensitivity to rapamycin (Figure 2-2B).

Nnk1 also exhibited an interaction with Ure2, a negative regulator of nitrogen catabolite repression (NCR)-sensitive genes that is thought to act by sequestering the Gln3 transcription factor in the cytoplasm during growth in preferred nitrogen sources (Cooper, 2002; Magasanik, 2005; Magasanik and Kaiser, 2002). The interaction between Nnk1 and Ure2 identified by mass spectrometry was confirmed by affinity capture-immunoblot (Breitkreutz, et al., 2010). Overexpression of *NNK1* induced rapid nuclear accumulation of Gln3 (Figure 2-2C) and specifically increased transcription of Gln3 target genes (Figure 2-2D), suggesting that Nnk1 activity may antagonize the Ure2-Gln3 interaction.

Nnk1's highest-confidence physical interactor identified by mass spectrometry (often having equivalent peptide coverage to Nnk1 itself) was Gdh2, a key enzyme of central nitrogen metabolism in yeast. Gdh2 encodes the NAD-linked glutamate dehydrogenase that catalyzes deamination of glutamate to α-ketoglutarate and ammonia (Magasanik and Kaiser, 2002):

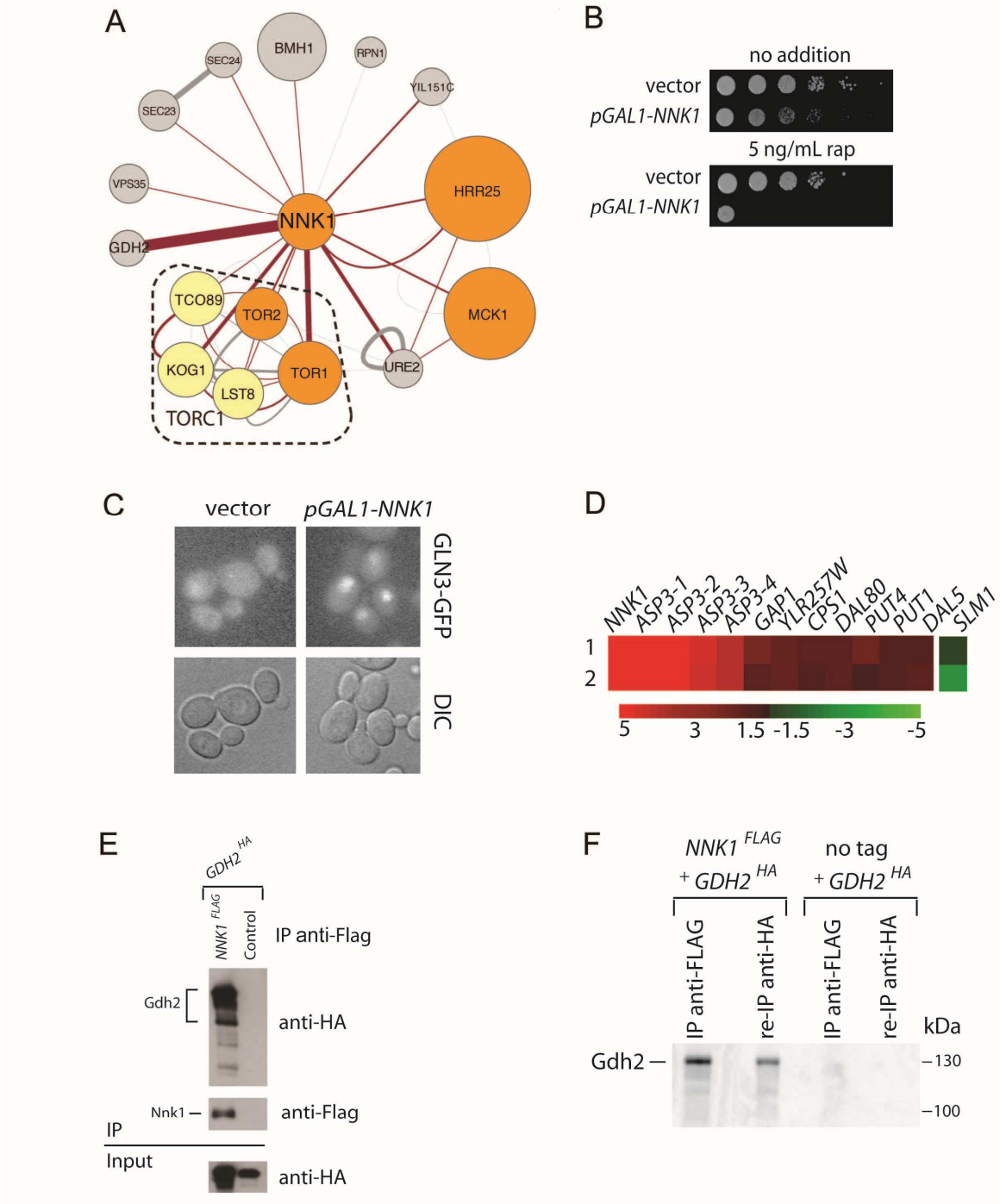
$$\text{glutamate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + NH_4^+ + NADH$$

The robust interaction between Nnk1 and Gdh2 was confirmed by affinity capture-immunoblot (Figure 2-2E) and Gdh2 was phosphorylated in Nnk1 immune complexes *in vitro* (Figure 2-2F).

In the course of investigating further connections between Nnk1 and Gdh2, I found that a *gdh2Δ* strain was resistant to rapamycin when glutamate, but not ammonium, was the sole nitrogen source (Figure 2-2G). In addition, *NNK1* overexpression caused relocalization of Gdh2 from a diffuse cytoplasmic localization to one discrete focus per cell, and this focus appeared distinct from the nucleus and vacuole (Figure 2-2H). Gdh2 foci were also present during respiratory growth and their formation under these conditions required *NNK1*, but foci did not occur in response to carbon starvation or rapamycin treatment (Figure 2-2I).

Figure 2-2. Nnk1 links TORC1 to nitrogen sensing and metabolism. (A) Interaction network for Ykl171w/Nnk1. See legend to Figure 2-1 for details on colour scheme, line thickness, and node size. Figure constructed by Dr. Anne-Claude Gingras. **(B)** Overexpression of *NNK1* confers sensitivity to rapamycin. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-NNK1*^{FLAG}> plasmid (pMT4436) or empty vector (pMT3164) was spotted onto SC-leu +2% raffinose +2% galactose +5 ng/mL rapamycin and incubated at 30°C for 4 days (+rap) or 3 days (untreated control). **(C)** Overexpression of *NNK1* induces rapid nuclear accumulation of Gln3. A genomic *GLN3*^{GFP} strain (yMT4204) carrying either a <*pGAL1-NNK1*^{FLAG}> plasmid (pMT4436) or empty vector (pMT3164) was grown to early log phase in SC-leu +2% raffinose for >16 hours at 30°C. Galactose was added to 2% final concentration and cells visualized after 60 min. **(D)** Overexpression of *NNK1* specifically induces transcription of Gln3 target genes. A *gal1Δ* strain (yMT4164) carrying a <*pGAL1-NNK1*^{HA}> plasmid (pMT4492) or empty vector BG1805 (pMT4177) was grown to early log phase in minimal YNB raffinose media and induced with 0.2% galactose for 90 min. Color bar indicates fold increase (red) or decrease (green) relative to empty vector control. Two biological replicates are shown. **(E)** Validation of Nnk1-Gdh2 interaction by affinity capture-immunoblot. A genomic *GDH2*^{HA} strain (yMT4162) carrying either a <*pGAL1-NNK1*^{FLAG}> plasmid (pMT4436) or empty vector (pMT3164) was subject to anti-FLAG immunoprecipitation and co-precipitating Gdh2^{HA} detected with anti-HA antibody. Control lane carried no tagged bait protein. Experiment performed by Dr. Lorrie Boucher. **(F)** Gdh2 is phosphorylated in Nnk1 immune complexes *in vitro.* A genomic *GDH2*^{HA} strain (yMT4162) carrying either a <*pGAL1-NNK1*^{FLAG}> plasmid (pMT4436) or empty vector (pMT3164) was subject to FLAG immunoprecipitation. Immunopurified Nnk1^{FLAG} complexes were incubated with [^{33}P]- γ-ATP, then denatured, and radiolabeled Gdh2^{HA} species were reimmunoprecipitated with anti-HA antibody. **(G)** A *gdh2Δ* strain is resistant to rapamycin when glutamate is the sole nitrogen source. Congenic wild type (BY4700) and *gdh2Δ* (yMT4163) strains, each carrying an empty BG1805 plasmid (pMT4177) to cover *ura3Δ0*, were diluted into molten top agar and poured onto minimal YNB glucose plates (0.17% yeast nitrogen base, 2% glucose) containing either 0.5% ammonium sulfate or 0.1% glutamate as the sole nitrogen source. Filter discs containing 7 μg rapamycin were placed on solidified medium and incubated for 3 days at 30°C. **(H)** Overexpression of *NNK1* causes relocalization of Gdh2 from the cytoplasm to one focus per cell. A genomic *gal1Δ GDH2*^{GFP} strain (yMT4231) carrying either a

<*pGAL1-NNK1*^{HA}> plasmid (pMT4492) or empty vector BG1805 (pMT4177) was grown to early log phase in SC-ura +2% raffinose for >16 hours at 30°C. Galactose was added to 2% final concentration and cells visualized after 60 min. The nucleus was stained by the addition of 2 µg/mL DAPI for 15 min. **(I)** Gdh2 foci are present during respiratory growth and require *NNK1*, but do not occur in response to carbon starvation or rapamycin treatment. Congenic *GDH2*^{GFP} (yMT4230) and *nnk1Δ GDH2*^{GFP} (yMT4232) strains were grown to early log phase in SC +3% glycerol for >16 hours at 30°C. The genomic *GDH2*^{GFP} strain (yMT4230) was also grown to early log phase in SC +2% glucose for >16 hours at 30°C (glucose), and either treated with 200 ng/mL rapamycin for 2 hours (rapamycin), resuspended in the same media with no glucose for 2 hours (carbon starvation), or allowed to grow to saturation (saturated). Panels A-G are from: Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, X.S., Pawson, T., Gingras, A.-C., Nesvizhskii, A.I., and Tyers, M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* **328**, 1043-1046. Reprinted with permission from AAAS.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YIL151C
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VPS35
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NNK1
GDH2
TCO89
TOR2
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KOG1
TOR1
URE2
LST8
TORC1
B
no addition
vector
pGAL1-NNK1
5 ng/mL rap
vector
pGAL1-NNK1
C
vector
pGAL1-NNK1
GLN3-GFP
DIC
D
NNK1
ASP3-1
ASP3-2
ASP3-3
ASP3-4
GAP1
YLR257W
CPS1
DAL80
PUT4
PUT1
DAL5
SLM1
1
2
5
3
1.5
-1.5
-3
-5
E
GDH2 HA
NNK1 FLAG
Control
IP anti-Flag
Gdh2
anti-HA
Nnk1
anti-Flag
IP
Input
anti-HA
F
NNK1 FLAG
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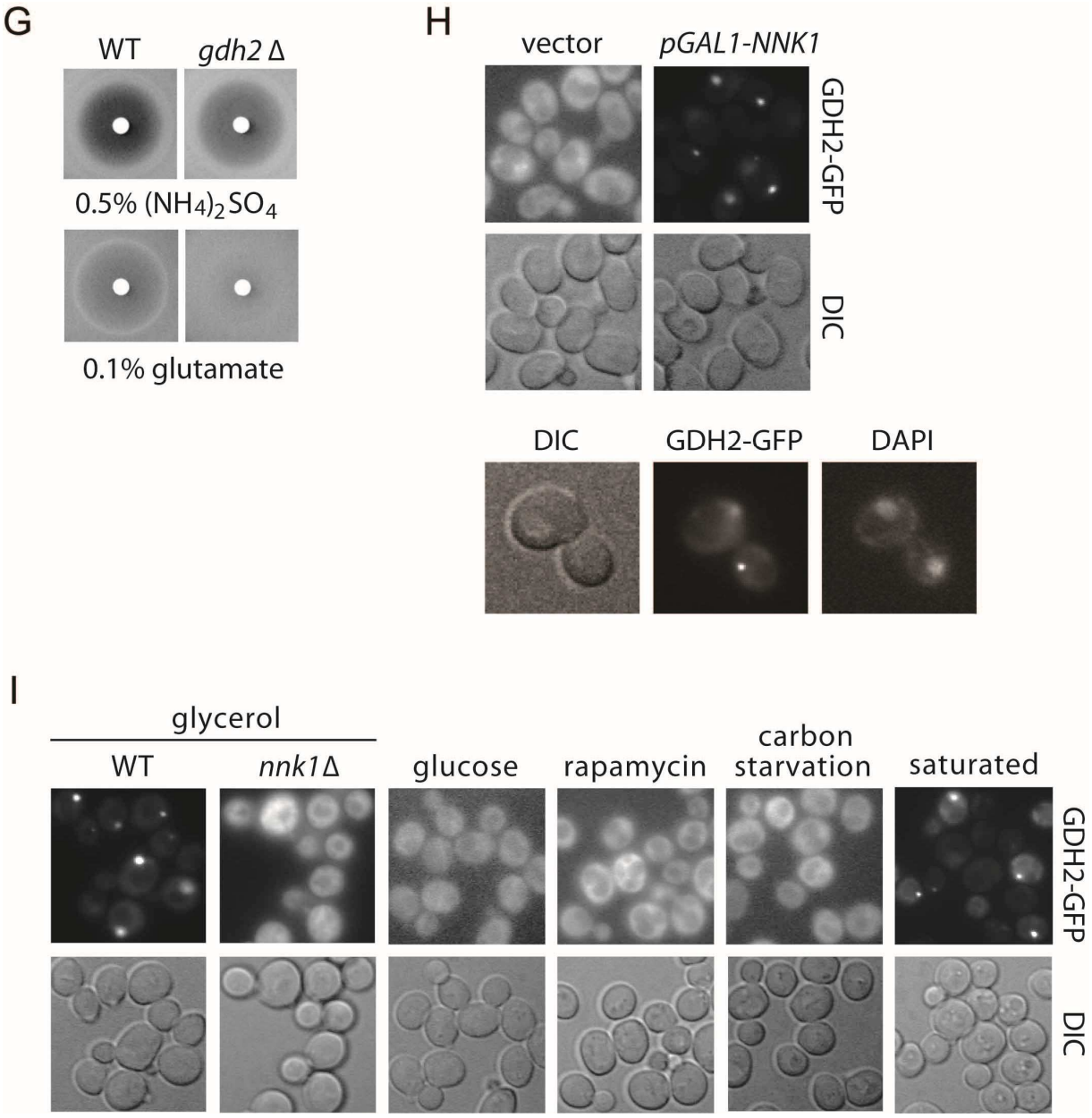
100


G
WT
gdh2 Δ
0.5% (NH4)2SO4
0.1% glutamate
H
vector
pGAL1-NNK1
GDH2-GFP
DIC
DIC
GDH2-GFP
DAPI
I
glycerol
WT
nnk1Δ
glucose
rapamycin
carbon starvation
saturated
GDH2-GFP
DIC

2.2.3 Fmp48 links TORC1 to mitochondrial function

Fmp48 is a kinase of unknown function that has been found in mitochondrial subcellular fractions (Reinders, et al., 2006; Sickmann, et al., 2003). In the KPI network generated by our laboratory, Fmp48 associated with all subunits of TORC1 (Figure 2-3A), and the interaction of Fmp48 with Tor1 was confirmed by affinity capture-immunoblot (Figure 2-1B). Consistent with the physical connection to TORC1, overexpression of *FMP48* conferred resistance to rapamycin (Figure 2-3B), and Fmp48-associated kinase activity was increased by rapamycin treatment (Figure 2-3C).

Fmp48 was found to regulate mitochondrial function, in that low-level ectopic expression of *FMP48* caused a growth defect on a non-fermentable carbon source (Figure 2-3D) and abnormal mitochondrial morphology (Figure 2-3E). Transcriptional profiling showed that ectopic expression of *FMP48* induced Msn2/4-and Hsf1-controlled genes involved in the response to stress, and repressed Hap2/3/4/5-controlled genes involved in respiratory metabolism (Figure 2-3F and Table 2-1).

Figure 2-3. Fmp48 links TORC1 to mitochondrial function. (A) Interaction network for Fmp48. See legend to Figure 2-1 for details on colour scheme, line thickness, and node size. Figure constructed by Dr. Anne-Claude Gingras. **(B)** Overexpression of *FMP48* confers resistance to rapamycin. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-FMP48*^{FLAG}> plasmid (pMT4434) or empty vector (pMT3164) was spotted onto SC-leu +2% raffinose +2% galactose +7 ng/mL rapamycin and incubated at 30°C for 5 days (+rap) or 3 days (untreated control). **(C)** Fmp48-associated kinase activity is increased by rapamycin treatment. A *pep4Δ* strain (yMT2398) carrying either a <*pGAL1-FMP48*^{FLAG}> plasmid (pMT4434) or a <*pGAL1-SCH9*^{FLAG}> plasmid (pMT4493) was treated (or not) with 200 ng/mL rapamycin for 30 min. Fmp48^{FLAG} and Sch9^{FLAG} complexes were immunopurified, incubated with [^{33}P]- γ-ATP, and radiolabeled species were resolved by SDS–polyacrylamide gel electrophoresis. Non-regulated Sch9-associated kinase activity served as a negative control. Anti-FLAG immunoblot is shown below. **(D)** Low-level ectopic expression of *FMP48* causes a growth defect on a non-fermentable carbon source. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-FMP48*^{FLAG}> plasmid (pMT4434) or empty vector (pMT3164) was spotted onto: SC-leu +2% raffinose +0.02% galactose and incubated at 30°C for 3 days; or SC-leu +3% glycerol +0.02% galactose and incubated at 30°C for 5 days. **(E)** *FMP48* overexpression causes abnormal mitochondrial morphology. A genomic *ILV3*^{GFP} strain (Ilv3^{GFP} localizes to the mitochondrial matrix and was used to visualize mitochondrial morphology) carrying either a <*pGAL1-FMP48*^{HA}> plasmid (pMT4494) or empty vector BG1805 (pMT4177) was grown to early log phase in SC-ura +2% raffinose for >16 h at 30°C. Galactose was added to 2% final concentration and cells visualized after 6 h. **(F)** Transcriptional effects of ectopic *FMP48* expression. A *gal1Δ* strain (yMT4164) carrying a <*pGAL1-FMP48*^{HA}> plasmid (pMT4494) or empty vector BG1805 (pMT4177) was grown to early log phase in minimal YNB raffinose media (0.17% yeast nitrogen base, 2% raffinose, 0.5% ammonium sulfate). Expression was induced with 0.2% galactose and cells harvested after 6 hours. Heatmap shows the results of 2 independent biological replicates and their average. The set of genes induced or repressed by more than 1.5-fold was assessed for statistically enriched Munich Information Center for Protein Sequences (MIPS) functional classifications using FunSpec (Robinson, et al., 2002). Panels A-E are from: Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, X.S., Pawson, T.,

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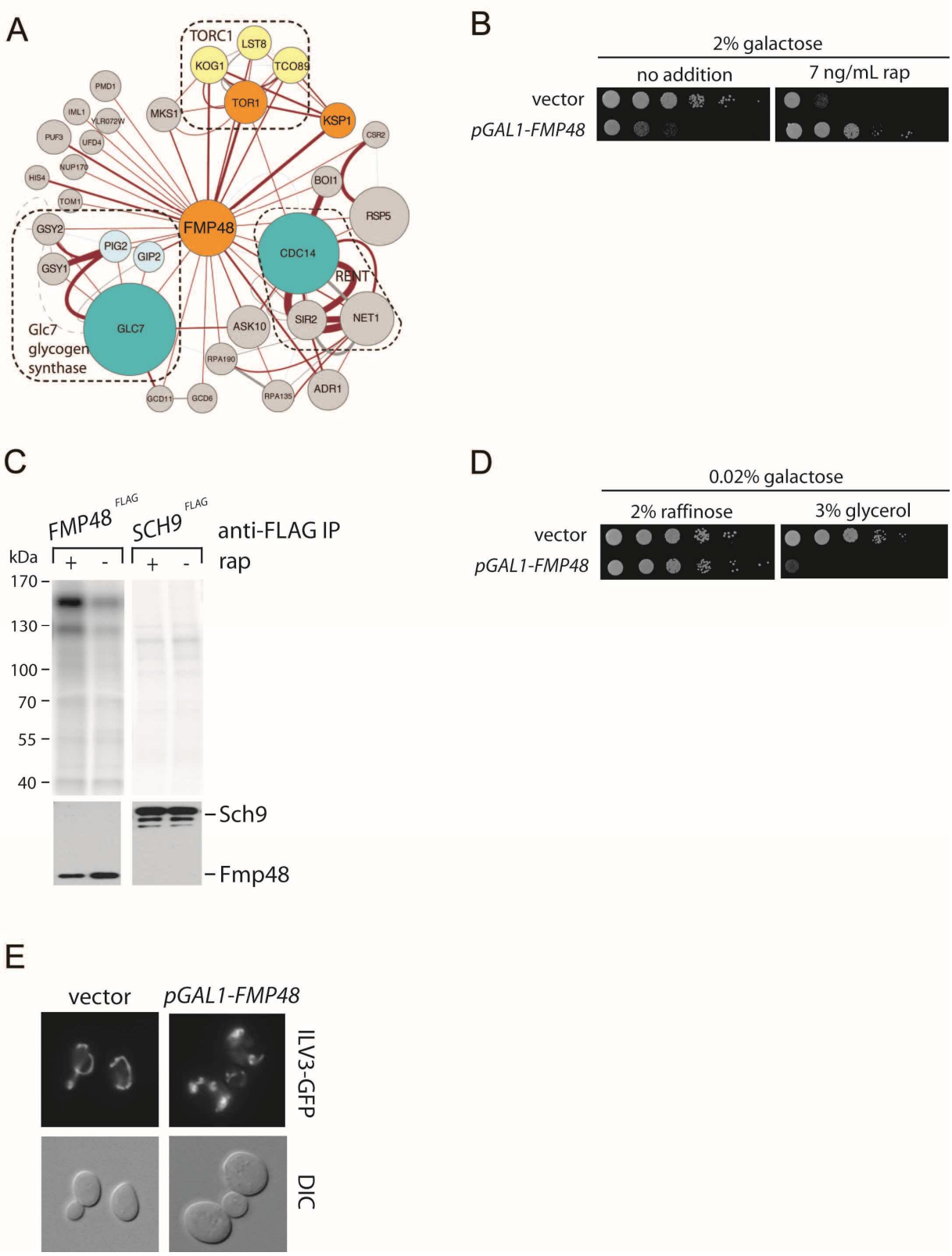
A
TORC1
LST8
KOG1
TCO89
TOR1
KSP1
MKS1
PMD1
IML1
YLR072W
PUF3
UFD4
NUP170
HIS4
TOM1
CSR2
BOI1
RSP5
FMP48
GSY2
PIG2
GIP2
GSY1
CDC14
RENT
GLC7
Glc7
glycogen
synthase
ASK10
SIR2
NET1
RPA190
ADR1
GCD11
GCD6
RPA135
B
2% galactose
no addition
7 ng/mL rap
vector
pGAL1-FMP48
C
FMP48 FLAG
SCH9 FLAG
anti-FLAG IP
kDa
+ - + -
rap
170
130
100
70
55
40
Sch9
Fmp48
D
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vector
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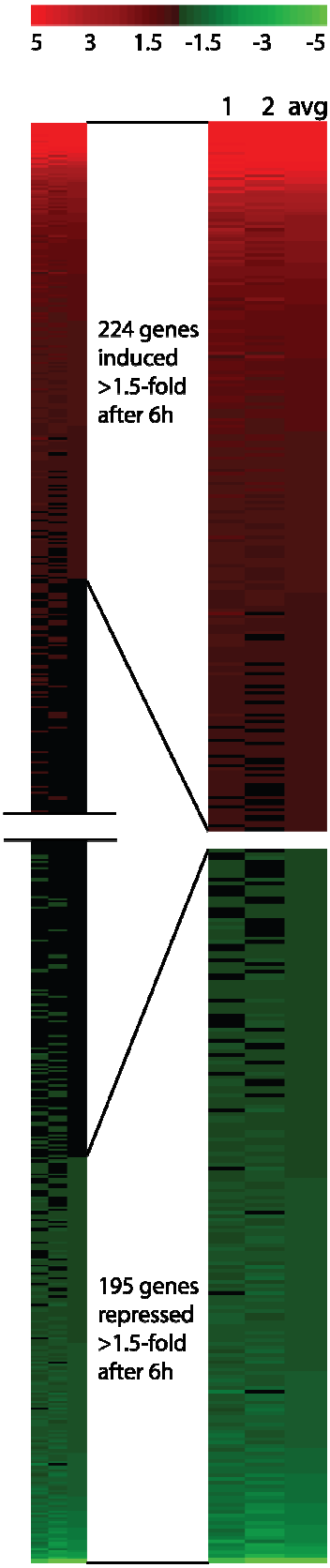
E
vector
pGAL1-FMP48
ILV3-GFP
DIC

F



induced > 1.5-fold		
Category	**p-value**	**In Category from Cluster**
C-compound and carbohydrate metabolism [01.05]	9.67E-11	BDH2 ECM15 YBR056W GPD1 DIA3 YDL124W SFA1 NRG1 TPS2 YEL047C HOR2 HSP12 DAK2 HXK1 YGL157W UGA1 ENO1 GND2 ENO2 RHR2 AAD10 PDC1 YLR164W GLO1 DAK1 ALD3 ADH1 GCY1 ERR1 ERR2
oxidative stress response [32.01.01]	1.79E-10	GRX1 TRX3 TSA2 GRX2 HSP12 CTT1 TRR2 HYR1 GTT1 SOD1 GPX1 AHP1 GAD1 GRE2 GRE1
glycolysis and gluconeogenesis [02.01]	6.77E-07	CDC19 PGK1 GPM2 TDH3 ENO1 ENO2 GPM1 PGM2 ERR1 ERR2
stress response [32.01]	1.73E-06	SSA3 YRO2 TPS1 SSE2 TPS2 CPR1 CYC7 DAK2 STF2 YJL144W HSP104 DAK1 ALD3 DDR48 PAI3 HOR7 YDJ1 YGP1 DDR2
heat shock response [32.01.05]	2.36E-06	HSP12 GRE3 BCY1 GRE2 LSP1 YDC1 GRE1
sugar, glucoside, polyol and carboxylate catabolism [01.05.02.07]	2.44E-06	CDC19 TKL2 TPS1 PGK1 GPM2 NQM1 TDH3 ENO1 GRE3 ENO2 YJR096W GPM1 PGM2
peroxidase reaction [32.07.07.05]	4.02E-05	GRX1 GRX2 HYR1 GPX1
protein folding and stabilization [14.01]	5.82E-05	SSA1 SSA3 HSP26 SSE2 CPR1 HSP42 SSA4 SSA2 HSP104 ERO1 YDJ1 SSE1
metabolism of derivatives of dehydroquinic acid, shikimic acid and chorismic acid [01.20.15]	7.83E-05	ENO1 ENO2 ERR1 ERR2
PROTEIN FATE (folding, modification, destination) [14]	7.83E-05	HSP31 SNO4 HSP33 HSP32
detoxification by modification [32.07.03]	7.83E-05	GRX1 GRX2 GTT1 GRE2
unfolded protein response (e.g. ER quality control) [32.01.07]	9.00E-05	SSA1 HSP26 HSP42 HSP31 SSA4 SNO4 SIS1 HSP33 SSE1 HSP32
protease inhibitor [18.02.01.02.03]	3.57E-04	YHR138C TFS1 PAI3
glutathione conjugation reaction [32.07.07.03]	3.57E-04	GRX1 GRX2 GTT1
secondary metabolism [01.20]	4.98E-04	COQ6 GRE3 YJR096W GCY1
osmotic and salt stress response [32.01.03]	7.27E-04	GPD1 HOR2 HSP12 RHR2 SIP18 GRE2 GCY1 GRE1

repressed > 1.5-fold		
Category	**p-value**	**In Category from Cluster**
tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle) [02.10]	<1.00E-14	CIT2 IDP1 KGD2 LPD1 LSC2 MDH1 SDH3 SDH1 SDH2 IDP3 IDH1 CIT1 IDH2 GDH1 FUM1
electron transport and membrane-associated energy conservaion [02.11]	<1.00E-14	COR1 ATP1 ATP3 COX9 ATP5 QCR7 QCR6 PMA1 COX4 COX13 COX6 CYC1 ATP2 ATP7 SDH3 SDH1 SDH2 COX12 COX8 NDE1 COX7 COX5A ATP20
electron transport [20.01.15]	<1.00E-14	ATP1 ATP3 COX9 ATP5 PMA1 COX4 COX13 COX6 CYC1 ATP2 ATP7 SDH3 SDH1 SDH2 COX12 COX8 NDE1 COX7 COX5A VMA4 ATP20
aerobic respiration [02.13.03]	3.62E-12	PET9 COR1 COX9 RIB3 QCR7 QCR6 COX4 COX13 COX6 SDH3 SDH1 SDH2 COX12 COX8 NDE1 COX7 COX5A POR1
sugar, glucoside, polyol and carboxylate catabolism [01.05.02.07]	6.34E-08	GAL10 PSA1 KGD2 LSC2 SCW4 MDH1 SDH3 SDH1 SDH2 IDH1 SUN4 CIT1 IDH2 FUM1
biosynthesis of glutamate [01.01.03.02.01]	8.33E-08	CIT2 IDP1 PRO3 IDH1 CIT1 IDH2 GDH1
mitochondrial transport [20.09.04]	1.54E-06	PET9 ATP1 ATP3 AAC3 GGC1 ATP5 YAT2 SMF2 SFC1 ATP2 ATP7 POR1 CRC1 ODC2
glyoxylate cycle [02.04]	2.26E-06	CIT2 ICL1 AGX1 MLS1 MDH2
energy generation (e.g. ATP synthase) [02.45.15]	2.11E-05	ATP1 ATP3 ATP5 ATP2 ATP7 ATP20
antiporter [20.03.02.03]	2.27E-05	PET9 AAC3 SFC1 CRC1
respiration [02.13]	4.40E-05	ATP1 ATP3 ATP5 CYC1 YJR120W ATP2 ATP7 COX17 ATP20
homeostasis of protons [34.01.01.03]	5.25E-05	ATP1 ATP3 ATP5 PMA1 ATP2 ATP7 CWP2 VMA4
NAD/NADP binding [16.21.07]	6.43E-05	IDP1 PRO3 INO1 IDP3 IDH1 IDH2 GDH1
Fe/S binding [16.21.08]	2.32E-04	SDH3 SDH1 SDH2
metabolism of methionine [01.01.06.05]	2.75E-04	HOM2 SAM2 SAM1 MET17 MET16
biosynthesis of arginine [01.01.03.05.01]	4.03E-04	ARG4 ARG3 ARG1 ARG8
degradation of glycine [01.01.09.01.02]	4.54E-04	GCV3 LPD1 SHM2
metabolism of urea (urea cycle) [01.01.05.03]	4.54E-04	ARG4 ARG3 ARG1
cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.) [20.01.01.01]	7.32E-04	ATP1 ATP3 ATP5 PMA1 ATP2 ATP7 MEP2 VMA4
transport ATPases [20.03.22]	7.82E-04	ATP1 ATP3 ATP5 PMA1 ATP2 ATP7 VMA4

Table 2-1. Gene groups significantly induced or repressed upon ectopic expression of *FMP48*, as identified by T-profiler. [a]

(A) genes groups defined by the presence of a specific transcription factor consensus DNA-binding motif in their upstream region

Motif [b]	Name	t-value	E-value	Mean log_2(ratio)	ORFs
AGGGG	MSN2-4	8.90	< 1.0e-15	0.086	920
CCCCT	MSN2-4	7.42	1.70E-11	0.068	960
HRCCCYTWDT	MSN2/4	5.60	3.10E-06	0.121	226
TCTAGAA	HSF1	4.88	1.50E-04	0.114	193
TTCTRGAA	HSF1	4.69	4.00E-04	0.123	158
GAANNTTCNNGAA	HSF1	4.16	4.60E-03	0.228	41
TGNKAGCGCCG	Unknown	3.93	1.20E-02	0.459	11
CCGANNNTCGG	Novel Facilitat	-3.80	2.10E-02	-0.307	23
GAGTCA	GCN4	-4.35	2.00E-03	-0.099	296
TGACTCA	GCN4	-5.63	2.60E-06	-0.198	115

(B) genes groups defined by having their upstream region bound by a specific transcription factor in genome-wide chromatin immunoprecipitation (ChIP) experiments [c]

Transcription factor	Condition [d]	t-value	E-value	Mean log_2(ratio)	ORFs
HSF1	H2O2Lo	7.42	3.70E-11	0.269	91
MSN2	H2O2Hi	6.64	1.00E-08	0.276	70
MSN2	Acid	6.63	1.10E-08	0.437	30
MSN4	H2O2Hi	5.71	3.60E-06	0.250	63
HSF1	H2O2Hi	5.24	5.10E-05	0.165	114
MSN2	H2O2Lo	5.23	5.40E-05	0.293	40
GLN3	SM	-3.81	4.30E-02	-0.218	45
FKH1	YPD	-3.92	2.80E-02	-0.131	134
MBP1	H2O2Hi	-3.94	2.60E-02	-0.139	119
HAP5	SM	-3.98	2.20E-02	-0.254	36
ABF1	YPD	-4.12	1.20E-02	-0.101	256
MBP1	YPD	-4.27	6.20E-03	-0.148	122
FHL1	YPD	-4.50	2.20E-03	-0.129	179
GLN3	RAPA	-4.62	1.20E-03	-0.223	62
SIP4	SM	-4.84	4.20E-04	-0.430	19
HAP2	RAPA	-5.01	1.70E-04	-0.305	39
SWI4	YPD	-5.33	3.10E-05	-0.173	136
GCN4	YPD	-5.49	1.30E-05	-0.244	72
FKH2	YPD	-5.66	4.80E-06	-0.201	113
GCN4	SM	-6.13	2.80E-07	-0.174	176
GCN4	RAPA	-6.15	2.50E-07	-0.188	151
SWI6	YPD	-6.50	2.60E-08	-0.206	139
HAP4	YPD	-7.54	1.50E-11	-0.356	62

(C) genes groups defined by membership in a specific Gene Ontology (GO) category

Category	Aspect [e]	t-value	E-value	Mean log_2(ratio)	ORFs
heat shock protein activity	F	8.41	<1.0e-15	0.797	16
response to stress	P	7.07	2.20E-09	0.149	360
glycolysis	P	6.19	8.40E-07	0.586	16
oxidoreductase activity, acting on peroxide as acceptor	F	6.18	8.90E-07	0.634	14
response to dessication	P	5.32	1.40E-04	0.659	10
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	F	5.26	2.00E-04	0.225	63
ethanol metabolism	P	4.53	8.20E-03	0.566	9
amine catabolism	P	4.52	8.60E-03	0.300	27
glutamine family amino acid biosynthesis	P	-4.39	1.60E-02	-0.293	27
ribonucleoside triphosphate metabolism	P	-5.12	4.20E-04	-0.458	16
tricarboxylic acid cycle intermediate metabolism	P	-5.49	5.60E-05	-0.426	19
nucleosome	C	-6.27	5.00E-07	-0.653	12
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	P	-7.63	3.30E-11	-0.051	1265
cell growth and/or maintenance	P	-7.78	1.00E-11	-0.036	2109
structural molecule activity	F	-8.61	< 1.0e-15	-0.161	323
mitochondrial electron transport chain	C	-8.95	< 1.0e-15	-0.707	22
organic acid metabolism	P	-9.58	< 1.0e-15	-0.217	261

[a] Transcriptional profiles after 6 hours of ectopic *FMP48* expression were analyzed with T-profiler (Boorsma, et al., 2005). Induced gene groups are shaded in red, and repressed gene groups are shaded in green. See Material and Methods for details.

[b] Ambiguity Codes: S = C or G; W = A or T; R = A or G; Y = C or T; K = G or T; M = A or C; n = A, C, G or T.

[c] Genome-wide ChIP data is from (Harbison, et al., 2004).

[d] Conditions were: YPD = log phase growth in rich medium; H2O2Hi = treatment with 4 mM hydrogen peroxide for 30 minutes; H2O2Lo = treatment with 0.4 mM hydrogen peroxide for 30 minutes; Acid = treatment with 50 mM succinic acid for 30 minutes to reach a pH of 4.0; SM = treatment with 0.2 µg/ml sulfometuron methyl (an inhibitor of amino acid biosynthesis) for 2h;

RAPA = treatment with 100 nM rapamycin (an inhibitor of TORC1) for 20 minutes (Harbison, et al., 2004).

[e] Gene Ontology categories: F = molecular function; P = biological process; C = cellular component.

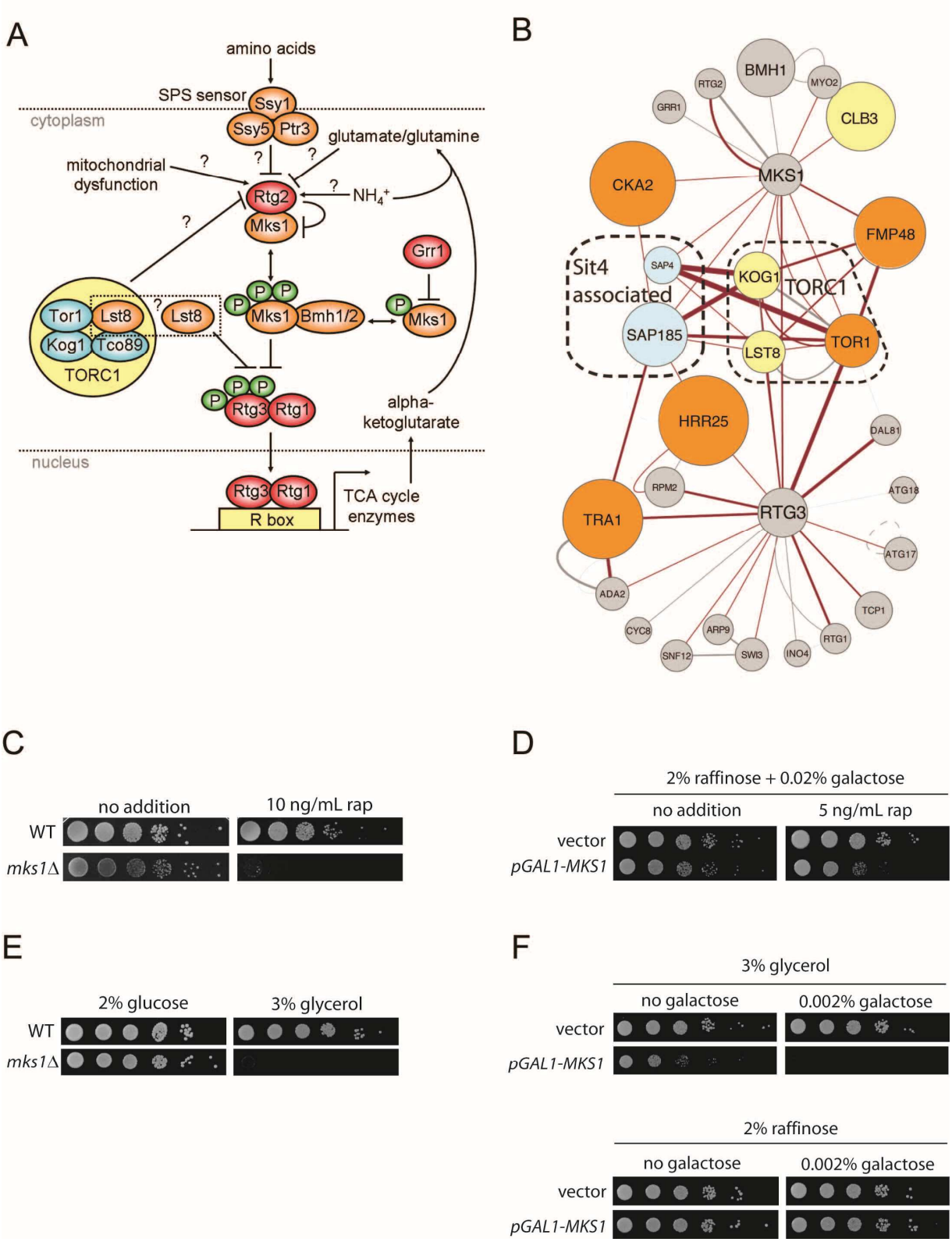
2.2.4 Mks1 links TORC1 to mitochondrial retrograde signaling

Mks1 has been previously characterized as a negative regulator of the mitochondria-to-nucleus retrograde (RTG) signaling pathway, which is activated in response to mitochondrial dysfunction, growth on poor nitrogen sources, or TORC1 inhibition by rapamycin (Figure 2-4A). In the KPI network generated by our laboratory, Mks1 associated with subunits of TORC1 (Figure 2-4B), and the interaction of Mks1 with Tor1 was confirmed by affinity capture-immunoblot (Figure 2-1B). Consistent with a connection to TORC1 signaling, both deletion and low-level ectopic expression of *MKS1* conferred sensitivity to rapamycin (Figure 2-4C and Figure 2-4D). Interestingly, the rapamycin sensitivity of an *mks1Δ* strain was only apparent on a non-fermentable carbon source (Figure 2-4C), and Mks1 also exhibited other links to mitochondrial function – both deletion and low-level ectopic expression of *MKS1* caused a severe growth defect on a non-fermentable carbon source (Figure 2-4E and Figure 2-4F).

Overexpression of *MKS1* caused severe growth inhibition that cannot be accounted for by current models of Mks1's function as a negative regulator of the RTG pathway. Indeed, the growth defect conferred by Mks1 overexpression was independent of *RTG1*, *RTG2*, and *RTG3* (Figure 2-4G). Consistent with the possibility that growth inhibition caused by overexpression of *MKS1* was due to disruption of TORC1 signaling, it was alleviated by mutations that confer rapamycin resistance (*npr1Δ*, *pmr1Δ*, *gln3Δ gat1Δ*, and *tap42-11*), and was exacerbated by deletion of *TOR1* (Figure 2-4G). Transcriptional profiling showed that ectopic expression of *MKS1* recapitulated a subset of the expression changes known to be induced by TORC1 inhibition by rapamycin, including induction of Gln3/Gcn4-controlled genes involved in amino acid metabolism and repression of ribosomal protein genes (Figure 2-4H and Table 2-2). Comparison of the transcriptional effects of ectopic *MKS1* and *FMP48* expression revealed partially overlapping but distinct profiles (Figure 2-4I).

Figure 2-4. Mks1 links TORC1 to mitochondrial retrograde signaling. (A) Schematic diagram of the yeast mitochondria-to-nucleus retrograde (RTG) signaling pathway. Negative regulators of RTG-dependent gene transcription are shown in orange and positive regulators in red. (**B)** Interaction network for Mks1 and Rtg3. See legend to Figure 2-1 for details on colour scheme, line thickness, and node size. Figure constructed by Dr. Anne-Claude Gingras. **(C)** Deletion of *MKS1* causes rapamycin sensitivity on a non-fermentable carbon source. Congenic wild type (BY4741) and *mks1Δ* (yMT4233) strains were spotted onto XY +3% glycerol +10 ng/mL rapamycin and incubated at 37°C for 6 days (+rap) or 4 days (no addition). **(D)** Low-level ectopic expression of *MKS1* causes rapamycin sensitivity. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-MKS1*^{FLAG}> plasmid (pMT4435) or empty vector (pMT3164) was spotted onto SC-leu +2% raffinose + 0.02% galactose and plates incubated at 37°C for 4 days (+rap) or 2 days (no addition). **(E)** Deletion of *MKS1* confers a growth defect on synthetic glycerol medium. Congenic wild type (BY4741) and *mks1Δ* (yMT4233) strains were spotted onto SC +2% glucose and incubated at 30°C for 2 days, or SC +3% glycerol and incubated at 30°C for 4 days. **(F)** Low-level ectopic expression of *MKS1* causes a growth defect on a non-fermentable carbon source. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-MKS1*^{FLAG}> plasmid (pMT4435) or empty vector (pMT3164) was spotted onto SC-leu medium containing the indicated carbon sources. Plates were incubated at 30°C for 5 days (glycerol) or 3 days (raffinose). **(G)** Growth inhibition conferred by *MKS1* overexpression is independent of the RTG signaling pathway, alleviated by mutations that confer rapamycin resistance, and exacerbated by *TOR1* deletion. Congenic wild type (BY4741), *rtg1Δ rtg2Δ rtg3Δ* (yMT4160), *gln3Δ gat1* (yMT4161), *pmr1Δ* (yMT4234), and *npr1Δ* (yMT4235) strains carrying either a <*pGAL1-MKS1*^{FLAG}> plasmid (pMT4435) or empty vector (pMT3164) were spotted onto SC-leu +2% raffinose +2% galactose and incubated at 30°C for 3 days. Congenic wild type (CY4907) and *tap42-11* (CY4908) strains carrying either a <*pGAL1-MKS1*^{HA}> plasmid (pMT4495) or empty vector BG1805 (pMT4177) were spotted onto SC-ura +2% raffinose +0.02% galactose and incubated at 25°C for 3 days (empty vector) or 5 days (*pGAL-MKS1*). Congenic wild type (BY4741) and *tor1Δ* strains carrying either a <*pGAL1-MKS1*^{FLAG}> plasmid (pMT4435) or empty vector (pMT3164) were spotted onto SC-leu +2% raffinose +2% galactose and incubated at 37°C for 3 days (empty vector) or 6 days (*pGAL1-MKS1*). **(H)** Transcriptional effects of ectopic *MKS1* expression. A *gal1Δ* strain (yMT4164) carrying a <*pGAL1-MKS1*^{HA}> plasmid (pMT4495) or

empty vector BG1805 (pMT4177) was grown to early log phase in minimal YNB raffinose media (0.17% yeast nitrogen base, 2% raffinose, 0.5% ammonium sulfate). Expression was induced with 0.2% galactose and cells harvested after 6 hours. Heatmap shows the results of 2 independent biological replicates and their average. The set of genes induced or repressed by more than 1.5-fold was assessed for statistically enriched Munich Information Center for Protein Sequences (MIPS) functional classifications using FunSpec (Robinson, et al., 2002). **(I)** Comparison of transcriptional profiles of strains overexpressing *FMP48* or *MKS1*. Retrograde-responsive (orange), mitochondrial (red), stress-responsive (green), and Gln3/Gcn4-responsive (blue) genes are marked. Mitochondrial genes shown in red have GO biological process category "tricarboxylic acid cycle", "mitochondrial electron transport chain", or "proton-transporting ATP synthase complex (sensu Eukarya)". Panels B-G and I are from: Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, X.S., Pawson, T., Gingras, A.-C., Nesvizhskii, A.I., and Tyers, M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* **328**, 1043-1046. Reprinted with permission from AAAS.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WT
mks1Δ
F
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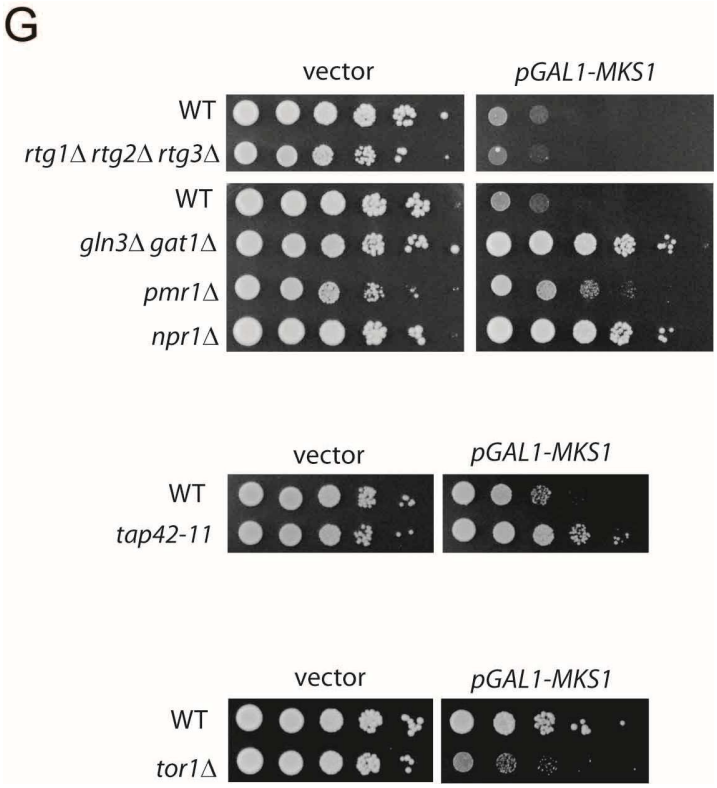
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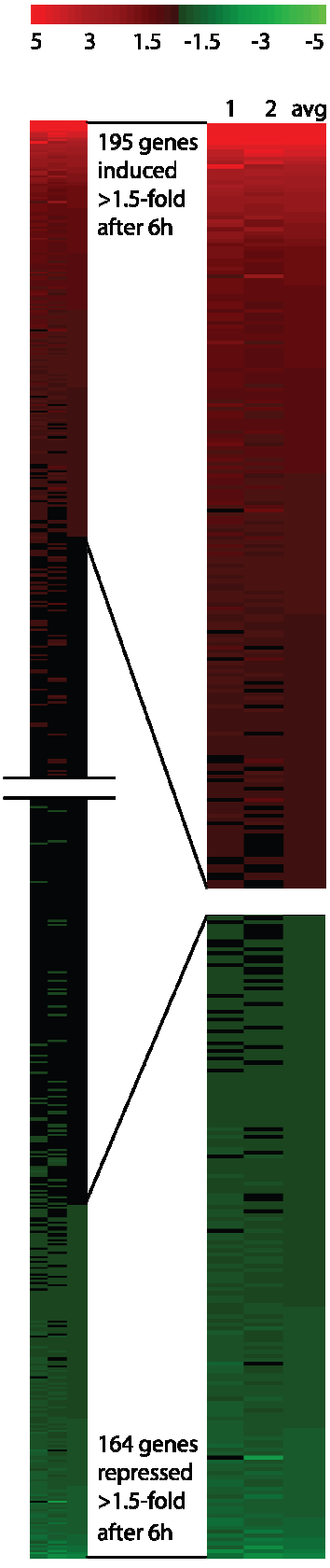


induced > 1.5-fold		
Category	**p-value**	**In Category from Cluster**
nitrogen, sulfur and selenium metabolism [01.02]	3.10E-14	GDH3 UGA3 GLT1 UGA4 AMD2 IRC7 STR3 UGA1 NIT1 CPS1 STR2 ALT1 ASP3-1 ASP3-2 ASP3-3 ASP3-4 ARG1
metabolism of aspartate [01.01.06.01]	3.05E-09	ARG4 ASP3-1 ASP3-2 ASP3-3 ASP3-4 ARG1
degradation of asparagine [01.01.06.02.02]	2.92E-06	ASP3-1 ASP3-2 ASP3-3 ASP3-4
anion transport [20.01.01.07]	6.59E-06	MEP1 DUR3 MEP2 SSU1 MEP3
cellular import [20.09.18]	6.65E-06	UGA4 CAN1 FCY2 MEP1 HXT5 DAL4 DAL5 GAP1 GAL2 FET4 MEP2 PUT4
biosynthesis of arginine [01.01.03.05.01]	1.75E-05	ARG4 CPA2 PUT1 ARG1 CPA1
nutrient starvation response [32.01.11]	3.91E-05	PRB1 ASP3-1 ASP3-2 ASP3-3 ASP3-4
C-compound and carbohydrate metabolism [01.05]	7.04E-05	ADH5 ILV6 HOR2 HSP12 UGA1 ENO1 GND2 BGL2 ENO2 RHR2 DAL7 PDC1 GAL2 PDC5 YLR164W ILV2 ALD3 YPL088W
catabolism of nitrogenous compounds [01.02.02.09]	2.08E-04	DAL1 DAL2 DAL7
amino acid/amino acid derivatives transport [20.01.07]	2.23E-04	STP4 UGA4 CAN1 GAP1 VBA1 PUT4 DIP5
alcohol fermentation [02.16.01]	3.50E-04	ADH5 PDC6 PDC1 PDC5
metabolism of urea (urea cycle) [01.01.05.03]	4.07E-04	ARG4 ARG1 CAR1
biosynthesis of valine [01.01.11.03.01]	4.07E-04	ILV6 BAT2 ILV2
stress response [32.01]	5.79E-04	YRO2 NSR1 SLT2 HSP150 HMS2 PIR3 SNZ1 ALD3 PAI3 HOR7 YGP1 ZWF1 DDR2

repressed > 1.5-fold		
Category	**p-value**	**In Category from Cluster**
ribosomal proteins [12.01.01]	<1.00E-14	RPS8A RPL32 RPS11B RPL19A RPS9B RPL21A RPL31A RPP1B RPL35B RPL41A RPL35A RPS11A RPS13 RPL12B RPS17B RPS18A RPL27B RPL12A RPS8B RPL23B RPS26B RPL2A RPL30 RPL7A RPL28 RPS2 RPL1B RPL9A RPS26A RPS25A RPL11B RPS23A RPS20 RPL27A RPS4B RPL2B RPL34B RPL40A RPS21B RPL39 RPS22A RPS5 RPS4A RPS21A RPL40B RPL15A RPS0B RPL22A RPL10 RPS31 RPL38 RPS25B RPS22B RPS29A RPS1A RPS18B RPS1B RPL15B RPS16A RPS10B RPL20A RPL9B RPS7B RPS3 RPL18B RPS19B RPP2A RPS15 RPL18A RPL25 RPL3 RPS7A RPS10A RPS12 RPL5 RPL1A RPL11A
electron transport and membrane-associated energy conservation [02.11]	1.08E-05	COR1 ATP3 QCR6 COX6 CYC1 MCR1 SDH2 COX12 COX7
glyoxylate cycle [02.04]	4.20E-05	CIT2 ICL1 MLS1 MDH2
tetrahydrofolate-dependent C-1-transfer [01.05.13.03]	2.20E-04	MTD1 SHM2 ADE17 CDC21
purine nucleotide/nucleoside/nucleobase anabolism [01.03.01.03]	6.51E-04	ADE1 HPT1 MTD1 ADE13 ADE17

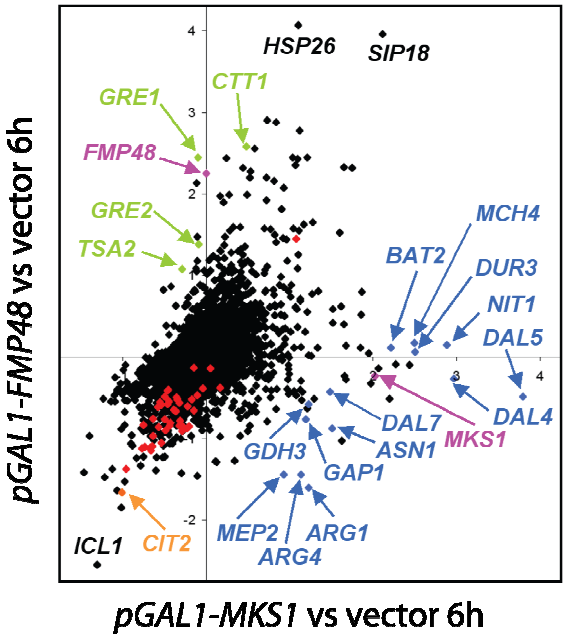

HSP26
SIP18
CTT1
GRE1
FMP48
GRE2
TSA2
MCH4
BAT2
DUR3
NIT1
DAL5
DAL4
DAL7
MKS1
GDH3
ASN1
GAP1
MEP2
ARG1
ARG4
ICL1
CIT2
4
3
2
-2
pGAL1-FMP48 vs vector 6h
pGAL1-MKS1 vs vector 6h

Table 2-2. Gene groups significantly induced or repressed upon ectopic expression of *MKS1*, as identified by T-profiler. [a]

(A) genes groups defined by the presence of a specific transcription factor consensus DNA-binding motif in their upstream region

Motif [b]	Name	t-value	E-value	Mean log_2(ratio)	ORFs
GATAAG	sporulation	8.20	3.20E-14	0.088	600
TGACTCA	GCN4	6.67	3.70E-09	0.185	115
CGGNNNNNNNNNCCG	PUT3	3.99	9.60E-03	0.096	136
TAWWWWTAGM	RLM1	3.89	1.50E-02	0.050	376
AGGGG	MSN2-4	3.69	3.20E-02	0.023	921
RMACCCA	RAP1	-3.71	3.00E-02	-0.072	366
CCRTACA	RAP1	-7.94	2.90E-13	-0.162	270

(B) genes groups defined by having their upstream region bound by a specific transcription factor in genome-wide chromatin immunoprecipitation (ChIP) experiments [c]

Transcription factor	Condition [d]	t-value	E-value	Mean log_2(ratio)	ORFs
GLN3	RAPA	16.09	< 1.0e-15	0.631	62
GCN4	RAPA	13.91	< 1.0e-15	0.340	151
DAL81	RAPA	12.80	< 1.0e-15	0.426	85
GCN4	SM	12.77	< 1.0e-15	0.287	176
GCN4	YPD	8.58	< 1.0e-15	0.311	72
DAL82	RAPA	6.81	3.10E-09	0.292	52
DAL82	SM	6.26	1.20E-07	0.259	55
GLN3	SM	4.45	2.70E-03	0.203	45
YAP7	H2O2Hi	4.30	5.50E-03	0.105	135
GAT1	RAPA	3.85	3.70E-02	0.243	25
BAS1	YPD	-4.14	1.10E-02	-0.234	37
SIP4	SM	-4.16	1.00E-02	-0.333	19
HAP4	YPD	-4.69	8.70E-04	-0.204	62
MBP1	YPD	-4.88	3.40E-04	-0.152	122
SFP1	SM	-8.44	< 1.0e-15	-0.428	43
RAP1	YPD	-10.01	< 1.0e-15	-0.263	157
FHL1	SM	-16.03	< 1.0e-15	-0.367	193
FHL1	RAPA	-17.08	< 1.0e-15	-0.404	180
FHL1	YPD	-18.80	< 1.0e-15	-0.442	179

(C) genes groups defined by membership in a specific Gene Ontology (GO) category

Category	Aspect [e]	t-value	E-value	Mean log_2(ratio)	ORFs
amine metabolism	P	7.11	1.60E-09	0.152	198
polyamine transporter activity	F	6.46	1.50E-07	0.645	11
nitrogen metabolism	P	5.48	5.90E-05	0.294	35
asparagine metabolism	P	4.96	9.80E-04	0.558	9
fermentation	P	4.29	2.50E-02	0.355	16

sulfur amino acid metabolism	P	-4.26	2.80E-02	-0.224	29
chromosome	C	-4.41	1.40E-02	-0.080	190
purine nucleotide biosynthesis	P	-4.43	1.30E-02	-0.211	35
protein metabolism	P	-4.56	7.10E-03	-0.028	1137
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	P	-4.81	2.10E-03	-0.036	1265
tricarboxylic acid cycle intermediate metabolism	P	-5.36	1.20E-04	-0.379	19
mitochondrial electron transport chain	C	-6.51	1.00E-07	-0.443	22
cytosolic ribosome (sensu Eukarya)	C	-21.87	< 1.0e-15	-0.551	152

[a] Transcriptional profiles after 6 hours of ectopic *MKS1* expression were analyzed with T-profiler (Boorsma, et al., 2005). Induced gene groups are shaded in red, and repressed gene groups are shaded in green. See Material and Methods for details.

[b] Ambiguity Codes: S = C or G; W = A or T; R = A or G; Y = C or T; K = G or T; M = A or C; N = A, C, G or T.

[c] Genome-wide ChIP data is from (Harbison, et al., 2004).

[d] Conditions were: YPD = log phase growth in rich medium; H2O2Hi = treatment with 4 mM hydrogen peroxide for 30 minutes; H2O2Lo = treatment with 0.4 mM hydrogen peroxide for 30 minutes; Acid = treatment with 50 mM succinic acid for 30 minutes to reach a pH of 4.0; SM = treatment with 0.2 μg/mL sulfometuron methyl (an inhibitor of amino acid biosynthesis) for 2h; RAPA = treatment with 100 nM rapamycin (an inhibitor of TORC1) for 20 minutes (Harbison, et al., 2004).

[e] Gene Ontology categories: F = molecular function; P = biological process; C = cellular component.

2.2.5 The Cdc14 phosphatase is physically and functionally linked to diverse cellular processes

In addition to characterizing novel TOR interactors, I also investigated potential functional links between the cell-cycle phosphatase Cdc14 and other cellular pathways as suggested by Cdc14's interactions in the KPI network. Although Cdc14 is not a focus of this chapter, these experiments are shown below for completeness.

The Cdc14 phosphatase antagonizes mitotic cyclin-dependent kinase (CDK) activity at the end of mitosis, thereby allowing re-establishment of G1 phase and loading of origins of DNA replication for the next S phase (Stegmeier and Amon, 2004). Cdc14 is sequestered in the nucleolus by the anchor protein Net1 until it is released in anaphase by the FEAR (fourteen early anaphase release) network and the MEN (mitotic exit network) (Stegmeier and Amon, 2004). In the KPI network generated by our laboratory, the Cdc14 phosphatase formed one of the largest single hubs in the network with 53 interaction partners. Many of these associations were shared with Net1 and the nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase Sir2, which along with Cdc14 collectively form the nucleolar RENT (regulator of nucleolar silencing and telophase exit) complex (Figure 2-5A).

To determine if these physical interactions reflected functional connections between Cdc14 and other cellular pathways, I examined strains with deregulated Cdc14 activity for relevant phenotypes. The interaction between Cdc14 and the DNA damage checkpoint kinases Chk1 and Dun1 prompted the finding that ectopic expression of *CDC14* caused sensitivity to the DNA-damaging agent methylmethane sulfonate (MMS), and reduced Cdc14 function conferred sensitivity to the ribonucleotide reductase inhibitor hydroxyurea (Figure 2-5B).

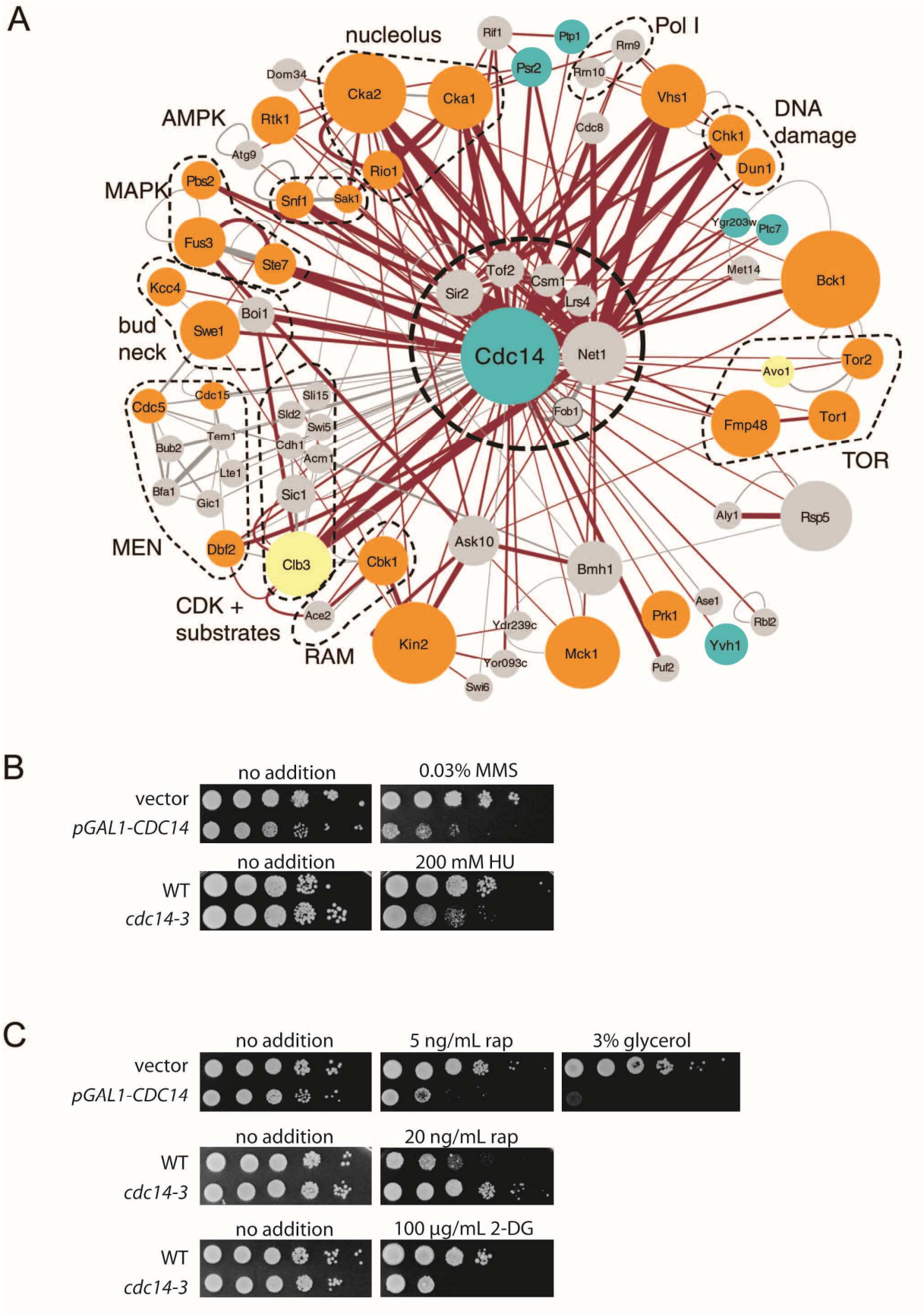
Cdc14 also exhibited connections with nutrient-sensing and metabolic pathways. The interaction between Cdc14 and Tor1 prompted the finding that ectopic expression of *CDC14* caused rapamycin sensitivity, whereas reduced Cdc14 function caused rapamycin resistance (Figure 2-5C). The interaction between Cdc14 and the adenosine 5'-monophosphate (AMP)–activated kinase (AMPK) Snf1 and its upstream kinase Sak1 prompted the finding that reduced Cdc14 function caused sensitivity to 2-deoxyglucose (Figure 2-5C). Ectopic expression of *CDC14* also caused a growth defect on a non-fermentable carbon source (Figure 2-5C).

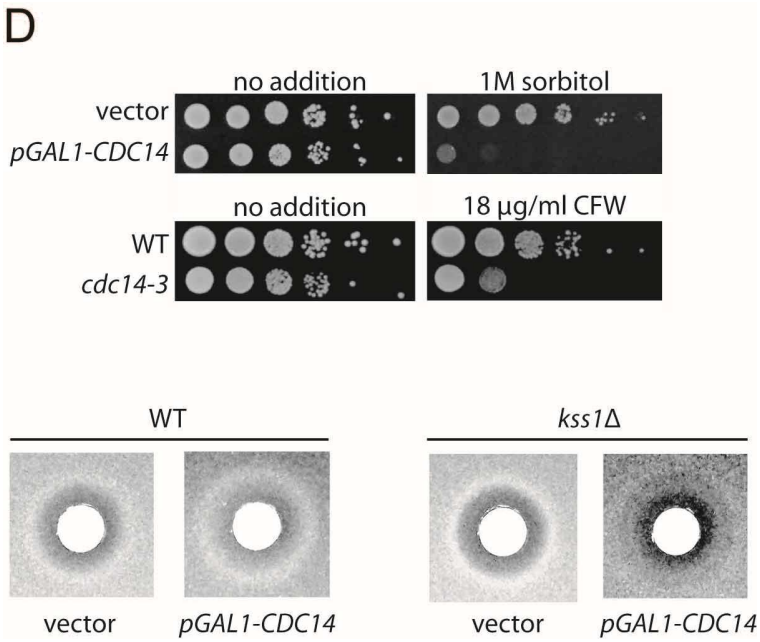
Cdc14 also exhibited connections with 3 different mitogen-activated protein kinase (MAPK) modules. First, the interaction between Cdc14 and the high osmolarity glycerol (HOG) pathway MAPK kinase Pbs2 prompted the finding that ectopic expression of *CDC14* caused sensitivity to osmotic stress (Figure 2-5D). Also, the interaction between Cdc14 and the cell wall integrity (CWI) pathway MAPK kinase Bck1 prompted the finding that reduced Cdc14 function caused sensitivity to the cell wall stress agent calcofluor white (Figure 2-5D). Finally, the interaction between Cdc14 and the pheromone MAPK pathway kinases Fus3 and Ste7 prompted the finding that ectopic expression of *CDC14* caused partial pheromone resistance (Figure 2-5D).

These observations suggest that Cdc14 has a role in nutrient, metabolic, and stress signaling in addition to its role in driving the cell cycle. These findings also serve to illustrate that the disruption of kinase or phosphatase function can cause cryptic phenotypes that are only apparent in directed studies prompted by physical interaction data.

Figure 2-5. The Cdc14 phosphatase is physically and functionally linked to diverse cellular processes. (A) Interaction network for the Cdc14-Net1-Sir2 (RENT) complex. Bold dashed circle indicates the nucleolar RENT (regulator of nucleolar silencing and telophase exit) complex and known associated proteins. MEN, mitotic exit network; CDK, cyclin-dependent kinase; RAM, regulation of Ace2p activity and cellular morphogenesis; TOR, target of rapamycin; AMPK, adenosine 5′-monophosphate (AMP)–activated kinase; MAPK, mitogen-activated protein kinase. See legend to Figure 2-1 for details on colour scheme, line thickness, and node size. Figure constructed by Dr. Anne-Claude Gingras. **(B)** Cdc14 exhibits functional connections to DNA damage signaling. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-CDC14*^{FLAG}> plasmid (pMT3887) or empty vector (pMT3164) was spotted onto SC-leu +2% raffinose +0.02% galactose +0.03% methyl methanesulfonate (MMS) and incubated at 30°C for 4 days (+MMS) or 3 days (untreated control). Congenic wild type (BY4741) and *cdc14-3* (yMT4206) strains were spotted onto XY +2% glucose +200 mM hydroxyurea (HU) and incubated at the semi-permissive temperature of 33°C for 4 days (+HU) or 2 days (untreated control). **(C)** Cdc14 exhibits functional connections to nutrient-sensing and metabolism. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-CDC14*^{FLAG}> plasmid (pMT3887) or empty vector (pMT3164) was spotted onto: SC-leu +2% raffinose +0.05% galactose +5 ng/mL rapamycin and incubated at 37°C for 4 days (+rap) or 3 days (untreated control); and SC-leu +3% glycerol +0.05% galactose and incubated at 37°C for 7 days. Congenic wild type (BY4741) and *cdc14-3* (yMT4206) strains were spotted onto: SC +2% glucose + 20 ng/mL rapamycin and incubated at the semi-permissive temperature of 33°C for 5 days (+rap) or 2 days (untreated control); and SC + 2% sucrose + 1 µg/mL antimycin A + 100 µg/mL 2-deoxyglucose (2-DG) and incubated at the semi-permissive temperature of 33°C for 7 days (+2-DG) or 2 days (untreated control). **(D)** Cdc14 exhibits functional connections to the high osmolarity glycerol (HOG) MAPK pathway, cell wall integrity (CWI) MAPK pathway, and pheromone MAPK pathway signaling. A *gal1Δ* strain (yMT4229) carrying either a <*pGAL1-CDC14*^{FLAG}> plasmid (pMT3887) or empty vector (pMT3164) was spotted onto SC-leu +2% raffinose +0.05% galactose +1 M sorbitol and incubated at 37°C for 4 days (+sorbitol) or 3 days (untreated control). Congenic wild type (BY4741) and *cdc14-3* (yMT4206) strains were spotted onto XY +2% glucose +18 µg/mL calcofluor white (CFW) and incubated at the semi-permissive temperature of 33°C for 2 days. Congenic wild type (BY4741) and *kss1Δ* (yMT4236) strains, each carrying either a <*pGAL1-*

CDC14^{FLAG}> plasmid (pMT3887) or empty vector (pMT3164), were diluted into top agar and poured onto plates of SC-leu +2% raffinose + 0.2% galactose. Filter discs containing 25 μg α-factor were placed on solidified medium and incubated for 3 days at 30°C. Panels A-D are from: Breitkreutz, A., Choi, H., Sharom, J.R., Boucher, L., Neduva, V., Larsen, B., Lin, Z.-Y., Breitkreutz, B.-J., Stark, C., Liu, G., Ahn, J., Dewar-Darch, D., Reguly, T., Tang, X., Almeida, R., Qin, X.S., Pawson, T., Gingras, A.-C., Nesvizhskii, A.I., and Tyers, M. (2010). A global protein kinase and phosphatase interaction network in yeast. *Science* **328**, 1043-1046. Reprinted with permission from AAAS.


A
nucleolus
Pol I
AMPK
DNA damage
MAPK
bud neck
MEN
CDK + substrates
RAM
TOR
Cdc14
Cka2
Cka1
Rtk1
Rio1
Pbs2
Snf1
Sak1
Fus3
Ste7
Kcc4
Swe1
Boi1
Cdc5
Cdc15
Sli15
Sld2
Swi5
Tem1
Cdh1
Bub2
Acm1
Lte1
Bfa1
Gic1
Sic1
Dbf2
Clb3
Cbk1
Ace2
Kin2
Ydr239c
Yor093c
Swi6
Mck1
Puf2
Prk1
Ase1
Rbl2
Yvh1
Ask10
Bmh1
Aly1
Rsp5
Fmp48
Tor1
Tor2
Avo1
Net1
Fob1
Tof2
Csm1
Lrs4
Sir2
Met14
Bck1
Ygr203w
Ptc7
Chk1
Dun1
Vhs1
Cdc8
Rrn10
Psr2
Rrn9
Ptp1
Rif1
Dom34
Atg9
B
no addition
0.03% MMS
vector
pGAL1-CDC14
no addition
200 mM HU
WT
cdc14-3
C
no addition
5 ng/mL rap
3% glycerol
vector
pGAL1-CDC14
no addition
20 ng/mL rap
WT
cdc14-3
no addition
100 µg/mL 2-DG
WT
cdc14-3


D
no addition
1M sorbitol
vector
pGAL1-CDC14
no addition
18 µg/ml CFW
WT
cdc14-3
WT
kss1Δ
vector
pGAL1-CDC14
vector
pGAL1-CDC14

2.3 Discussion

2.3.1 Nnk1 links TORC1 to nitrogen sensing and metabolism

Prior to this work, relatively little was known about the kinase encoded by *YKL171W* (here renamed *NNK1* for nitrogen network kinase). Cells deleted for *NNK1* had been found to have altered proteasome function (Cagney, et al., 2001) and aberrant levels of trace elements similar to vacuole-defective mutants (Eide, et al., 2005). Nnk1 had been reported to interact with the proteasome subunit Rpn1 by yeast two-hybrid assay (Cagney, et al., 2001), and with the 14-3-3 proteins Bmh1 and Bmh2 by immunoprecipitation-mass spectrometry (Kakiuchi, et al., 2007). Hinting at a role in nutrient sensing, *NNK1* transcript levels were known to increase during glycerol-based respiratory growth (Roberts and Hudson, 2006), the diauxic shift (DeRisi, et al., 1997), nitrogen depletion (Gasch, et al., 2000), entry into stationary phase (Gasch, et al., 2000), and TORC1 inhibition by rapamycin (Huang, et al., 2004). Nnk1 had also been predicted to contain a mitochondrial targeting sequence (Tomaska, 2000). This work presents evidence that physically and functionally links Nnk1 to TORC1 and two components of the nitrogen signaling apparatus in yeast: the nitrogen catabolite repression (NCR) regulator Ure2, and the NAD-linked glutamate dehydrogenase Gdh2.

Nnk1 physically interacted with TORC1 and overexpression of *NNK1* conferred sensitivity to rapamycin, suggesting that Nnk1 is a negative regulator of TORC1 signaling or acts in a cellular process that is negatively regulated by TORC1. Although the most conventional model would have Nnk1 acting as a downstream effector of TORC1 (Figure 2-6), it is worth noting that there is currently insufficient evidence to conclusively assign directionality to the TORC1-Nnk1 connection.

Nnk1 also physically interacted with Ure2, consistent with the results of another high-throughput study (Tarassov, et al., 2008). Ure2 is a negative regulator of nitrogen catabolite repression (NCR)-sensitive transcription that acts by binding and sequestering the Gln3 transcription factor. Overexpression of *NNK1* induced rapid nuclear accumulation of Gln3 and increased the transcript levels of Gln3 target genes. This effect was highly specific – of the 10 genes induced at least 2-fold after 90 minutes of *NNK1* overexpression, all were known Gln3 targets. These findings suggest that Nnk1 inhibits the Ure2-Gln3 interaction, potentially by

phosphorylation of one or both proteins (Figure 2-6). I speculate that Nnk1 may be the unidentified kinase reported to cause hyperphosphorylation of Gln3 during its nuclear accumulation in response to the glutamine synthetase inhibitor methionine sulfoximine (MSX) (Tate, et al., 2005). The physical interaction between TORC1, Nnk1, and Ure2 is intriguing given mounting evidence that a non-preferred nitrogen source and rapamycin induce NCR-sensitive gene expression via distinct mechanisms (Puria and Cardenas, 2008), and models suggesting that Ure2 may act as an intracellular sensor of nitrogen quality via direct binding of glutamine (Magasanik, 2005).

Finally, Nnk1 also physically interacted with Gdh2, the NAD^+-linked glutamate dehydrogenase that catalyzes deamination of glutamate to α-ketoglutarate and ammonia. This interaction appeared exceptionally robust by both mass spectrometry and affinity capture-immunoblot. Gdh2 was phosphorylated in Nnk1 immune complexes *in vitro*, although it is currently unknown whether this represents direct phosphorylation by Nnk1 or by a co-precipitating kinase. I speculate that Nnk1 may be the unidentified kinase activity reported to phosphorylate and inactivate Gdh2, first described over 30 years ago (Uno, et al., 1984). In the course of investigating further connections between Nnk1 and Gdh2, I found that a *gdh2Δ* strain was resistant to rapamycin when glutamate was the sole nitrogen source. This phenotype may be a reflection of this mutant having increased levels of intracellular glutamate and/or glutamine, which is though to be the key upstream activator of TORC1 (Crespo, et al., 2002). The ability of the *gdh2Δ* strain to grow on glutamate media at all is likely due to functional redundancy provided by the $NADP^+$-dependent glutamate dehydrogenases Gdh1 and Gdh3, which normally catalyze the reverse reaction to Gdh2 but which may be able to substitute for it under certain conditions (DeLuna, et al., 2001; Magasanik and Kaiser, 2002; Miller and Magasanik, 1990).

Interestingly, *NNK1* overexpression was sufficient to cause relocalization of Gdh2 from a diffuse cytoplasmic localization to one discrete focus per cell. Gdh2 foci were also present during respiratory growth and their formation under these conditions was Nnk1-dependent, but foci were not seen during carbon starvation or rapamycin treatment. The identity of the Gdh2 focus remains an open question, but several potential candidates exist. For instance, RNA processing bodies (P-bodies) are cytoplasmic granules known to harbour mRNA and protein components involved in mRNA metabolism (Parker and Sheth, 2007). Although P-bodies are generally found 2 or 3 per cell, they form one punctuate focus per cell under certain conditions

(Brengues and Parker, 2007). Other punctate cytoplasmic strctures have also been described, including EGP bodies (Hoyle, et al., 2007), TAM bodies (Gill, et al., 2006), T-bodies (Malagon and Jensen, 2008), foci containing eIF2B and eIF2 (Campbell, et al., 2005), and the pre-autophagosomal structure (PAS) – a focus adjacent to the vacuolar membrane involved in autophagic vesicle nucleation (Kawamata, et al., 2008). Alternatively, Gdh2 foci could represent a novel structure.

Interestingly, while this work was in progress, another study found that a large number of metabolic enzymes – including Gdh2 – formed reversible punctate foci when yeast cells were grown into stationary phase (Narayanaswamy, et al., 2009). The mechanism of foci formation and dissolution seemed enzyme-specific – for instance, Ade4-GFP foci were induced by adenine depletion in a cyloheximide-sensitive manner and dispersed by re-addition of adenine in a cyloheximide-insensitive manner, whereas Gln1-GFP foci were induced by glucose depletion in a cyloheximide-insensitive manner and dispersed by re-addition of glucose in a cyloheximide-sensitive manner (Narayanaswamy, et al., 2009). Thus, Gdh2 foci may represent part of a more general phenomenon in which enzymes are reversibly sequestered in protein assemblies, either for storage, or to enable efficient channeling of substrates between enzymes in a metabolic pathway (Srere, 1987). Indeed, a multi-protein complex composed of enzymes involved in purine biosynthesis (the "purinosome") has been previously described in mammalian cells (An, et al., 2008; An, et al., 2010), and a recent study found mammalian glutamate dehydrogenase in a supramolecular complex with enzymes involved in branched-chain amino acid catabolism (the branched-chain amino acid "metabolon") (Islam, et al., 2010).

The physical connection between TORC1, Nnk1, and Gdh2 is intriguing when viewed in the larger context of emerging evidence from higher eukaryotes that suggests an intimate link between TOR signaling, metabolism, and cancer. Often deregulated in human malignancies, the TOR signaling network is currently a major target for cancer therapeutics (Chiang and Abraham, 2007; Easton and Houghton, 2006; Faivre, et al., 2006; Guertin and Sabatini, 2005; Guertin and Sabatini, 2007; Lane and Breuleux, 2009; Sabatini, 2006). In recent years, an increasing body of evidence has pointed to a critical role for metabolic transformation in cancer, and changes in energy metabolism are now widely recognized as a near-universal attribute of malignant cells (DeBerardinis, et al., 2008a; Deberardinis, et al., 2008b; Fritz and Fajas, 2010; Hsu and Sabatini, 2008; Jones and Thompson, 2009; Kroemer and Pouyssegur, 2008; Tennant, et al., 2009;

Tennant, et al., 2010; Thompson, 2009; Vander Heiden, et al., 2009). Cancer cells may become dependent on these metabolic changes, leading to "glucose addiction" (Shaw, 2006) or "glutamine addiction" (DeBerardinis and Cheng, 2010; Wise and Thompson, 2010). In particular, recent work has highlighted the central role of glutamine, glutamate, and α-ketoglutarate in the metabolic alterations found in transformed cells.

In addition to the longstanding observation of increased aerobic glycolysis in cancer cells (the "Warburg effect"), recent work indicates that transformed cells exhibit a high rate of glutamine metabolism that exceeds that required for protein and nucleotide synthesis (DeBerardinis, et al., 2007). In these cells, glutamine is metabolized by glutaminolysis, in which the sequential action of glutaminase (GLS) and glutamate dehydrogenase (GDH) converts glutamine to glutamate and then to α-ketoglutarate, which can in turn enter the TCA cycle. Interestingly, the MYC oncogene has been reported to promote glutaminolysis through the induction of a suite of genes, including mitochondrial glutaminase 1 (GLS1) (Gao, et al., 2009; Wise, et al., 2008). Indeed, glutamine depletion in Myc-transformed human cells has been shown to cause loss of TCA cycle intermediates and subsequent apoptotic cell death (Yuneva, et al., 2007), suggesting that deregulated MYC may promote "glutamine addiction". Paradoxically, the tumour suppressor p53 has also recently been shown to promote glutaminolysis, but through the induction of mitochondrial glutaminase 2 (GLS2) (Hu, et al., 2010; Suzuki, et al., 2010), suggesting that the regulation and consequences of glutaminolysis are complex. Cross-talk between glutaminolysis and other metabolic pathways has also been noted. For instance, glutaminolysis has been found to stimulate glucose uptake and glycolysis via repression of the MondoA transcription factor (Kaadige, et al., 2009), which may act as a sensor of mitochondrial energy status (Sans, et al., 2006). Glutaminolysis and GDH activity are critical for the survival after glucose deprivation of MYC-transformed cells (Yang, et al., 2009) and *TSC1/2 -/-* cells with constitutively high mTORC1 activity (Choo, et al., 2010). Interestingly, ammonia produced as a product of glutaminolysis has recently been identified as a diffusible factor that stimulates autophagy (Eng, et al., 2010), a process known to have both positive and negative roles in regulating tumorigenesis (Morselli, et al., 2009). Understanding the complex interplay between TOR signaling, glucose, glutamine, α-ketoglutarate, and other metabolites may facilitate the development of cancer therapeutics that selectively exploit the metabolic deregulation characteristic of transformed cells.

Although intracellular glutamine is thought to be the primary regulator of TORC1 in yeast (Crespo, et al., 2002), in higher eukaryotes mTORC1 is thought to be activated primarily by leucine (Avruch, et al., 2009). However, a recent report showed that glutamine is also a critical regulator of mTORC1, albeit indirectly – it was found that the rate-limiting step for mTORC1 activation was cellular uptake of glutamine and its subsequent export in exchange for import of leucine and other essential amino acids via the SLC7A5 antiporter (Nicklin, et al., 2009). Interestingly, since MYC has been reported to drive transcription of both the glutamine transporter SLC1A5 and the glutamine antiporter SLC7A5 (Gao, et al., 2009), MYC may indirectly activate mTORC1 via this mechanism.

Alternatively, it is also worth noting that leucine – based on its ability to act as an allosteric activator of GDH (Li, et al., 2003a; Sener and Malaisse, 1980) – has been proposed to activate mTORC1 in an indirect manner by stimulating glutaminolysis (Tennant, et al., 2009). In this view, the downstream product of glutaminolysis – the TCA cycle intermediate α-ketoglutarate – is actually the key molecule regulating mTORC1. The level of α-ketoglutarate is proposed to act as a proxy for the level of amino acids, and to control the activity of prolyl hydroxylases (PHDs), enzymes that use α-ketoglutarate as a substrate (Boulahbel, et al., 2009). Although the level of α-ketoglutarate has been shown to regulate PHD activity *in vivo* (MacKenzie, et al., 2007), the above hypothesis is speculative and awaits evidence that PHDs regulate mTORC1. Still, this model is unique in that unlike all previously proposed models for amino acid sensing by mTORC1 – including those involving the Rheb GTPase (Long, et al., 2005b), the MAP kinase MAP4K3 (Findlay, et al., 2007), the class III phosphoinositide 3-kinase hVps34 (Gulati, et al., 2008), and the Rag GTPases (Kim, et al., 2008; Sancak, et al., 2008) – this model offers a specific molecular mechanism for how amino acids are sensed in the cell (Boulahbel, et al., 2009). The intimate link between glutamine/α-ketoglutarate flux and TORC1 hints that the physical connection between TORC1, Nnk1, and Gdh2 observed in yeast may be relevant to higher eukaryotes. Indeed, the remarkable evolutionary conservation of TOR signaling has meant that, historically, findings in yeast have often foreshadowed and informed discoveries in higher eukaryotes (Crespo and Hall, 2002; De Virgilio and Loewith, 2006a).

2.3.2 Fmp48 links TORC1 to mitochondrial function

Prior to this work, relatively little was known about the kinase encoded by *FMP48*. Cells deleted for *FMP48* had been found to be sensitive to high levels of copper (van Bakel, et al., 2005), and the Fmp48 protein had been detected in purified mitochondria (Reinders, et al., 2006; Sickmann, et al., 2003), consistent with its predicted mitochondrial targeting sequence (Tomaska, 2000). As expected from the presence of an Msn4-binding site in its 5' upstream region, *FMP48* expression had been found to be induced in response to a variety of stresses, including DNA damage (Dardalhon, et al., 2007), high osmolarity (Hohmann, 2002), the diauxic shift (DeRisi, et al., 1997), high calcium or sodium (Yoshimoto, et al., 2002), reoxygenation following anaerobic growth (Lai, et al., 2006), diamide treatment (Gasch, et al., 2000), heat shock (Gasch, et al., 2000), and entry into stationary phase (Aragon, et al., 2008; Gasch, et al., 2000). This work presents evidence that physically and functionally links Fmp48 to TORC1 signaling.

Fmp48 physically interacted with TORC1 and overexpression of *FMP48* conferred resistance to rapamycin, suggesting that Fmp48 is a positive regulator of TORC1 signaling or acts in a cellular process that is positively regulated by TORC1. However, interpretation of the rapamycin resistance phenotype is complicated by the finding that Fmp48 overexpression also inhibits growth on a non-fermentable carbon source (see below), because a large number of mitochondrial deletions and perturbations that block respiration also exhibit rapamycin resistance (Butcher, et al., 2006; Chan, et al., 2000; Fournier, et al., 2010; Xie, et al., 2005). *In vitro* immune-complex kinase assays showed that Fmp48-associated kinase activity was consistently increased by rapamycin treatment. Although a co-precipitating kinase cannot currently be ruled out, a preliminary model would place TORC1 as an upstream inhibitor of Fmp48 (Figure 2-6). Fmp48 was also linked to mitochondrial function, in that *FMP48* overexpression caused a growth defect on a non-fermentable carbon source and abnormal mitochondrial morphology.

The findings presented here suggest a preliminary model in which Fmp48 is a negative regulator of respiration that is in turn negatively regulated by TORC1 (Figure 2-6). However, it should be noted that previous studies assessing the relationship between TOR and mitochondrial function have yielded a nuanced picture. In yeast, TOR and its effector Sch9 have been reported to be negative regulators of respiratory function (Bonawitz, et al., 2007; Hardwick, et al., 1999;

Lavoie and Whiteway, 2008; Pan and Shadel, 2009; Shamji, et al., 2000), while in mammalian cells, mTOR signaling has been shown to positively regulate respiration (Bentzinger, et al., 2008; Chen, et al., 2008; Cunningham, et al., 2007; Ramanathan and Schreiber, 2009; Schieke, et al., 2006). The difference between yeast and mammalian cells likely reflects yeast's unusual preference for fermentative metabolism over respiratory metabolism in the presence of glucose and oxygen (Diaz-Ruiz, et al., 2009; Vander Heiden, et al., 2009). However, paradoxically, other studies in yeast have consistently found a cryptic positive role for TOR and Sch9 in respiratory function (Chen and Powers, 2006; Cheng, et al., 2007a; Cheng, et al., 2007b; Ge, et al., 2010; Piper, et al., 2006; Smets, et al., 2008; Wei, et al., 2009a). Further complicating matters, in adipocytes, mTOR and its effector S6K appear to negatively regulate mitochondrial function (Polak, et al., 2008; Um, et al., 2004). Thus, the relationship between TOR and respiration is complex and appears to vary depending on the cellular context.

2.3.3 Mks1 links TORC1 to mitochondrial retrograde signaling

The yeast retrograde (RTG) mitochondria-to-nucleus signaling pathway is activated in response to mitochondrial dysfunction, growth on poor nitrogen sources, or TORC1 inhibition by rapamycin (Liu and Butow, 2006). The KPI network generated by our laboratory confirmed physical connections between many components of the RTG pathway that had initially been identified genetically (compare Figure 2-4A and Figure 2-4B). Although the RTG pathway regulators Mks1 and Rtg3 were known to be dephosphorylated under conditions where the pathway is activated, including TORC1 inhibition by rapamycin (Dilova, et al., 2002; Sekito, et al., 2002), no direct links with TORC1 had been reported. This work's finding that Mks1 and Rtg3 physically interact with TORC1 suggests that these proteins may in fact be direct substrates of TORC1.

In this work, Mks1 was functionally linked to TORC1 signaling. Both deletion and low-level ectopic expression of *MKS1* conferred sensitivity to rapamycin. The rapamycin sensitivity of an *mks1Δ* strain was apparent on a non-fermentable (but not fermentable) carbon source, which may explain its invisibility in previous genome-wide screens for rapamycin sensitivity conducted under glucose media conditions (Chan, et al., 2000; Parsons, et al., 2004; Xie, et al., 2005). Such a respiration-specific rapamycin phenotype has been noted at least once before (Camougrand, et al., 2003). As noted in previous studies (Edskes, et al., 1999; Gelperin, et al.,

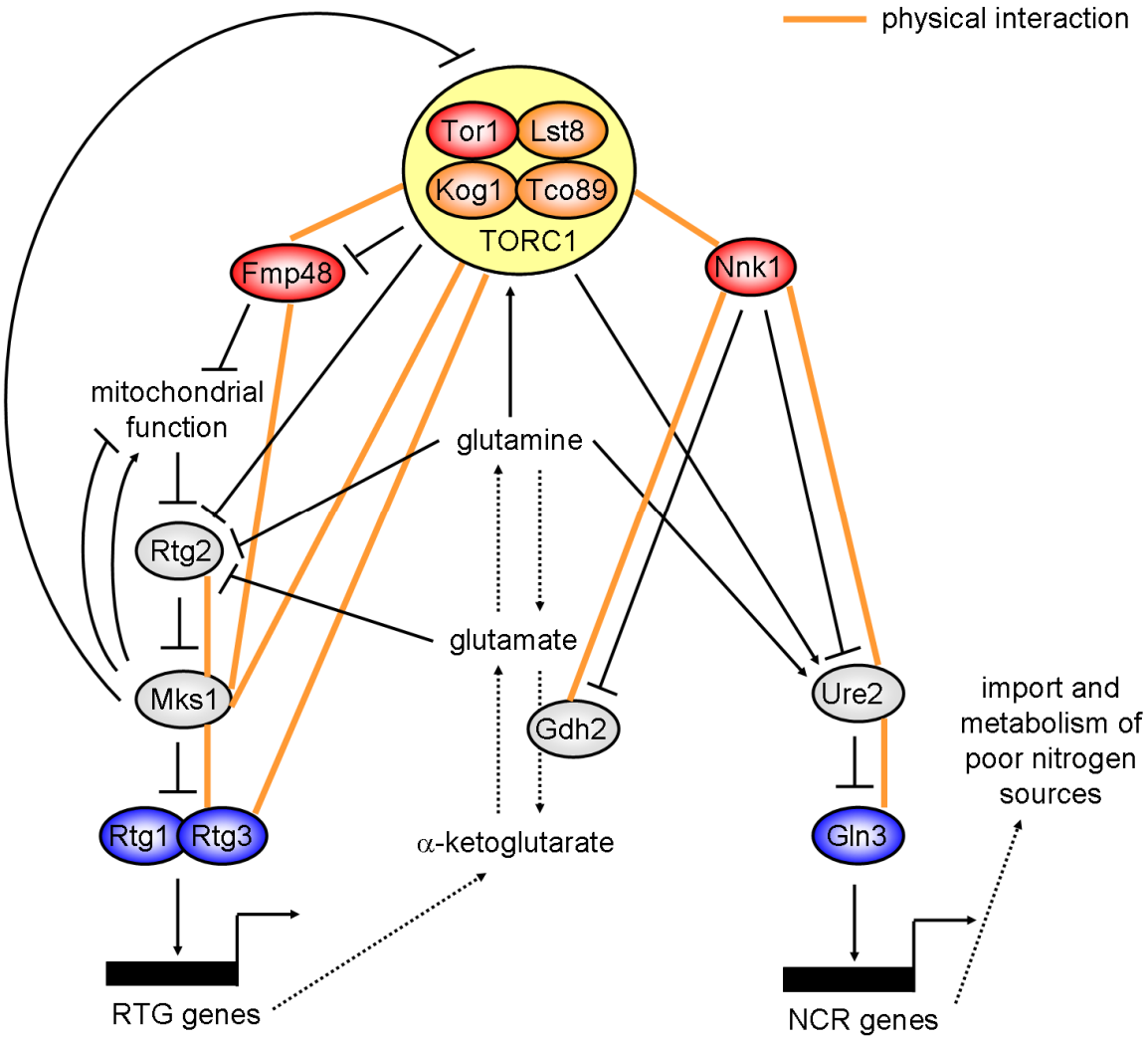
2005; Sopko, et al., 2006), overexpression of *MKS1* caused severe growth inhibition. This effect cannot be explained by the role of Mks1 as negative regulator of the Rtg1/3 transcription factor, and indeed was found to be independent of *RTG1*, *RTG2*, and *RTG3*. In contrast, consistent with the model that growth inhibition caused by *MKS1* overexpression is due to disruption of TORC1 signaling, it could be alleviated by mutations that confer rapamycin resistance (*npr1Δ*, *pmr1Δ*, *gln3Δ gat1Δ*, and *tap42-11*) and exacerbated by deletion of *TOR1*. This model is consistent with previous reports that overexpression of *MKS1* caused a G1 cell-cycle delay (Stevenson, et al., 2001) and de-repression of the NCR-sensitive gene *DAL5* (Edskes, et al., 1999). It is also foreshadowed by the previous isolation of Mks1 as a putative negative regulator of the PKA pathway (Matsuura and Anraku, 1993), which has partially overlapping roles with the TOR pathway in driving growth in response to nutrient availability (De Virgilio and Loewith, 2006a).

Although the role of Mks1 in the mitochondria-to-nucleus RTG signaling pathway has been well documented, its connection to mitochondrial respiratory function was unknown. Both deletion and low-level ectopic expression of *MKS1* were sufficient to block growth on a non-fermentable carbon source, suggesting that levels of Mks1 are critical for proper respiratory function. The inability of an *mks1Δ* strain to grow on a non-fermentable carbon source was apparent on synthetic (but not rich) glycerol medium, which may explain its invisibility in previous genome-wide screens for respiratory function conducted under rich media conditions (Dimmer, et al., 2002; Steinmetz, et al., 2002). Mks1 and Fmp48 interact in the KPI network, and low-level ectopic expression of either protein confers a severe growth defect on a non-fermentable carbon source. Comparison of the genome-wide expression profiles of strains overexpressing *MKS1* and *FMP48* showed similarities – for instance, repression of the canonical retrograde response gene *CIT2* and genes involved in respiratory metabolism, including TCA cycle enzymes, electron transport chain components, and subunits of the ATP synthase. However, substantial differences between the profiles also suggested non-overlapping functions. For instance, overexpression of *MKS1* but not *FMP48* caused strong induction of NCR-sensitive genes. Conversely, overexpression of *FMP48* but not *MKS1* caused strong induction of stress response genes.

The two known negative regulators of the RTG pathway, Lst8 and Mks1, were isolated genetically based on their ability to bypass the requirement for *RTG2* for retrograde gene expression (Liu, et al., 2001; Sekito, et al., 2002). Lst8 was subsequently found to be a

component of TOR complexes (Chen and Kaiser, 2003; Loewith, et al., 2002; Wedaman, et al., 2003). The finding that Mks1 is also physically and functionally connected with TORC1 suggests that the TOR and RTG pathways are deeply integrated at the molecular level via the sharing of common components.

Figure 2-6. Preliminary model showing connections between novel TORC1 interactors, mitochondrial function, and nitrogen signaling in yeast. Orange lines indicate physical interactions from the KPI network and the literature. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. Dashed arrows represent metabolic conversions. Transcription factors are shown in blue, kinases in read, and TORC1 components in orange. RTG, retrograde-responsive genes; NCR, nitrogen catabolite repression-sensitive genes.


physical interaction
Tor1
Lst8
Kog1
Tco89
TORC1
Fmp48
Nnk1
mitochondrial function
glutamine
Rtg2
glutamate
Mks1
Gdh2
Ure2
import and metabolism of poor nitrogen sources
Rtg1
Rtg3
α-ketoglutarate
Gln3
RTG genes
NCR genes

2.4 Material and Methods

2.4.1 Yeast media, strains, and plasmids

Yeast culture and genetics was performed according to standard techniques (Amberg, et al., 2005). Synthetic complete media (SC) is 0.2% amino acid mix, 0.5% ammonium sulfate, 0.17% yeast nitrogen base; where indicated, specific amino acids or uracil were omitted to select for plasmid maintenance. Rich media (XY) is 2% bactopeptone, 1% yeast extract, 0.01% adenine, 0.02% tryptophan. All chemicals were from Sigma-Aldrich (St. Louis MO) unless otherwise stated. Deletion strains were obtained from the genome-wide *MAT***a** deletion collection (EUROSCARF) or the genome-wide *MAT***α** deletion collection (Research Genetics) (Giaever, et al., 2002); strains bearing GFP-tagged proteins were obtained from the Yeast-GFP collection (Invitrogen) (Huh, et al., 2003); strains bearing TAP-tagged proteins were obtained from the Yeast TAP-tagged collection (Open Biosystems) (Gavin, et al., 2006; Gavin, et al., 2002; Ghaemmaghami, et al., 2003; Krogan, et al., 2006). Yeast strains were constructed using standard PCR-based methods (Goldstein and McCusker, 1999; Longtine, et al., 1998). Plasmid-borne FLAG-tagged genes were obtained by inserting ORFs into plasmid pMT3164 <*pGAL1-ORF*^{FLAG} *LEU2 CEN*> via Gateway recombination-based cloning (Invitrogen) as described previously (Ho, et al., 2002). HA-tagged ORFs in plasmid BG1805 <*pGAL1-ORF*$^{HA\text{-}PrA}$ *URA3 2μm*> were obtained from the Yeast ORF Collection (Open Biosystems) (Gelperin, et al., 2005). Strains and plasmids used in this work are listed in Table 2-3 and Table 2-4, respectively.

2.4.2 Affinity capture-immunoblot

Strains transformed with <*pGAL1-ORF*^{FLAG}> or <*pGAL1-ORF*$^{HA\text{-}PrA}$> expression plasmids were grown in appropriate synthetic selective medium in the presence of 2% raffinose to early log phase (A_{600} = ~0.4), and induced by the addition of 2% galactose for 2 hours prior to harvesting and flash-freezing in liquid nitrogen (final A_{600} = ~0.8). Cells were disrupted by bead lysis with HEN buffer [200 mM NaCl, 5 mM EDTA, 50 mM HEPES pH 7.5, 1 mM DTT, and EDTA-free protease inhibitor tablet (Roche)]. Immunoprecipitations were performed on 150 µg of total protein with 10 µL (2 µg) anti-HA (F-7) antibody (Santa Cruz) or 10 µL (20 µg) anti-M2-FLAG antibody (Sigma) pre-bound to Dynabead Protein A (Invitrogen), separated by SDS-PAGE, and transferred to Immobilon-P (PVDF) membranes (Millipore). HA-tagged proteins were detected using anti-HA monoclonal antibody (Santa Cruz) at 1:2000 dilution, followed by

incubation with anti-mouse IgG-HRP (Sigma) at 1:10,000 dilution in 3% milk powder/TBST solution. FLAG-tagged proteins were detected using anti-FLAG-HRP (Sigma A8592) at 1:1,000 dilution in 1% milk powder/TBS-T solution. All proteins were detected using the West Pico chemiluminescence detection system (Thermo-Fisher).

2.4.3 Immune-complex kinase assays

Immunoprecipitations were carried out in the same manner as for immunoblot analysis above, except that the amount of Dynabead Protein A bound to anti-HA or anti-FLAG antibody used to collect the bait protein was increased three-fold. Precipitated immune complexes were washed once with lysis buffer, transferred to a fresh tube and washed once with kinase reaction buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT), essentially as described (Tyers and Futcher, 1993). After aspiration of residual buffer, 5 µL of kinase reaction cocktail (15 µCi [^{33}P]-γ-ATP, 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) was added to the beads and incubated at 30°C for 30 minutes. Reactions were stopped by the addition of 2X SDS-PAGE sample buffer and radio-labeled products either resolved by SDS-PAGE or reimmunoprecipitated as detailed below.

Reimmunoprecipitation of [^{33}P]-labeled proteins was performed as described (Tyers and Futcher, 1993) by diluting the denatured kinase reaction mixtures 1:100 in reimmunoprecipitation buffer (1% Triton X-100, 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 5 mg/mL BSA), removing the magnetic beads from the first immunoprecipitation, and incubating the supernatant with Protein A beads conjugated to anti-HA antibody for 2 h at 4^{o}C. The beads were washed once with re-immunoprecipitation buffer, boiled in 10 µL 2X SDS-PAGE sample buffer and subjected to SDS-PAGE. Kinase reaction and reimmunoprecipitated products were visualized on either X-ray film or by phosphorimager.

2.4.4 Halo assays

Saturated overnight yeast cultures were washed once in distilled water, diluted 1:200 in molten top agar at 42°C, poured onto solid medium, and allowed to recover for 2 hours before addition of filter discs.

2.4.5 Cell viability spot assays

Saturated overnight cultures were washed once, diluted 1:10 in distilled water, and serially diluted in 10-fold steps. Volumes of 4 μL or 6 μL were spotted onto synthetic- or rich-media plates, respectively.

2.4.6 Live-cell microscopy

DIC and fluorescence microscopy were performed with an Eclipse E600FN microscope (Nikon) and imaged on an Orca II CCD camera (Hamamatsu, Bridgewater, NJ). Metamorph Software (Universal Imaging, West Chester, PA) was used to capture and process images. To visualize cells, an aliquot of culture was pelleted by centrifugation for 5 seconds, supernatant removed, and cells resuspended in residual liquid were mounted on a glass slide. Total time from incubation to visualization was < 3 min.

2.4.7 Genome-wide transcriptional profiles

A *gal1Δ* strain (yMT4164) carrying a <*pGAL1-FMP48*^{HA}> plasmid (pMT4494), a <*pGAL1-MKS1*^{HA}> plasmid (pMT4495), or empty vector BG1805 (pMT4177), was grown to early log phase in minimal YNB raffinose media (0.17% yeast nitrogen base, 2% raffinose, 0.5% ammonium sulfate). Deletion of *GAL1* renders strains incapable of metabolizing galactose, thereby minimizing changes in the metabolic state of the cell due to galactose addition (Wang, et al., 2004). Media lacking amino acids was used because their presence has previously been shown to mask some mitochondrial transcriptional effects (Hall, et al., 2004). Expression was induced with 0.2% galactose for 1.5 and 6 hours, after which cells were harvested by centrifugation at room temperature and frozen in liquid nitrogen. RNA was extracted by glass-bead lysis in equilibrated phenol, 3 rounds phenol-chloroform extraction, and precipitation. RNA from overexpression strains was competitively hybridized to RNA from an identically treated strain carrying empty vector. Two biological replicates were performed for each overexpressed gene.

Sample labeling, hybridization, and image analysis were performed by the University Health Network Microarray Centre (Toronto, Canada). Briefly, RNA samples were labeled using indirect aminoallyl labeling. Ten μg of total RNA were labeled with cyanine dyes (Cy5 and Cy3, Amersham Biosciences) and hybridized to Yeast 6.4K arrays. Hybridization was performed on

the Advalytix SlideBooster™ (Advalytix, Germany) using AdvaHybC hybridization solution at 42°C. Slides were scanned on the Agilent G2565BA scanner and quantified using Array Vision v.8.0 (Imaging Research Inc.) The quantified data from each array were then loaded into Midas (Microarray Data Analysis System, TM4) version 2.19 for normalization using Locfit (the equivalent of Lowess, sub-grid method). The data plotted was the average of 2 independent arrays (biological replicates), each of which bears 2 duplicate array features for each yeast ORF. All microarray data are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-2171.

Heat-maps visualizing genes whose expression changed over 1.5-fold were generated using AFM 4.0 (Breitkreutz, et al., 2001). To assess statistical overrepresentation of Munich Information Center for Protein Sequences (MIPS) (Mewes, et al., 2002) functional classifications in the set of up-regulated or down-regulated genes, FunSpec (http://funspec.med.utoronto.ca/) (Robinson, et al., 2002) was used, with a p-value cut-off of 1×10^{-3} and no Bonferroni correction. For an analysis approach that does not require the selection of an arbitrary 1.5-fold-change threshold, T-profiler (http://www.t-profiler.org/) (Boorsma, et al., 2005) was used. T-profiler can score a pre-defined group of genes as being significantly induced or repressed even if the expression of none of its individual members changes significantly (the method is sensitive to small-amplitude coordinate changes in the expression of groups of genes). The pre-defined groups of genes that were queried included: (i) genes containing a specific transcription factor DNA-binding motif in their upstream region (Kellis, et al., 2004; Roven and Bussemaker, 2003; Zhu and Zhang, 1999); (ii) genes bound by a specific transcription factor in a series of genome-wide ChIP experiments (Harbison, et al., 2004); and (iii) genes belonging to a specific Gene Ontology (GO) category (Ashburner, et al., 2000).

2.4.8 Cytoscape network representations

Visualization of interaction networks was performed using Cytoscape 2.6.0 (Shannon, et al., 2003). A master Cytoscape file was built by merging all KPI network interactions, low-throughput (LTP) interactions from the literature, and high-throughput - high-confidence (HTP-HC) interactions [previously reported in two or more HTP studies; see (Breitkreutz, et al., 2010) for details]. Kinases are shown in orange, kinase-associated proteins in yellow, phosphatases in dark blue, phosphatase-associated proteins in light blue, and other proteins in gray. Red

connecting lines indicate KPI network interactions, gray lines indicate LTP interactions, and gray dashed lines indicate HTP-HC interactions. Line thickness is proportional to the evidence for an interaction and node size is proportional to the total number of connections for a node. Normalized evidence counts were used to define line thickness as follows. When repeat purifications for a bait were available, the spectral counts were normalized (sum of spectra across all purifications divided by number of purifications). This number was further normalized by dividing the modified spectral count by 208, the highest spectral count for a prey in the KPI dataset. For literature-derived interactions, the number of evidence counts for an interaction – defined by the number of entries in the BioGRID database (Breitkreutz, et al., 2008) – was normalized by dividing by 28, the maximum number of evidence counts in this dataset. To generate each Cytoscape view, the indicated node(s) and its first neighbors were selected, and connections between the proteins displayed. The edge-weighed, spring-embedded Cytoscape layout was applied, using the normalized spectral count or evidence count as the weight factor, followed by manual adjustment.

Table 2-3. Yeast strains.

strain	relevant genotype	background	*MAT*	source
BY4741 (yMT1448)	*his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	S288C	**a**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
BY4742 (yMT1449)	*his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	S288C	**α**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
BY4743 (yMT1450)	*his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0*	S288C	**a/α**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
yMT2398	*pep4Δ::kanR*	S288C	**a**	EUROSCARF
yMT4204	*gln3::GLN3^{GFP}-his5+*	S288C	**a**	Invitrogen
yMT4205	*ilv3::ILV3^{GFP}-his5+*	S288C	**a**	Invitrogen
yMT4160	*rtg1Δ::URA3 rtg2Δ::natR rtg3Δ::kanR*	S288C	**a**	this work
yMT4161	*gln3Δ::natR gat1Δ::URA3*	S288C	**a**	this work
yMT4162	*gdh2::GDH2^{3HA}-his5+*	S288C	**a**	this work
yMT4206	*cdc14*::*cdc14-3-URA3 can1Δ mfa1::pMFA1-HIS3*	S288C	**α**	Lorrie Boucher
yMT4229	*gal1Δ::kanR*	S288C	**a**	EUROSCARF
yMT4230	*gdh2::GDH2^{GFP}-his5+*	S288C	**a**	Invitrogen
yMT4231	*gal1Δ::natR gdh2::GDH2^{GFP}-his5+*	S288C	**a**	this work
yMT4232	*nnk1Δ::kanR gdh2::GDH2^{GFP}-his5+*	S288C	**a**	this work
yMT4233	*mks1Δ*::*kanR*	S288C	**a**	EUROSCARF
yMT4234	*pmr1Δ*::*kanR*	S288C	**a**	EUROSCARF
yMT4235	*npr1Δ*::*kanR*	S288C	**a**	EUROSCARF

yMT4236	*kss1Δ*::*kanR*	S288C	**a**	EUROSCARF
BY4700 (yMT4254)	*ura3Δ0*	S288C	**a**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
BY4709 (yMT4255)	*ura3Δ0*	S288C	**α**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
yMT4163	*gdh2Δ::kanR ura3Δ0*	S288C	**a**	this work
yMT4164	*gal1Δ::natR ura3Δ0*	S288C	**a**	this work
CY4907 (yMT2894)	*tap42Δ::TRP1 <TAP42 LEU2 CEN>*	W303	**a**	Di Como, C.J. and Arndt, K.T. (1996). *Genes Dev* **10**, 1904-1916.
CY4908 (yMT2895)	*tap42Δ::TRP1 <tap42-11 LEU2 CEN>*	W303	**a**	Di Como, C.J. and Arndt, K.T. (1996). *Genes Dev* **10**, 1904-1916.

All S288C strains except for yMT4163 and yMT4164 are congenic with BY4741/BY4742 and thus carry *ura3Δ0*, *his3Δ1*, and *leu2Δ0*; note that *met15Δ0* and *lys2Δ0* were generally not followed in crosses and their status is unknown. yMT4163 and yMT4164 are congenic with BY4700/BY4709 and thus carry *ura3Δ0* only.

Table 2-4. Plasmids.

plasmid	relevant insert	yeast marker	vector type	source
pMT3164	empty *pGAL1-ORF*^{FLAG}	*LEU2*	*CEN*	Ho, Y. *et al*. (2002). *Nature* **415,** 180-183.
pMT4434	*pGAL1-FMP48*^{FLAG}	*LEU2*	*CEN*	Tyers lab collection
pMT4435	*pGAL1-MKS1*^{FLAG}	*LEU2*	*CEN*	Tyers lab collection
pMT4436	*pGAL1-NNK1*^{FLAG}	*LEU2*	*CEN*	Tyers lab collection
pMT3887	*pGAL1-CDC14*^{FLAG}	*LEU2*	*CEN*	Tyers lab collection
pMT4493	*pGAL1-SCH9*^{FLAG}	*LEU2*	*CEN*	Tyers lab collection
BG1805 (pMT4177)	empty *pGAL1-ORF*$^{HA\text{-}PrA}$	*URA3*	*2µm*	Open Biosystems
pMT4492	*pGAL1-NNK1*$^{HA\text{-}PrA}$	*URA3*	*2µm*	Open Biosystems
pMT4494	*pGAL1-FMP48*$^{HA\text{-}PrA}$	*URA3*	*2µm*	Open Biosystems
pMT4495	*pGAL1-MKS1*$^{HA\text{-}PrA}$	*URA3*	*2µm*	Open Biosystems
pMT4173	*pTOR1-*^{HA}*TOR1*	*HIS3*	*CEN*	gift of Yoshinori Ohsumi

Chapter 3 – The Sch9 kinase is a regulator and sensor of mitochondrial function

Contributions:

I designed and performed all the experiments in this chapter.

3.1 Abstract

The AGC-family kinase Sch9 is a powerful regulator of cell growth and lifespan that appears to occupy a position in yeast analogous to ribosomal protein S6 kinase (S6K) or protein kinase B (PKB)/Akt in higher eukaryotes. Here, I demonstrate that Sch9 appears to act as both an upstream regulator and downstream sensor of mitochondrial function. Loss of Sch9 conferred a respiratory growth defect, a defect in mitochondrial DNA (mtDNA) transmission, lower mitochondrial membrane potential, and decreased levels of reactive oxygen species (ROS). Conversely, loss of mtDNA caused loss of Sch9 enrichment at the vacuolar membrane, loss of Sch9 phospho-isoforms, and small cell size suggestive of reduced Sch9 activity. The ability of a protonophore – but not other interventions that inhibit respiration – to recapitulate the above effects suggested that the proton gradient across the inner mitochondrial membrane may be the molecular signal to which Sch9 responds. Epistasis analysis suggested that reduced Sch9 activity may account for the small size of cells lacking mtDNA. The ability of loss of Sch9 to confer small cell size was unaffected by the absence of all known upstream regulators of the G1/S transcriptional machinery, or by fixing the level of G1 cyclin by heterologous transcriptional control.

I also show that Sch9 exhibits dynamic relocalization in response to stress. Live cell imaging of GFP-tagged Sch9 showed that some cellular stresses caused sequential enrichment of Sch9 in the nucleus, followed by the nuclear envelope, and eventually in cycloheximide-inhibitable cytoplasmic foci that may correspond to messenger ribonucleoprotein (mRNP) granules. Sch9 also became enriched at mitochondria under conditions that have previously been shown to generate reactive oxygen species (ROS) and induce apoptosis in yeast.

Taken together, these findings implicate Sch9 in many cellular processes. In particular, the intimate connection between Sch9 and mitochondrial homeostasis has potential implications for the mechanism by which Sch9 controls lifespan.

3.2 Results

Our laboratory has investigated the function of Sch9 in nutrient sensing and cell size control (Jorgensen, et al., 2002; Jorgensen, et al., 2004). In previous work, we made several key observations: Sch9 was found to be enriched at the vacuolar membrane in log phase cells and

this enrichment was lost upon carbon starvation; Sch9 phospho-isoforms were found to be decreased by carbon starvation or rapamycin treatment; and Sch9 was found to control transcription of the ribosomal protein (RP) and ribosome biogenesis (RiBi) regulons (see Appendix 2) (Jorgensen, et al., 2004).

3.2.1 Sch9 regulates mitochondrial function

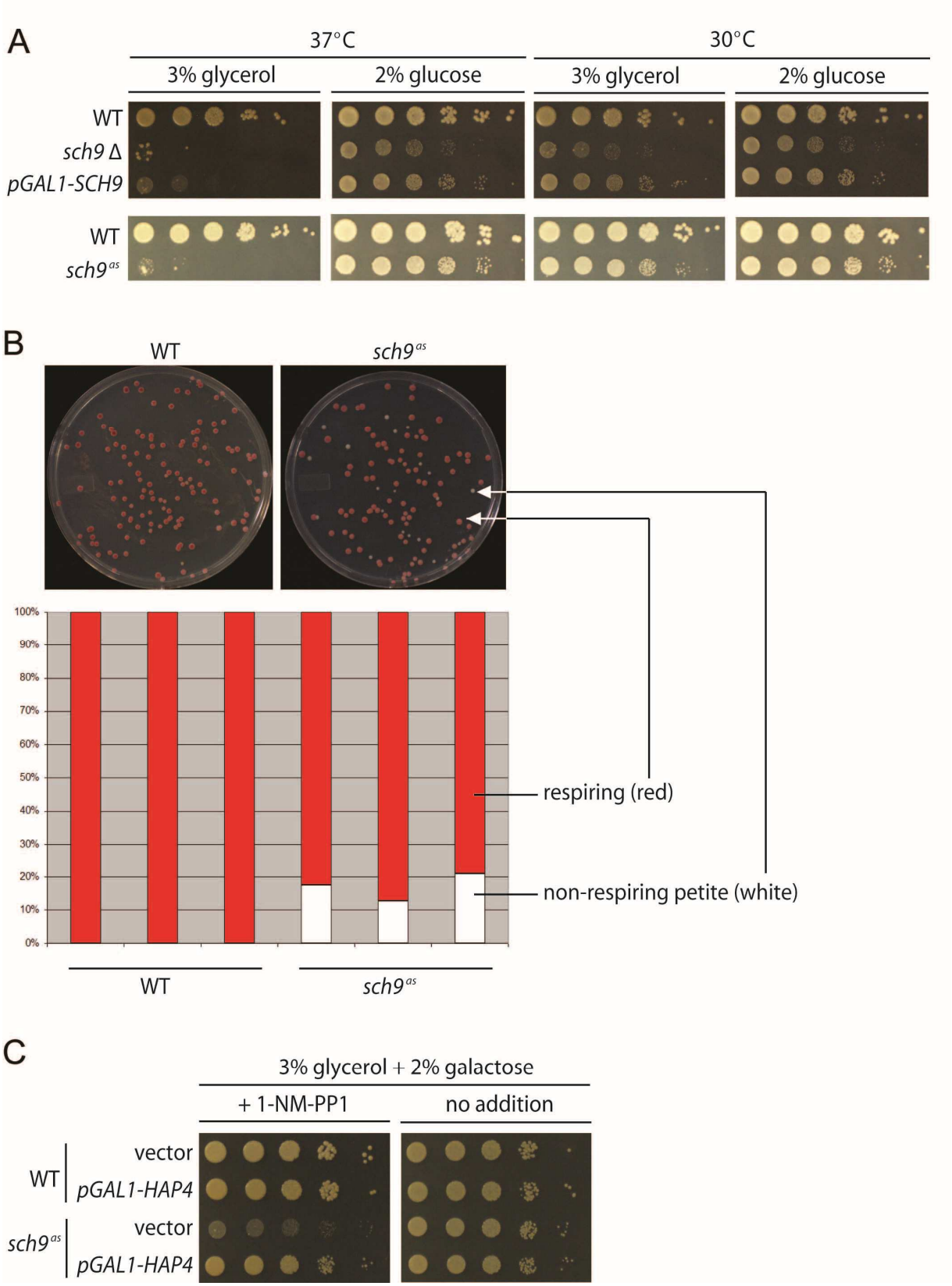
To discover novel functions of Sch9, cells lacking Sch9 kinase activity were assessed for their ability to grow under various nutrient and stress conditions. In the course of this work, I found that loss of Sch9 conferred a severe growth defect on a non-fermentable carbon source that was only apparent at 37°C. This phenotype was observed with a *sch9Δ* deletion strain, with a strain in which *SCH9* was under the control of the *GAL1* promoter, and with a strain bearing an analogue-sensitive (as) allele of Sch9 that is specifically inhibited by the chemical inhibitor 1-naphthylmethyl-PP1 (1-NM-PP1) (Figure 3-1A). The cell-size and mitochondrial functions of Sch9 appear to be separable, as the $sch9^{as}$ allele is sufficient to confer most of the small cell size of the *sch9Δ* deletion, but exhibits no growth defect on glycerol in the absence of 1-NM-PP1.

In yeast, the mitochondrial genome is non-essential for growth on a fermentable carbon source and can be lost to generate a rho^0 petite. To investigate if the inability to grow on a non-fermentable carbon source at 37°C was due to a defect in mtDNA (mtDNA) transmission under these conditions, a strain lacking Sch9 activity was assessed for frequency of petite generation. Loss of Sch9 activity caused a reproducible but modest increase in petite frequency at 37°C (Figure 3-1B).

Since this modest increase in petite generation was unlikely to be sufficient to account for the severe growth defect observed on glycerol medium, the mitochondrial effects conferred by loss of Sch9 were characterized in further detail. The growth defect on a non-fermentable carbon source conferred by loss of Sch9 could be partially suppressed by overexpression of *HAP4*, a transcriptional activator of respiratory genes (Figure 3-1C). To assess the effect of Sch9 on mitochondrial membrane potential ($\Delta\psi_m$), cells were stained with MitoTracker Red CMXRos, a dye whose uptake is dependent on membrane potential. Marking one of the strains with an unrelated GFP fusion protein allowed cells lacking Sch9 kinase activity to be directly compared to WT in the same field. Loss of Sch9 kinase activity resulted in lower mitochondrial membrane

potential (Figure 3-1D). In addition, staining with the reactive oxygen species (ROS)-sensitive dye dihydrorhodamine 123 showed that loss of Sch9 decreased levels of ROS (Figure 3-1E).

Figure 3-1. Sch9 regulates mitochondrial function. (A) Cells lacking Sch9 activity exhibit a thermosensitive growth defect on a non-fermentable carbon source. Congenic WT (BY4741), *pGAL1-SCH9* (yMT2422), and *sch9Δ* (yMT2290) strains were spotted onto SC +2% glucose and incubated for 2 days, or spotted onto SC +3% glycerol and incubated for 5 days (30°C) or 7 days (37°C). Congenic WT (BY4741) and $sch9^{as}$ (yMT2594) strains were spotted onto SC +2% glucose + 1 μM 1-NM-PP1 and incubated for 2 days, or spotted onto SC +3% glycerol + 1 μM 1-NM-PP1 and incubated for 5 days (30°C) or 7 days (37°C). **(B)** Loss of Sch9 causes a modest increase in the frequency of petites. Congenic WT (BY4741) and $sch9^{as}$ (yMT4238) strains were grown for ~18 generations at 37°C in SC +2% glucose + 200 nM 1-NM-PP1 and assessed for petite frequency by 2,3,5-triphenyltetrazolium chloride (TTC) overlay assay. A typical plate showing respiring (red) and non-respiring petite (white) colonies is shown. Respiring and non-respiring colonies (n>400) were scored for 3 independent replicates for each strain. **(C)** The growth defect on a non-fermentable carbon source conferred by loss of Sch9 can be partially suppressed by overexpression of *HAP4*. Congenic *gal1Δ* (yMT4229) and *gal1Δ* $sch9^{as}$ (yMT4239) strains carrying either a <*pGAL1-HAP4*> plasmid (pMT4496) or empty vector pRS316 (pMT273) were spotted onto SC-ura +3% glycerol +2% galactose +1 μM 1-NM-PP1 and incubated at 30°C for 7 days (+1-NM-PP1) or 6 days (no addition). **(D)** Loss of Sch9 causes reduces mitochondrial membrane potential. WT cells carrying an unrelated $Rpa190^{GFP}$ fusion protein (yMT4240) and $sch9^{as}$ cells (yMT2594) were grown to early log phase in SC +2% raffinose +200 nM 1-NM-PP1 for >16 h at 30°C and the two cultures were mixed for side-by-side comparison by microscopy. In the converse experiment, WT cells (BY4741) and $sch9^{as}$ cells carrying an unrelated $Rpa190^{GFP}$ fusion protein (yMT4241) were grown to early log phase in SC +2% raffinose +200 nM 1-NM-PP1 for >16 h at 30°C and the two cultures were mixed. Mitochondrial membrane potential was assessed in the mixed population of cells by staining with MitoTracker Red CMXRos. GFP-marked cells are circled in red. Enlargement shows detail of MitoTracker Red CMXRos staining for WT and $sch9^{as}$ cells. **(E)** Loss of Sch9 decreases levels of reactive oxygen species (ROS). Congenic WT (BY4741) and $sch9^{as}$ (yMT4238) strains were grown to saturation in SC +2% raffinose +200 nM 1-NM-PP1 +5 μg/mL dihydrorhodamine 123 for >16 h at 30°C in the dark.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30°C
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2% glucose
3% glycerol
2% glucose
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sch9 Δ
pGAL1-SCH9
WT
sch9as
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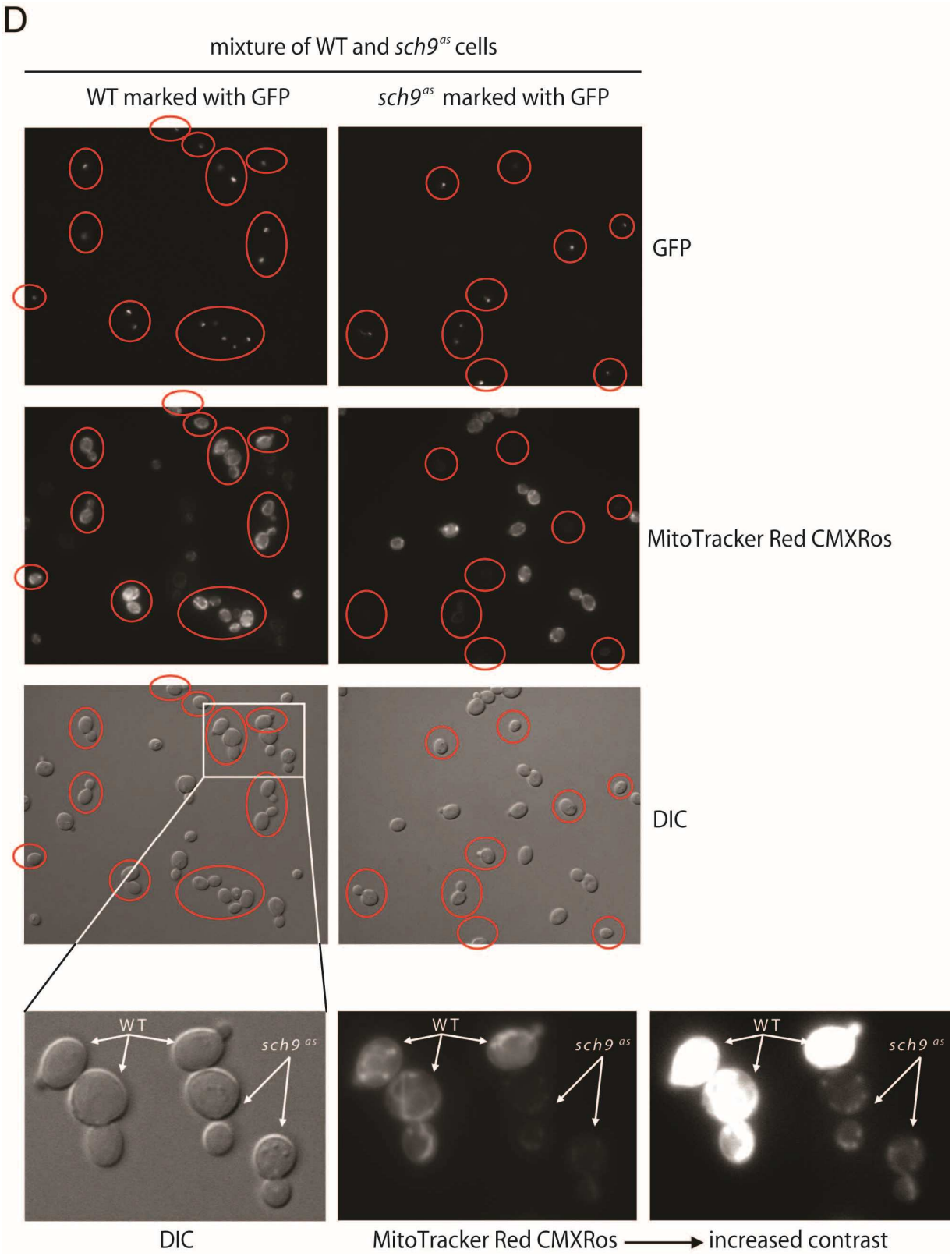
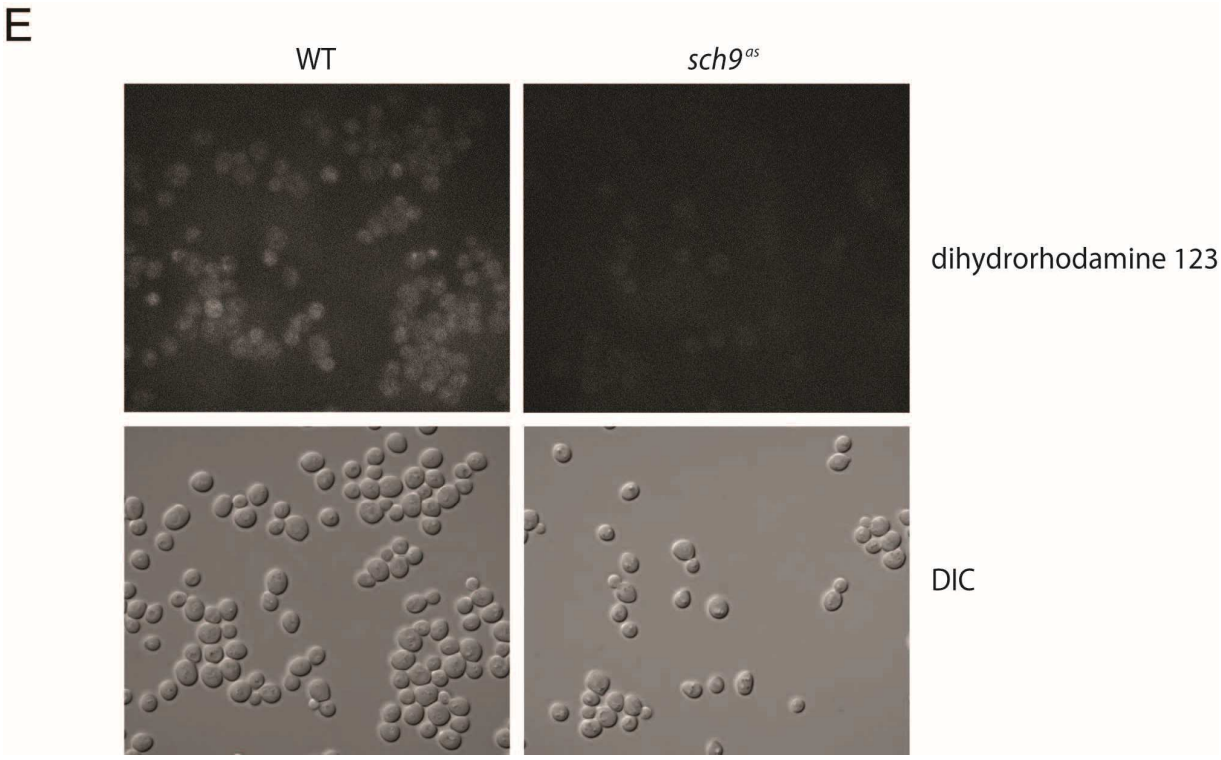
WT
sch9as
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
respiring (red)
non-respiring petite (white)
WT
sch9as
C
3% glycerol + 2% galactose
+ 1-NM-PP1
no addition
WT
vector
pGAL1-HAP4
sch9as
vector
pGAL1-HAP4

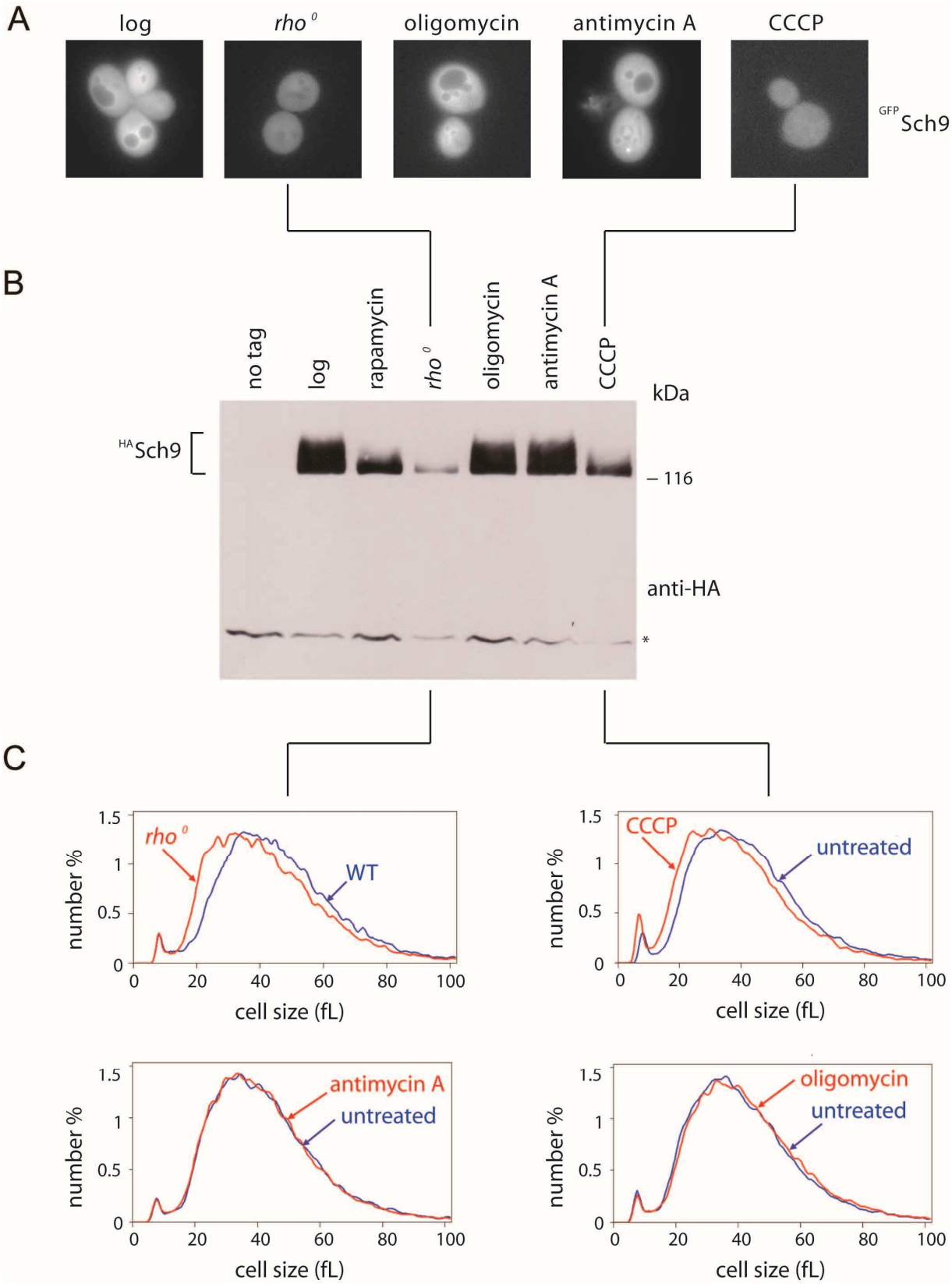

D
mixture of WT and sch9as cells
WT marked with GFP
sch9as marked with GFP
GFP
MitoTracker Red CMXRos
DIC
WT
sch9 as
WT
sch9 as
WT
sch9 as
DIC
MitoTracker Red CMXRos
increased contrast


E
WT
sch9as
dihydrorhodamine 123
DIC

3.2.2 Sch9 senses mitochondrial function

In a parallel series of experiments, I found that Sch9 also appeared to be a downstream sensor of mitochondrial function. Loss of mtDNA caused loss of ^{GFP}Sch9 vacuolar membrane enrichment (Figure 3-2A), loss of ^{HA}Sch9 phospho-isoforms (Figure 3-2B), and small cell size (Figure 3-2C). The yeast mitochondrial genome encodes 4 components of the electron transport chain (*COB*, *COX1*, *COX2*, and *COX3*), 3 components of the F_0 portion of the ATP synthase (*ATP6*, *ATP8*, and *ATP9*), 1 mitochondrial ribosomal protein (*VAR1*), mitochondrial rRNAs, mitochondrial tRNAs, and intron-encoded maturases involved in mitochondrial splicing reactions (Pon and Schatz, 1991). To probe what particular function encoded by mtDNA might be impacting Sch9, chemical inhibitors were used to disrupt specific mitochondrial processes: antimycin A, which inhibits the electron transport chain; oligomycin, which inhibits the ATP synthase; and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which allows protons to flow across the mitochondrial inner membrane and thus dissipates the proton gradient (Epstein, et al., 2001). The electron transport chain inhibitor antimycin A and the ATP synthase inhibitor oligomycin had no effect on ^{GFP}Sch9 vacuolar membrane enrichment (Figure 3-2A), no effect on ^{HA}Sch9 phospho-isoforms (Figure 3-2B), and no effect on cell size (Figure 3-2C). In contrast, the protonophore CCCP caused loss of ^{GFP}Sch9 vacuolar membrane enrichment (Figure 3-2A), collapse of ^{HA}Sch9 phospho-isoforms (Figure 3-2B), and small cell size (Figure 3-2C).

Figure 3-2. Sch9 senses mitochondrial function. (A) Sch9 vacuolar membrane enrichment is disrupted by loss of mtDNA and by the protonophore CCCP, but not by the electron transport chain inhibitor antimycin A or the ATP synthase inhibitor oligomycin. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C in the presence of either 1 μg/mL antimycin A, 3 μg/mL oligomycin, or 3 μg/mL CCCP. A *rho*^{0} strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT4242) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C. **(B)** Sch9 phospho-isoforms are collapsed by loss of mtDNA and by the protonophore CCCP, but not by the electron transport chain inhibitor antimycin A or the ATP synthase inhibitor oligomycin. A strain carrying ^{HA}Sch9 expressed from its endogenous promoter (yMT3989) was grown to log phase in SC-ura +2% glucose and treated with either 1 μg/mL antimycin A, 3 μg/mL oligomycin, or 3 μg/mL CCCP for 60 min. Treatment with 200 ng/mL rapamycin for 60 min was used as a positive control for Sch9 dephosphorylation. A *rho*^{0} strain carrying ^{HA}Sch9 expressed from its endogenous promoter (yMT4243) was grown to log phase in SC-ura +2% glucose. Strains were subject to TCA lysis and immunoblotting with anti-HA antibody. A strain carrying untagged Sch9 (yMT2558) was used a negative control. Asterisk (*) indicates a cross-reactive background band used as a loading control. **(C)** Small cell size is caused by loss of mtDNA and by treatment with the protonophore CCCP, but not by treatment with the electron transport chain inhibitor antimycin A or the ATP synthase inhibitor oligomycin. A WT strain (BY4741) was grown to early log phase in SC +2% glucose in the presence of either 1 μg/mL antimycin A, 3 μg/mL oligomycin, or 3 μg/mL CCCP, and cell size distributions were measured with a Coulter Channelizer. A *rho*^{0} strain (yMT4244) was grown to early log phase in SC-ura +2% glucose and cell size distributions were measured with a Coulter Channelizer.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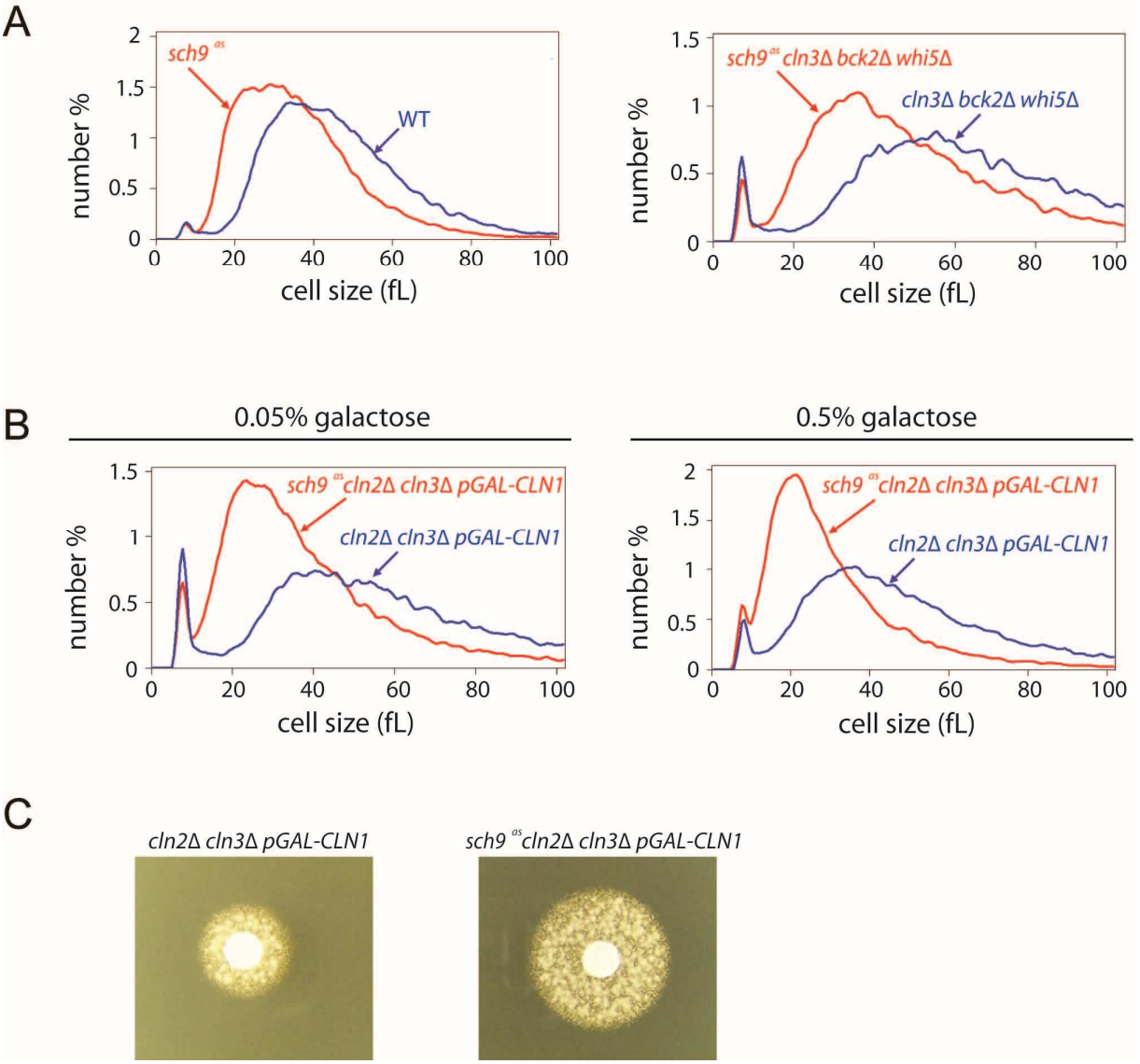
log
rho 0
oligomycin
antimycin A
CCCP
GFP Sch9
B
no tag
log
rapamycin
rho 0
oligomycin
antimycin A
CCCP
kDa
HA Sch9
116
anti-HA
*
C
rho 0
WT
number %
cell size (fL)
CCCP
untreated
antimycin A
untreated
oligomycin
untreated

3.2.3 Connections between Sch9, mitochondrial function, and cell size

Our laboratory has shown that loss of Sch9 kinase activity causes cells to commit to cell division at a smaller cell size, as measured by the onset of G1/S transcription (Jorgensen, et al., 2004). To further investigate the mechanism by which Sch9 links cell size to initiation of the G1/S transition, its relationship with other known G1/S regulators was examined. The small cell size conferred by loss of Sch9 was independent of the known upstream regulators of the G1/S transcriptional machinery – *CLN3*, *BCK2*, and *WHI5* (Figure 3-3A). The effects of loss of Sch9 were also examined in a strain in which the expression of G1 cyclin is under the control of the *GAL1* promoter and can be modulated by the concentration of galactose in the media (Schneider, et al., 2004). Loss of Sch9 activity caused small cell size at any given level of G1 cyclin (Figure 3-3B) and appeared to decrease the amount of G1 cyclin required for viability (Figure 3-3C).

Given that loss of mtDNA caused loss of Sch9 vacuolar membrane enrichment and phospho-isoforms (Figure 3-2A and Figure 3-2B), decreased Sch9 activity may account for the small size of cells lacking mtDNA. Like the small cell size conferred by loss of Sch9, the small cell size conferred by loss of mtDNA was independent of the known upstream regulators of the G1/S transcriptional machinery (Figure 3-3D). Importantly, loss of mtDNA conferred no further cell size decrease in cells lacking Sch9 activity (Figure 3-3E). The same was not true for cells lacking *WHI5*, which are almost as small as cells lacking Sch9 activity (Figure 3-3E). Still, to rule out the possibility that cells lacking Sch9 are at a minimum cell size (median size 27 fL) that cannot be further decreased, the $sch9^{as}$ allele was combined with other mutations known to confer small cell size. The resulting strain – which simultaneously carries the hypomorphic $sch9^{as}$ allele, the hypomorphic $sfp1^{hER}$ allele, deletion of *WHI5*, and overexpresses the hypermorphic *CLN3-1* allele – had a median cell size of 16 fL (Figure 3-3F), suggesting that cells lacking Sch9 are not at a minimum size barrier.

Figure 3-3. Connections between Sch9, mitochondrial function, and cell size. (A) The small cell size conferred by loss of Sch9 is independent of all known upstream regulators of the G1/S transcriptional machinery. Congenic *cln3Δ bck2Δ whi5Δ* (yMT2643) and *sch9^{as} cln3Δ bck2Δ whi5Δ* (yMT4245) strains were grown to early log phase in XY +2% glucose and cell size distributions were measured with a Coulter Channelizer. **(B)** Loss of Sch9 causes small cell size when the level of G1 cyclin is fixed by heterologous transcriptional control. Congenic *cln2Δ cln3Δ pGAL-CLN1* (BS111) and *sch9^{as} cln2Δ cln3Δ pGAL-CLN1* (yMT4247) strains were grown to early log phase in XY +2% raffinose in the presence of 0.05% or 0.5% galactose and cell size distributions were measured with a Coulter Channelizer. **(C)** Loss of Sch9 activity appears to decrease the amount of G1 cyclin required for viability. Congenic *cln2Δ cln3Δ pGAL-CLN1* (BS111) and *sch9^{as} cln2Δ cln3Δ pGAL-CLN1* (yMT4247) strains were diluted into molten top agar and poured onto XY +2% raffinose plates. Filter discs containing 5 µL of 20% galactose solution were placed on solidified medium and incubated for 3 days at 30°C. **(D)** The small cell size conferred by loss of mtDNA is independent of all known upstream regulators of the G1/S transcriptional machinery. Congenic *cln3Δ bck2Δ whi5Δ* (yMT2643) and *rho^{0} cln3Δ bck2Δ whi5Δ* (yMT4248) strains were grown to early log phase in XY +2% glucose and cell size distributions were measured with a Coulter Channelizer. **(E)** The decrease in cell size conferred by loss of mtDNA is Sch9-dependent. Congenic *sch9^{as}* (yMT4237) and *rho^{0} sch9^{as}* (yMT4249) strains were grown to early log phase in XY +2% glucose +200 nM 1-NM-PP1 and cell size distributions were measured with a Coulter Channelizer. Congenic *whi5Δ* (yMT2641) and *rho^{0} whi5Δ* (yMT4250) strains were grown to early log phase in XY +2% glucose and cell size distributions were measured with a Coulter Channelizer. **(F)** Combining multiple mutations known to confer small cell size results in an extremely small cell. Congenic WT (BY4741) and *sch9^{as} sfp1^{hER} whi5Δ pGAL1-CLN3-1* (yMT4252) strains were grown to early log phase in XY +2% galactose and cell size distributions were measured with a Coulter Channelizer.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sch9 as cln3Δ bck2Δ whi5Δ
cln3Δ bck2Δ whi5Δ
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0.5% galactose
sch9 as cln2Δ cln3Δ pGAL-CLN1
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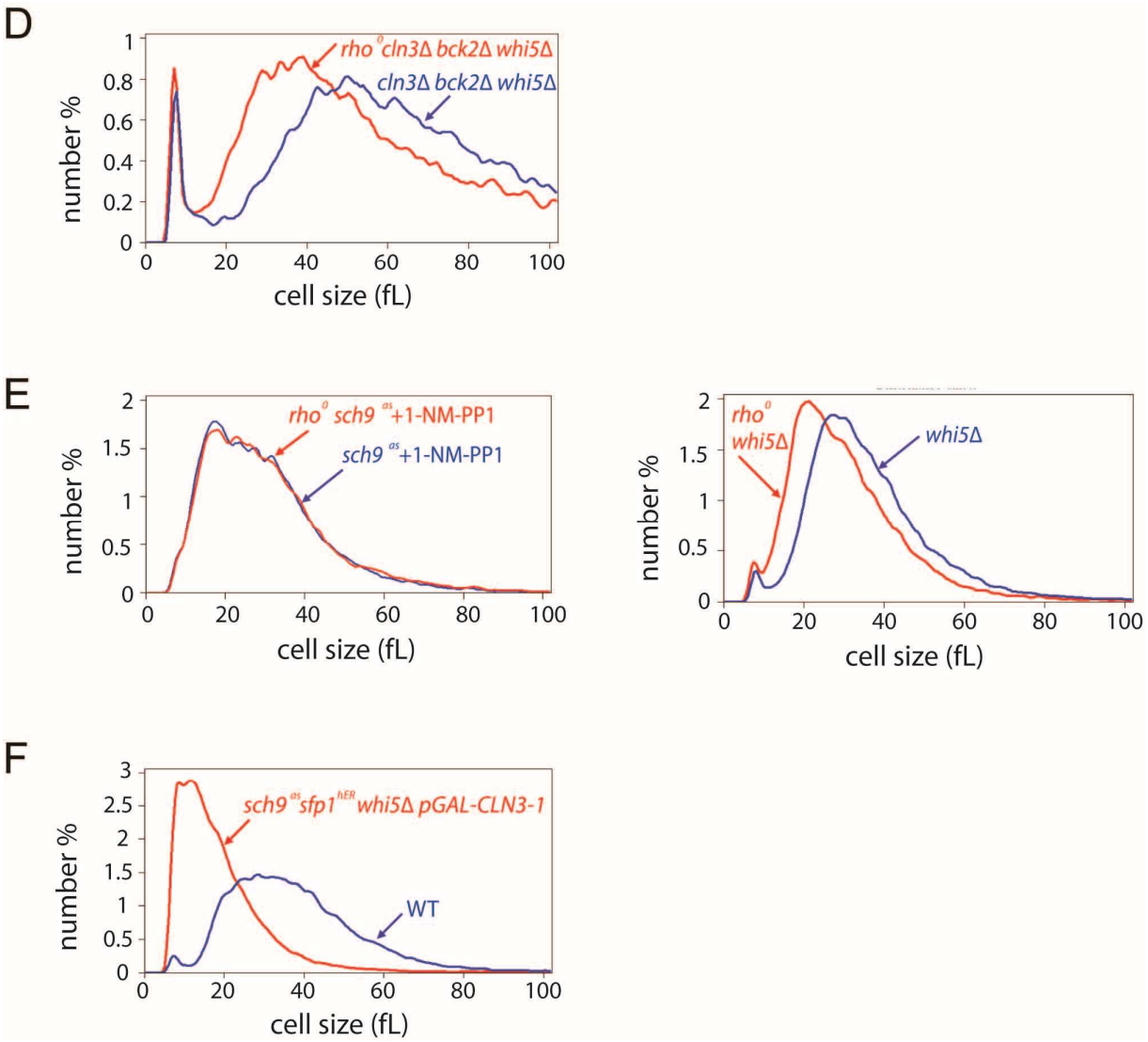
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number %
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sch9as+1-NM-PP1
rho0 whi5Δ
whi5Δ
F
number %
cell size (fL)
sch9as sfp1hER whi5Δ pGAL-CLN3-1
WT

3.2.4 Sch9 exhibits dynamic localization

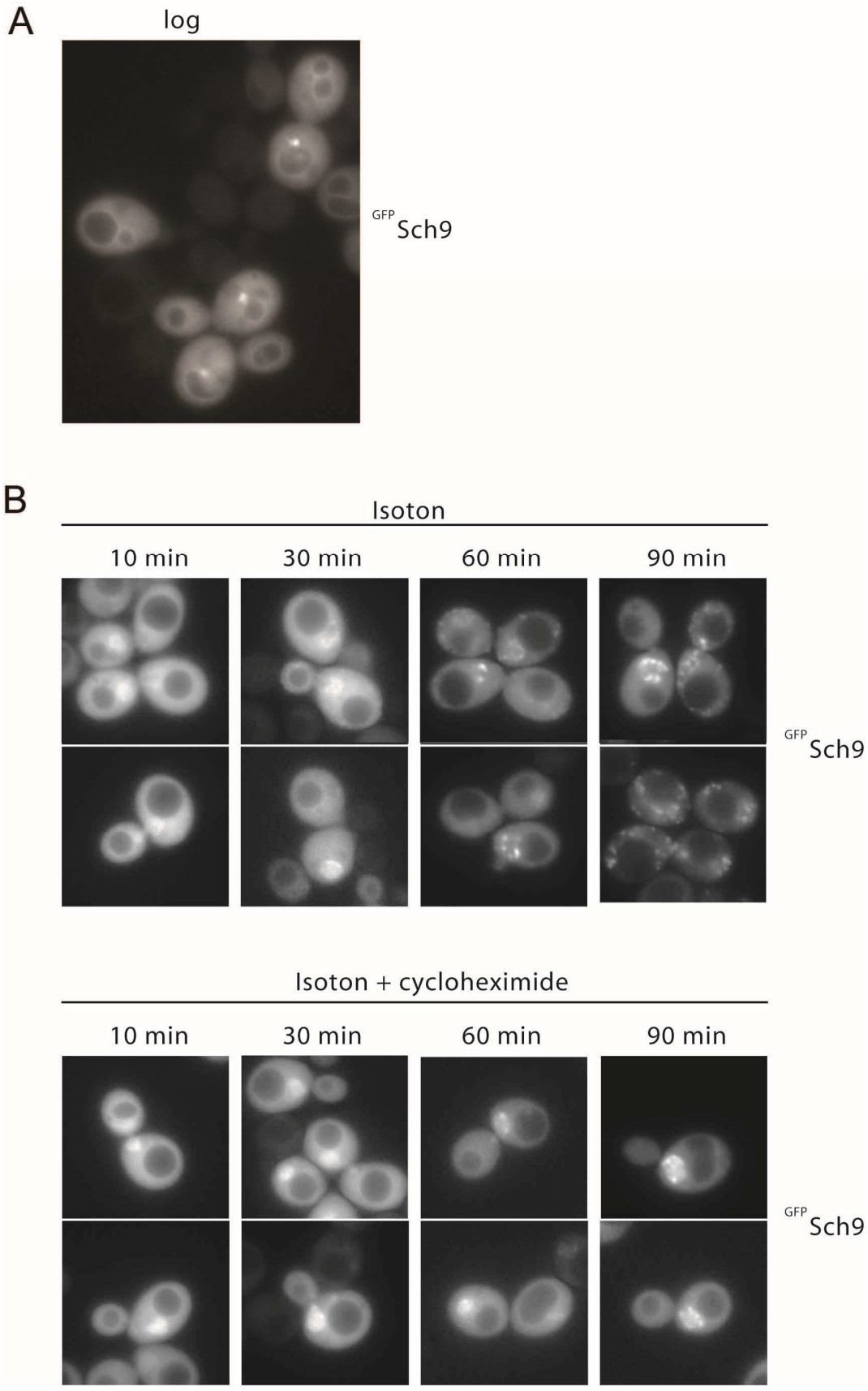
In previous work, our laboratory showed that Sch9 was enriched at the vacuolar membrane in log-phase cells and that this enrichment was lost upon carbon starvation (Jorgensen, et al., 2004). To further explore what signals regulate Sch9, I monitored ^{GFP}Sch9 localization in response to a variety of genetic and environmental perturbations.

During log-phase growth, Sch9 was enriched at the vacuolar membrane as previously reported (Jorgensen, et al., 2004). Sch9 also appeared to be consistently enriched in a focus adjacent to the vacuolar membrane that resembles the pre-autophagosomal structure (PAS) (Kawamata, et al., 2008) involved in autophagic vesicle nucleation (Huang and Klionsky, 2002) (Figure 3-4A).

In the course of assessing various environmental stresses for their effect on Sch9 localization, I unexpectedly found that transfer of cells to Isoton II diluent (typically used to dilute cells for analysis by Coulter Channelizer) caused sequential enrichment of ^{GFP}Sch9 in the nucleus, followed by the nuclear envelope, and eventually in punctate cytoplasmic foci (Figure 3-4B). Treatment with 7% ethanol similarly caused ^{GFP}Sch9 enrichment at the nuclear envelope and cytoplasmic foci (Figure 3-4C). Sch9 enrichment at cytoplasmic foci – but not nuclear enrichment – was abolished in the presence of the translation inhibitor cycloheximide (Figure 3-4B and Figure 3-4C).

Treatment of cells with acetic acid caused Sch9 to become enriched in foci that appeared qualitatively different from those induced by ethanol stress (Figure 3-4D). Co-staining with the mitochondrial dye MitoTracker Red showed that acetic acid-induced ^{GFP}Sch9 foci corresponded to mitochondria (Figure 3-4D). Unlike Sch9 foci caused by ethanol stress, acetic acid-induced enrichment of ^{GFP}Sch9 at mitochondria was unaffected by the presence of cycloheximide (Figure 3-4E). Acetic acid has been shown to trigger apoptosis in yeast (Ludovico, et al., 2001), and other stresses known to cause apoptosis – including H_20_2 treatment (Madeo, et al., 1999) and hyper-activation of the PKA pathway in poor nutrients (Gourlay and Ayscough, 2006) – also caused Sch9 enrichment at mitochondria (Figure 3-4F).

Figure 3-4. Sch9 exhibits dynamic localization. (A) Sch9 is consistently enriched in a focus adjacent to the vacuolar membrane. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C. **(B)** Transfer of cells to Isoton II diluent causes sequential enrichment of ^{GFP}Sch9 in the nucleus, at the nuclear envelope, and in punctate cytoplasmic foci. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C and treated (or not) with 10 μM cycloheximide for 15 min. Cells were pelleted, resuspended in Isoton II diluent (with or without cycloheximide), and visualized after 10, 30, 60, and 90 min. **(C)** Ethanol stress causes sequential enrichment of ^{GFP}Sch9 at the nuclear envelope and in cytoplasmic foci. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C and treated (or not) with 10 μM cycloheximide for 15 min. Ethanol was added to 7% final concentration and cells visualized after 10, 30, 60, and 90 min. **(D)** Treatment with acetic acid causes Sch9 to become enriched at mitochondria. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C, treated 50 mM acetic acid for 60 min, and mitochondria visualized with the dye MitoTracker Red CMXRos. **(E)** Acetic acid-induced enrichment of Sch9 at mitochondria is reversible and independent of new protein synthesis. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was pretreated with 10 μM cycloheximide for 10 min, treated with 50 mM acetic acid for 60 min, and then washed and resuspended in fresh medium without acetic acid (but still containing 10 μM cycloheximide) for 30 min. **(F)** Sch9 enrichment at mitochondria is also induced H_20_2 treatment and by hyper-activation of the PKA pathway under poor nutrient conditions. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter (yMT3106) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C, and treated with 30 mM H_2O_2 for 60 min. A strain carrying ^{GFP}Sch9 expressed from its endogenous promoter and bearing a *pde2Δ* deletion (yMT4253) was grown to early log phase in SC-ura +2% glucose for >16 h at 30°C, and resuspended in medium containing 5 mM cAMP and no glucose for 60 min. Deletion of *PDE2*, which eliminates the high-affinity cAMP phosphodiesterase, renders the cell responsive to exogenous cAMP.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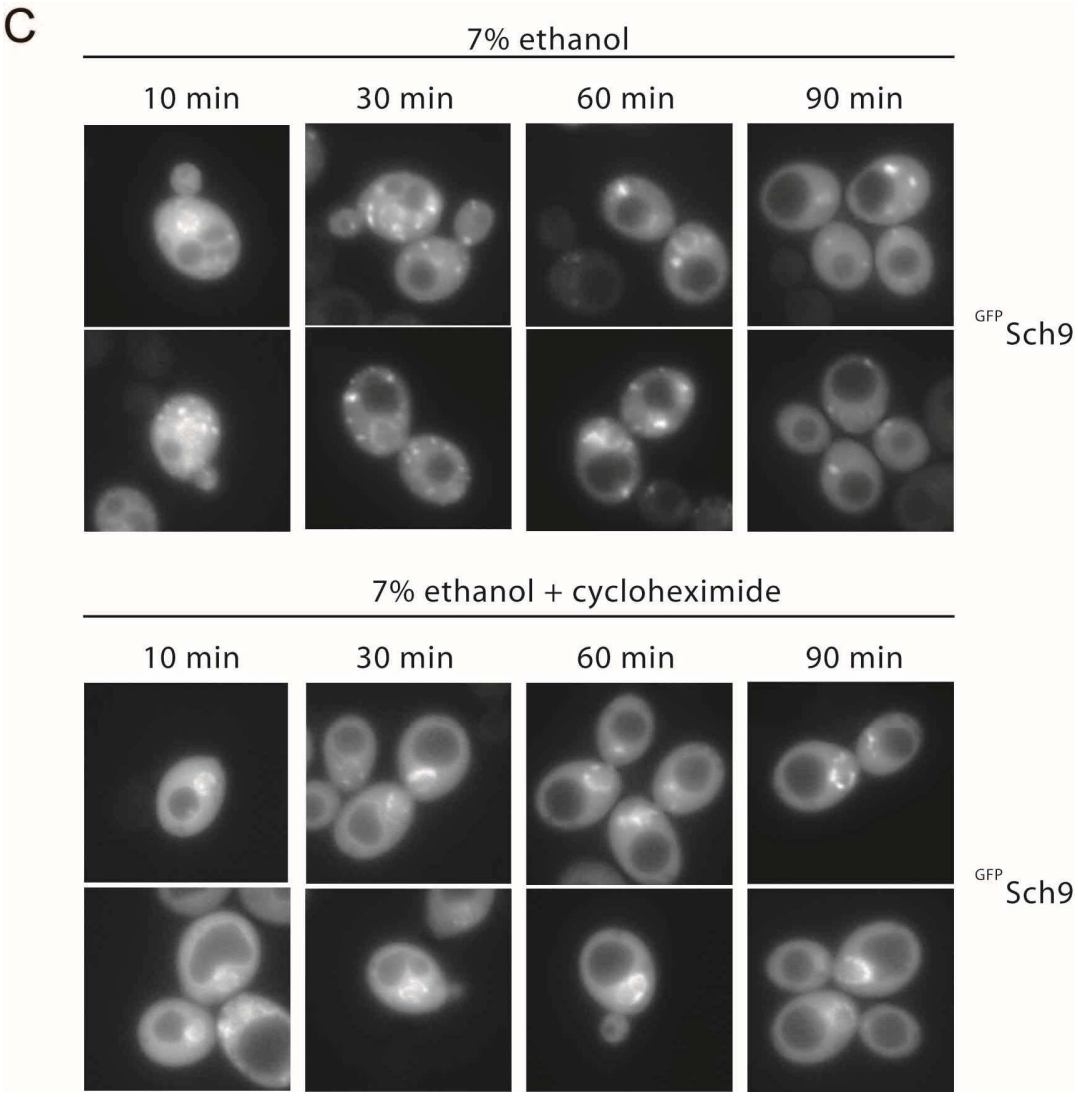
10 min
30 min
60 min
90 min
GFP Sch9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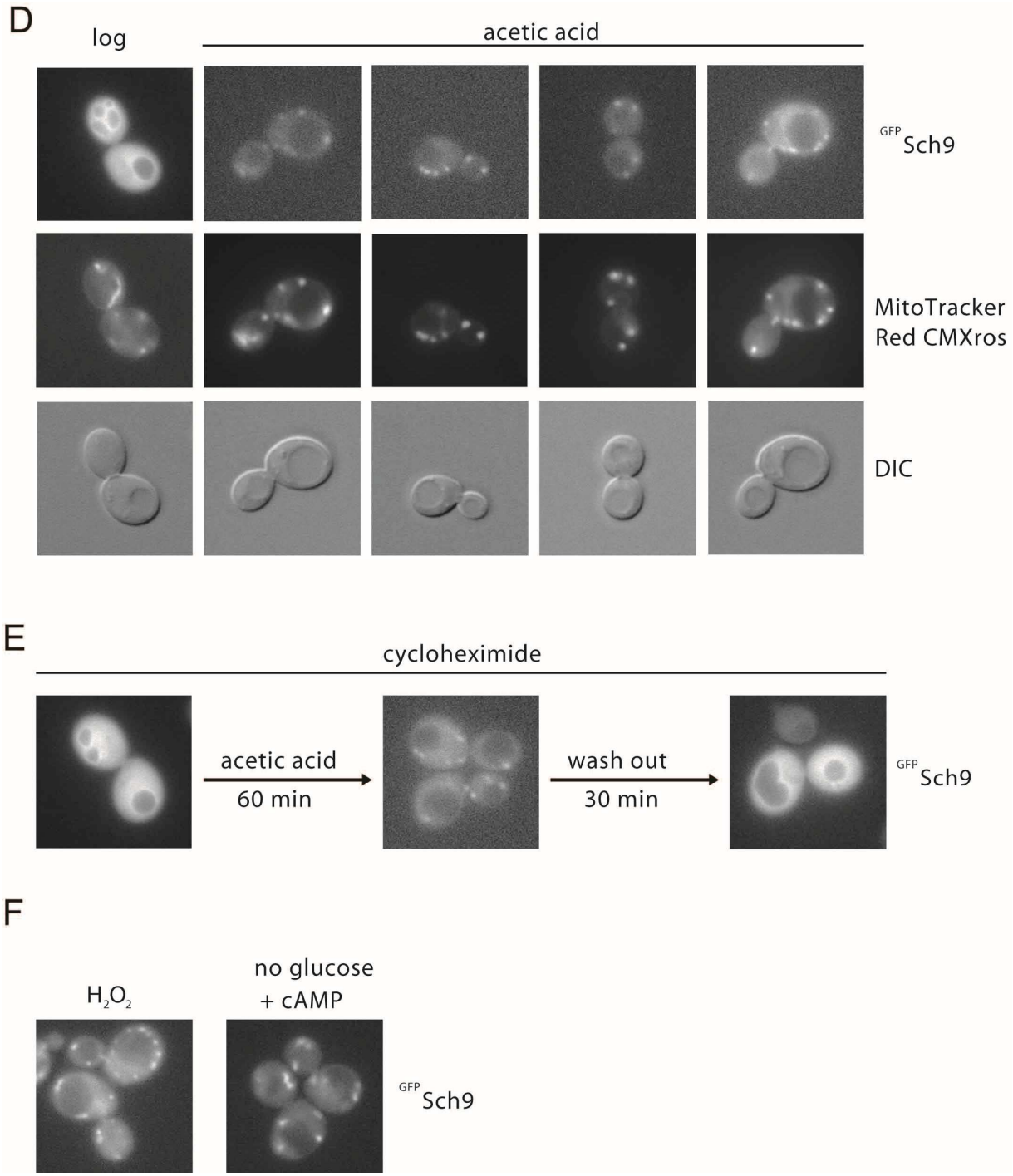
30 min
60 min
90 min
GFP Sch9
7% ethanol + cycloheximide
10 min
30 min
60 min
90 min
GFP Sch9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acetic acid
GFP Sch9
MitoTracker
Red CMXros
DIC
E
cycloheximide
acetic acid
60 min
wash out
30 min
GFP Sch9
F
H2O2
no glucose
+ cAMP
GFP Sch9

3.3 Discussion

3.3.1 TOR/Sch9 is a regulator of mitochondrial function

While this work was in progress, another study identified Sch9 as a major TORC1 substrate and effector, placing it in a position analogous to S6K in higher eukaryotes (Powers, 2007; Urban, et al., 2007) [although it should be noted that Sch9 still shares many similarities with PKB/Akt (Sobko, 2006)]. This work presents evidence that Sch9 – and presumably by extension, TORC1 – is both an upstream regulator and downstream sensor of mitochondrial function.

Loss of Sch9 conferred a severe growth defect on a non-fermentable carbon source and an increase in petite frequency that were only apparent at 37°C. These defects were likely uncovered by the increased strain that high temperature places on mitochondrial function (Sakaki, et al., 2003). The growth defect on a non-fermentable carbon source could be partially suppressed by overexpression of *HAP4*, a transcriptional activator of respiratory genes (Buschlen, et al., 2003; Lascaris, et al., 2003). Loss of Sch9 kinase activity also resulted in lower mitochondrial membrane potential and lower ROS levels. The mechanism by which Sch9 impacts mitochondria remains an open question.

The relationship between TOR signaling and mitochondrial function in yeast is complex. TORC1 and Sch9 have previously been reported to be negative regulators of respiratory function, in that loss of Tor1 or Sch9 increased oxygen consumption, mitochondrial translation rates, and steady-state levels of electron transport chain proteins (Bonawitz, et al., 2007; Lavoie and Whiteway, 2008; Pan and Shadel, 2009). In addition, levels of nuclear-encoded mitochondrial transcripts have been found to increase in response to rapamycin treatment (Cardenas, et al., 1999; Hardwick, et al., 1999; Shamji, et al., 2000) and *SCH9* deletion (Lavoie and Whiteway, 2008). However, interpretation of the latter observation is complicated by the fact that mitochondrial transcripts are apparently also upregulated in a compensatory response to mitochondrial dysfunction (Bourges, et al., 2005; Hughes, et al., 2000; Laun, et al., 2005; Traven, et al., 2001).

Paradoxically, other studies have found a cryptic positive role for TOR and Sch9 in mitochondrial function. Transcriptional profiling of aging cells lacking Tor1 or Sch9 has

consistently found decreased levels of nuclear-encoded mitochondrial transcripts (Cheng, et al., 2007a; Cheng, et al., 2007b; Ge, et al., 2010; Smets, et al., 2008; Wei, et al., 2009a). Similarly, another study found that rapamycin treatment repressed nuclear-encoded mitochondrial transcripts, an effect that was only apparent in a strain deleted for all 3 catalytic subunits of PKA (Chen and Powers, 2006). Also, in contrast to the increased lifespan conferred by loss of Sch9 under standard assay conditions in glucose media, the absence of Sch9 actually shortens lifespan when cells are grown in a non-fermentable carbon source (Piper, et al., 2006). In the time since the work in this chapter was completed (2005-2007), others have confirmed that loss of Sch9 confers a growth defect on a non-fermentable carbon source (Smets, et al., 2008) and reduces mitochondrial membrane potential (Pan and Shadel, 2009).

The findings presented here are intriguing when viewed in the larger context of the evolutionarily conserved role of TOR signaling in regulating lifespan (Kapahi, et al., 2010). Decreased signaling through TOR and Sch9 has been shown to increase replicative (Kaeberlein, et al., 2005b) and chronological (Fabrizio, et al., 2001; Powers, et al., 2006) lifespan in yeast, and the analogous pathways have been shown to regulate lifespan in worms (Hansen, et al., 2007; Jia, et al., 2004; Pan, et al., 2007; Vellai, et al., 2003), flies (Bjedov, et al., 2010; Kapahi, et al., 2004; Luong, et al., 2006), and mice (Harrison, et al., 2009; Selman, et al., 2009). Most of the increased chronological lifespan conferred by loss of Sch9 has been attributed to increased stress resistance resulting from downstream activation of the kinase Rim15, transcription factor Gis1, and mitochondrial superoxide dismutase Sod2 (Fabrizio, et al., 2003; Fabrizio, et al., 2001; Wei, et al., 2008).

In light of the findings presented in this chapter, I speculate that control of mitochondrial function may be an additional mechanism by which loss of Sch9 extends chronological lifespan in yeast (Figure 3-5). Indeed, increased respiration has recently been proposed to underlie the ability of caloric restriction to confer increased chronological (Oliveira, et al., 2008; Smith, et al., 2007) and replicative (Lin, et al., 2002) lifespan in yeast [although the claim with respect to replicative lifespan has been disputed (Kaeberlein, et al., 2005a; Lavoie and Whiteway, 2008)]. Increased respiration has also been linked to extended longevity in worms (Bishop and Guarente, 2007; Schulz, et al., 2007) and flies (Zid, et al., 2009). I found that loss of Sch9 decreased mitochondrial membrane potential and ROS levels, and other recent studies have reported that loss of Sch9 leads to increased oxygen consumption (Lavoie and Whiteway, 2008; Pan and

Shadel, 2009). These observations are consistent with the so-called "uncoupling to survive" hypothesis, which predicts that uncoupled respiration – in which proton leakage through the inner mitochondrial membrane dissipates the mitochondrial membrane potential in the absence of ATP synthesis – will result in lower ROS generation (Korshunov, et al., 1997; Skulachev, 1996) and increased lifespan (Brand, 2000).

First proposed over a decade ago, the "uncoupling to survive" hypothesis has accumulated some experimental support in recent years (Dietrich and Horvath, 2010; Mookerjee, et al., 2010; Wolkow and Iser, 2006). For instance, mild uncoupling of respiration with protonophores like CCCP and 2,4-dinitrophenol has been shown to increase lifespan in yeast (Barros, et al., 2004), worms (Lemire, et al., 2009), flies (Padalko, 2005), and mice (Caldeira da Silva, et al., 2008). Uncoupled respiration has been correlated with increased lifespan in individual mice (Speakman, et al., 2004), and reduced age-associated mitochondrial dysfunction of human muscle cells (Amara, et al., 2007). Endogenous uncoupling proteins (UCPs) play an important role in mediating uncoupled respiration *in vivo* (Brand and Esteves, 2005; Echtay, 2007; Krauss, et al., 2005), and their genetic manipulation has been shown to affect ROS production (Brand, et al., 2002; Vidal-Puig, et al., 2000), lifespan (Andrews and Horvath, 2009; Fridell, et al., 2005), and age-related disease (Gates, et al., 2007). However, other studies of uncoupled respiration have produced conflicting results (Humphrey, et al., 2009; Kontani, et al., 2005; McDonald, et al., 2008; Stockl, et al., 2007), and whether uncoupled respiration is relevant to the increased lifespan conferred by caloric restriction remains an open question (Mookerjee, et al., 2010).

Indeed, on a larger level, studies examining the effect of mitochondrial function and ROS on the lifespan of model organisms have produced complex and seemingly contradictory findings. Consistent with the free radical theory of aging (Harman, 1956), respiration has been found to be anti-correlated with lifespan in worms (Chen, et al., 2007; Cristina, et al., 2009; Curran and Ruvkun, 2007; Dillin, et al., 2002; Kayser, et al., 2004; Lee, et al., 2003; Rea, et al., 2007; Van Raamsdonk, et al., 2010; Yang and Hekimi, 2010), flies (Copeland, et al., 2009), and rodents (Lapointe and Hekimi, 2008). But respiration has also been found to be *correlated* with lifespan in yeast (Lin, et al., 2002; Oliveira, et al., 2008; Smith, et al., 2007), worms (Bishop and Guarente, 2007; Rea, et al., 2007; Schulz, et al., 2007), and flies (Rera, et al., 2010; Tong, et al., 2007; Walker, et al., 2006; Zid, et al., 2009). Consistent with the free radical theory of aging,

ROS have been found to be anti-correlated with lifespan in worms (Feng, et al., 2001; Ishii, et al., 1998), flies (Tong, et al., 2007; Walker, et al., 2006), and rodents (Pinton, et al., 2007). But ROS have also been found to be *correlated* with lifespan in worms (Heidler, et al., 2010; Schulz, et al., 2007), and rodents (Andziak, et al., 2006; Lapointe and Hekimi, 2008), and other studies have found no correlation in flies (Miwa, et al., 2004; Sanz, et al., 2010) and rodents (Jang, et al., 2009). Consistent with the free radical theory of aging, high levels of antioxidants/antioxidant enzymes have been found to be correlated with lifespan in worms (Melov, et al., 2000), flies (Orr and Sohal, 1994; Tong, et al., 2007), and rodents (Schriner, et al., 2005). But high levels of antioxidants/antioxidant enzymes have also been found to be *anti-correlated* with lifespan in worms (Schulz, et al., 2007; Van Raamsdonk and Hekimi, 2009; Yang, et al., 2007), and other studies have found no correlation in worms (Doonan, et al., 2008; Keaney, et al., 2004; Yang, et al., 2007), flies (Sanz, et al., 2010; Seto, et al., 1990), and rodents (Jang, et al., 2009; Perez, et al., 2009b). Interestingly, transgenic mice carrying a proofreading-deficient mtDNA polymerase have been shown to accumulate point mutations in mtDNA and exhibit decreased lifespan (Trifunovic, et al., 2004) – however, subsequent work has shown that this accumulation of mtDNA mutations has no effect on ROS (Kujoth, et al., 2005) and in any event is unlikely to be relevant to the natural lifespan of mice (Khrapko, et al., 2006; Vermulst, et al., 2007).

To account for such paradoxical results, some have invoked the concept of mitochondrial hormesis (or "mitohormesis"), which holds that increased ROS from mitochondria lead to a compensatory upregulation of stress resistance mechanisms that ultimately increase lifespan (Gems and Partridge, 2008; Le Bourg, 2009; Ristow and Zarse, 2010). Others have suggested that the free radical theory of aging remains unproven or has been falsified by a critical mass of evidence (Gems and Doonan, 2009; Howes, 2006; Lapointe and Hekimi, 2010; Muller, et al., 2007; Perez, et al., 2009a). It has also been proposed that TOR signaling – not ROS-mediated damage – is actually the ultimate cause of aging (Blagosklonny, 2006; Blagosklonny, 2008; Blagosklonny and Hall, 2009). Clearly, further studies will be needed to clarify the link between TOR, mitochondrial function, ROS, and lifespan.

3.3.2 TOR/Sch9 is a sensor of mitochondrial function

This work also presents evidence that TOR/Sch9 is a downstream sensor of mitochondrial function. Loss of mtDNA caused loss of Sch9 vacuolar membrane enrichment,

loss of Sch9 phospho-isoforms, and a small cell size phenotype consistent with reduced Sch9 activity. These effects could be recapitulated by treating cells with the protonophore CCCP, but not with the electron transport chain inhibitor antimycin A or the ATP synthase inhibitor oligomycin. Although Sch9 is phosphorylated at one site in its activation loop by the PDK1-like kinases Pkh1 and Pkh2 in addition to being phosphorylated at six C-terminal sites by TORC1, essentially all of the phospho-isoforms visible by SDS-PAGE are attributable to TORC1 rather than Pkh1/2 (Urban, et al., 2007). Thus, the decreased Sch9 phospho-isoforms observed in this work are most likely a reflection of decreased TORC1 activity towards Sch9.

Taken together, these findings suggest a paradoxical model in which TOR/Sch9 signaling is both an upstream regulator and downstream sensor of mitochondrial function (Figure 3-5). Interestingly, an analogous model has previously been proposed for mammalian TOR (Schieke and Finkel, 2006). In mammalian cells, mTOR has been shown to regulate mitochondrial function (Bentzinger, et al., 2008; Chen, et al., 2008; Cunningham, et al., 2007; Polak, et al., 2008; Ramanathan and Schreiber, 2009; Schieke, et al., 2006; Um, et al., 2004) and to respond to disruption of mitochondrial function (Desai, et al., 2002; Kim, et al., 2002; Sarbassov and Sabatini, 2005; Vander Haar, et al., 2007; Xu, et al., 2001). Thus, TOR signaling in both yeast and higher eukaryotes appears to be intimately linked with mitochondria, underscoring the central role of TOR in cell growth signaling.

The finding that loss of mtDNA is sufficient to cause loss of Sch9 phosphorylation, loss of Sch9 vacuolar membrane enrichment, and small cell size, suggests that Sch9 is affected by one or more gene products encoded by the mitochondrial genome. However, this is counter-intuitive, because all known proteins and RNAs encoded by mtDNA are thought to function in respiration, and inhibition of respiration does not recapitulate the above effects on Sch9 phosphorylation, Sch9 localization, or cell size. The mitochondrial genome encodes few proteins: only 4 components of the electron transport chain (*COB*, *COX1*, *COX2*, and *COX3*), 3 components of the F_0 portion of the ATP synthase (*ATP6*, *ATP8*, and *ATP9*), and 1 mitochondrial ribosomal protein (*VAR1*). All other mitochondrial gene products form part of the mitochondrial transcription and translation apparatus required for synthesis of the above proteins – this apparatus includes mitochondrial rRNAs, mitochondrial tRNAs, and intron-encoded maturases involved in mitochondrial splicing reactions (Pon and Schatz, 1991). It is possible that TOR/Sch9 may be responding to the physical presence of mtDNA or associated factors.

Alternatively, the relevant signal may be a function, distinct from respiration, enabled by a gene product encoded by the mitochondrial genome. Indeed, previous studies have noted mitochondrial proteins that appear to be multi-functional. The mtDNA-encoded protein Atp6 is a subunit of the F_0 portion of the ATP synthase, and has been suggested to have an additional role in maintenance of the mitochondrial genome (Contamine and Picard, 2000). A point mutation in the nuclear-encoded *ATP2* gene, which encodes a subunit of the F_1 portion of the ATP synthase, has been reported to result in defective mitochondrial segregation at division and reduced replicative lifespan due to daughter cells being born "old" (Lai, et al., 2002). Nuclear-encoded mitochondrial ribosomal proteins Mrps17 and Mrpl37 have been suggested to have a specific role in meiosis (Hanlon, et al., 2004), while deletion of the mitochondrial ribosomal protein Mrpl25 has been reported to confer increased lifespan (which, interestingly, appears to involve a novel form of mitochondrial "back-signaling" to TOR) (Heeren, et al., 2009).

Loss of mtDNA appears to cause many important changes in cellular physiology in addition to loss of respiration, but most remain poorly characterized. In addition to triggering the classic retrograde response involving the RTG genes (Liao, et al., 1991; Liu and Butow, 2006), loss of mtDNA has been reported to affect a host of other processes, including: *PDR3*-dependent transcription and pleiotropic drug resistance (Hallstrom and Moye-Rowley, 2000; Panwar and Moye-Rowley, 2006), rDNA transcription and formation of extrachromosomal ribosomal DNA circles (ERCs) (Conrad-Webb and Butow, 1995), glycogen accumulation (Enjalbert, et al., 2000), aerobic *COX* gene transcription (Dagsgaard, et al., 2001), mitochondrial morphology (Xu, et al., 2005), *GAL4*-dependent transcription (Jelicic, et al., 2005), Ty1 transposition (Stoycheva, et al., 2007), transcriptional repression by the Ssn6-Tup1 complex (Conlan, et al., 1999), and replicative lifespan in some strain backgrounds (Kaeberlein, et al., 2005a; Kirchman, et al., 1999). Interestingly, at least some of the above physiological changes cannot be recapitulated by inhibition of respiration (Dagsgaard, et al., 2001; Kaeberlein, et al., 2005a; Kirchman, et al., 1999; Xu, et al., 2005; Zhang and Moye-Rowley, 2001). Some studies have employed mutations and chemical inhibitors to test the requirement for specific mitochondrial functions. For instance, by employing a panel of mutants with specific defects in nuclear-encoded mitochondrial components to characterize the molecular signal that induces *PDR5* transcription in *rho*^{0} cells, it was found that only mutations that affect the F_0 subunit of the ATP synthase induced *PDR5* expression (Zhang and Moye-Rowley, 2001). Similarly, by using chemical inhibitors of different

mitochondrial processes to investigate the connection between respiration and cardiolipin biosynthesis, it was found that the proton gradient across the inner mitochondrial membrane was required, but not the trans-membrane electrical gradient or ATP synthesis (Gohil, et al., 2004).

In this work, I found that the protonophore CCCP, like loss of mtDNA, caused loss of Sch9 phosphorylation, loss of Sch9 vacuolar membrane enrichment, and small cell size. This finding suggests that the proton gradient across the inner mitochondrial membrane may be the molecular signal sensed by TOR/Sch9 (Figure 3-5). This hypothesis is consistent with reports that *rho*^{0} cells have reduced mitochondrial membrane potential compared to WT *rho*$^{+}$ cells (Dupont, et al., 1985; Rikhvanov, et al., 2005). Even in the absence of respiration, maintenance of the mitochondrial membrane potential is essential due to its role in mitochondrial protein import, which is in turn required for the biosynthesis of iron-sulfur proteins that are required for cell viability (Kispal, et al., 2005). Because mtDNA encodes 4 components of the electron transport chain and 3 components of the F_0 portion of the ATP synthase, *rho*^{0} cells cannot generate their mitochondrial membrane potential via the usual mechanisms – by coupling electron transport to the pumping of protons into the intermembrane space, or by reversing the activity of the F_1F_0 ATP synthase (coupling ATP hydrolysis to the pumping of protons into the intermembrane space). In fact, *rho*^{0} cells generate their membrane potential via an alternate mechanism in which the mitochondrial adenine nucleotide translocator (ANT) carries out electrogenic exchange of ATP^{4-} generated by glycolysis for ADP^{3-} produced by ATP hydrolysis by the remaining F_1 portion of the ATP synthase (Chen and Clark-Walker, 1999; Dupont, et al., 1985; Giraud and Velours, 1997; Kominsky, et al., 2002; Lefebvre-Legendre, et al., 2003). The lower membrane potential generated by this alternate mechanism in *rho*^{0} cells may be the relevant signal sensed by TOR/Sch9.

However, a potential caveat should be noted – in addition to dissipating the pH gradient across the mitochondrial inner membrane, CCCP has also been shown to affect the pH gradient across the plasma membrane and vacuolar membrane (Beauvoit, et al., 1991). For instance, treatment of cells with CCCP and other protonophores at the low extracellular pH typical of yeast synthetic media has been reported to cause intracellular acidification, leading to activation of the PKA pathway (Colombo, et al., 1998; Noshiro, et al., 1987; Purwin, et al., 1986; Thevelein, 1984; Thevelein and de Winde, 1999) and an increase in intracellular calcium that activates the plasma membrane ATPase (Eilam, et al., 1990; Pereira, et al., 2008b). Curiously, a

more recent study has shown that intracellular alkalinization – rather than acidification – is associated with activation of the PKA pathway, and that such pH changes are relevant to the glucose-sensing mechanism in yeast (Dechant, et al., 2010). Regardless, it is clear that the effects of CCCP should be interpreted with caution given its lack of specificity for the mitochondrial inner membrane. Given that Sch9 is dephosphorylated in response to a wide variety of stresses (Urban, et al., 2007), non-mitochondrial effects of CCCP cannot be ruled out. Further work will be needed to clarify the nature of the mitochondrial signal to which TOR/Sch9 responds.

3.3.3 Reduced Sch9 activity may account for the small cell size phenotype conferred by loss of mtDNA

In a previous genome-wide screen for single-gene deletions that affect cell size, our laboratory found that small cell size was conferred by deletion of many mitochondrial components – indeed, the largest class of small-cell-size mutants was those that conferred a respiratory defect (Jorgensen, et al., 2002). In retrospect, the common feature unifying this class of mutants may be lack of rho^{+} mtDNA – importantly, all known mutations in nuclear or mitochondrial genes that directly or indirectly block mitochondrial translation are known to invariably lead to the production of rho^{-} or rho^{0} cells (Contamine and Picard, 2000; Myers, et al., 1985). It is also worth noting that the heterogeneity of the small-cell-size phenotype observed for non-respiring mutants in the genome-wide size screen may have been due to second-site mutations present in the systematic yeast deletion collection, as loss of mtDNA has been shown to lead to nuclear genome instability (Rasmussen, et al., 2003; Veatch, et al., 2009). Interestingly, recent studies have noted links between mitochondrial function and the G1/S transition in higher eukaryotes, although in these systems respiration appears to be a positive regulator of G1/S. Disruption of the mitochondrial electron transport chain was reported to delay the cell cycle at the G1/S transition in flies (Owusu-Ansah, et al., 2008), and mTOR-regulated mitochondrial function has been implicated in a G1 metabolic checkpoint in mammalian cells (Schieke, et al., 2008). These results are consistent with the growing recognition of the role played by metabolism and reactive oxygen species in cell cycle progression (Burhans and Heintz, 2009).

In this work, I found that loss of mtDNA, but not inhibition of respiration, was sufficient to cause small cell size. Loss of mtDNA caused loss of Sch9 vacuolar membrane enrichment and phospho-isoforms, and epistasis analysis showed that loss of mtDNA conferred no further cell

size decrease in the absence of Sch9 activity. These observations suggest that decreased Sch9 activity may account for the small size of cells lacking mtDNA. The possibility that cells lacking Sch9 are at a minimum cell size (median size 27 fL) seems unlikely given that combining several known small-size mutations could generate a much smaller cell. (In fact, the *sch9*^{as} *sfp1*^{hER} *whi5Δ pGAL1-CLN3-1* strain's median cell size of 16 fL is likely an underestimate, since its size distribution falls below the lower detection limit of the Coulter Channelizer.)

If the small cell size of *rho*^{0} cells is a reflection of decreased Sch9 activity, this in turn raises the question of how Sch9 controls cell size. In earlier work, our laboratory found that loss of Sch9 kinase activity caused cells to commit to division at a smaller cell size, as measured by the onset of G1/S transcription (Jorgensen, et al., 2004). In this work, it was found that the small cell size conferred by loss of Sch9 was independent of the known upstream regulators of the G1/S transcriptional machinery (*CLN3*, *BCK2*, and *WHI5*), and that loss of Sch9 activity caused small cell size even when the level of G1 cyclin was fixed by heterologous transcriptional control. Sch9 activity appeared to decrease the amount of G1 cyclin required for viability. The mechanism by which Sch9 modulates this cyclin threshold remains an open question.

A reduced cell size threshold for the G1/S transition in yeast has been linked to poor nutrient conditions, slow growth, and mutants with decreased signaling through nutrient-sensing pathways (Jorgensen, et al., 2002; Jorgensen, et al., 2004; Jorgensen and Tyers, 2004). Decreasing the cell-size threshold for division is thought to confer a competitive advantage to yeast by allowing them to minimize their generation time under conditions where nutrients are limiting for growth (Jorgensen and Tyers, 2004). Interestingly, a recent study experimentally uncoupled the effects of growth rate and nutrient sensing using an *adh1Δ* strain, which – in contrast to WT – grows faster in glycerol medium than in glucose medium (Levy, et al., 2007). Importantly, it was found that cell size correlated with the external nutrient conditions and not the actual growth rate – like WT, the *adh1Δ* strain exhibited larger cell size in glucose than in glycerol, despite a higher growth rate in glycerol (Levy, et al., 2007). The same correlation was observed at the level of the transcriptome – like WT, the *adh1Δ* strain exhibited higher levels of ribosomal protein (RP) and ribosome biogenesis (RiBi) transcripts and lower levels of stress transcripts in glucose than in glycerol, despite a higher growth rate in glycerol (Levy, et al., 2007). Indeed, other recent transcriptional profiling experiments have shown that activation of nutrient sensing pathways can recapitulate the transcriptional signature associated with growth in

the absence of any actual nutrient metabolism or increase in growth rate (Slattery, et al., 2008; Wang, et al., 2004; Zaman, et al., 2009). These observations suggest that the cell-size threshold for division may be actively set by Sch9 and other regulators of nutrient signaling based on external environmental conditions. Such an evolutionary strategy would offer yeast cells a selective advantage by enabling them to rapidly anticipate and prepare for future environmental changes based on perceived nutrient status, instead of passively waiting for nutrient metabolism to eventually affect growth rate.

3.3.4 Sch9 exhibits dynamic localization

During log-phase growth, Sch9 was enriched at the vacuolar membrane as previously reported (Jorgensen, et al., 2004), and was also consistently enriched in a focus adjacent to the vacuolar membrane that resembles the pre-autophagosomal structure (PAS) (Kawamata, et al., 2008) involved in autophagic vesicle nucleation. Although confirmation that this structure is the PAS awaits co-localization of Sch9 with known PAS markers, such a possibility would be consistent with the recent identification of Sch9 as a regulator of autophagy (Yorimitsu, et al., 2007).

This work unexpectedly found that certain stress conditions – including treatment with 7% ethanol or resuspension of cells in Isoton II diluent – caused sequential enrichment of Sch9 in the nucleus, followed by the nuclear envelope, followed by punctate cytoplasmic foci whose formation could be blocked by the translation inhibitor cycloheximide. The observed nuclear localization of Sch9 is consistent with a recent report that Sch9 acts as transcription elongation factor that can be cross-linked along the coding region of HOG pathway osmostress genes (Pascual-Ahuir and Proft, 2007). I note in passing that the relevant physiological change induced by Isoton II diluent – the solution routinely used to dilute cells for analysis by Coulter Channelizer – is unclear. According to the manufacturer, Isoton is an isotonic electrolyte solution of pH ~7 containing 154 mM sodium chloride, 1.4 mM disodium EDTA, 1.6 mM monosodium phosphate, 14mM disodium phosphate, 7.1 mM sodium fluoride, and 5.4 mM potassium chloride (Beckman Coulter, personal communication). Curiously, a solution of the above composition made using standard laboratory reagents did not recapitulate the effects on Sch9 localization, suggesting that the relevant agent in Isoton may be a trace contaminant introduced during manufacturing or storage.

I speculate that sequential localization of Sch9 at the nuclear envelope and cycloheximide-inhibitable foci may reflect physical association of Sch9 with messenger ribonucleoprotein particles (mRNPs) and a potential role for Sch9 in mRNA metabolism. First, accumulation of Sch9 at the nuclear membrane in response to treatment with 7% ethanol would fit with the reported ability of ethanol stress to block bulk poly(A)$^+$ mRNA export (Takemura, et al., 2004). Second, the finding that Sch9 localization to cytoplasmic foci could be inhibited by cycloheximide – an inhibitor of translation elongation that traps mRNAs in polysomes – suggests that either Sch9 requires ongoing translation for its localization to these foci, or that the foci themselves disassemble when mRNAs are prevented from exiting polysomes. Indeed, Sch9 cytoplasmic foci closely resemble RNA processing bodies (P-bodies) and stress granules – cytoplasmic foci composed of non-translating mRNAs and proteins involved in mRNA metabolism (Anderson and Kedersha, 2006; Eulalio, et al., 2007; Parker and Sheth, 2007). Current models of mRNA fate envision a dynamic equilibrium between translation in polysomes, storage in stress granules, and transcript degradation in P-bodies (Balagopal and Parker, 2009; Buchan and Parker, 2009). The assembly of P-bodies requires non-translating mRNAs, and all known components of P-bodies fail to assemble into foci when mRNAs are trapped in polysomes with cycloheximide (Teixeira, et al., 2005). I speculate that Sch9 cytoplasmic foci may represent P-bodies or related mRNP granules. Indeed, a recent study unexpectedly found that the PKA catalytic subunits Tpk2 and Tpk3 accumulated in P-bodies and EGP-bodies during stationary phase, glucose starvation, and hyperosmotic shock (Tudisca, et al., 2010). Interestingly, TORC1 signaling has previously been reported to control mRNA stability (Albig and Decker, 2001), and a recent study implicated the kinase Rim15 – a known substrate of Sch9 (Wanke, et al., 2008) – in mRNA degradation via the Igo1/2 proteins and Dhh1 (Talarek, et al., 2010).

Sch9 has been shown to regulate translation in yeast (Urban, et al., 2007), and S6K – Sch9's putative counterpart in mammalian cells – has been reported to physically interact with mRNPs and the eukaryotic initiation factor 3 (eIF3) translation preinitiation complex (Holz, et al., 2005; Ma, et al., 2008). If Sch9 is localized to mRNP granules as hypothesized above, this would imply a model in which Sch9 follows an mRNA throughout the process of gene expression, from transcription in the nucleus, to export in an mRNP, to translation on cytosolic ribosomes. In fact, a similar model has recently been proposed for the polymerase II C-terminal domain (CTD) kinase Ctk1, which – in addition to being implicated in transcription by RNA

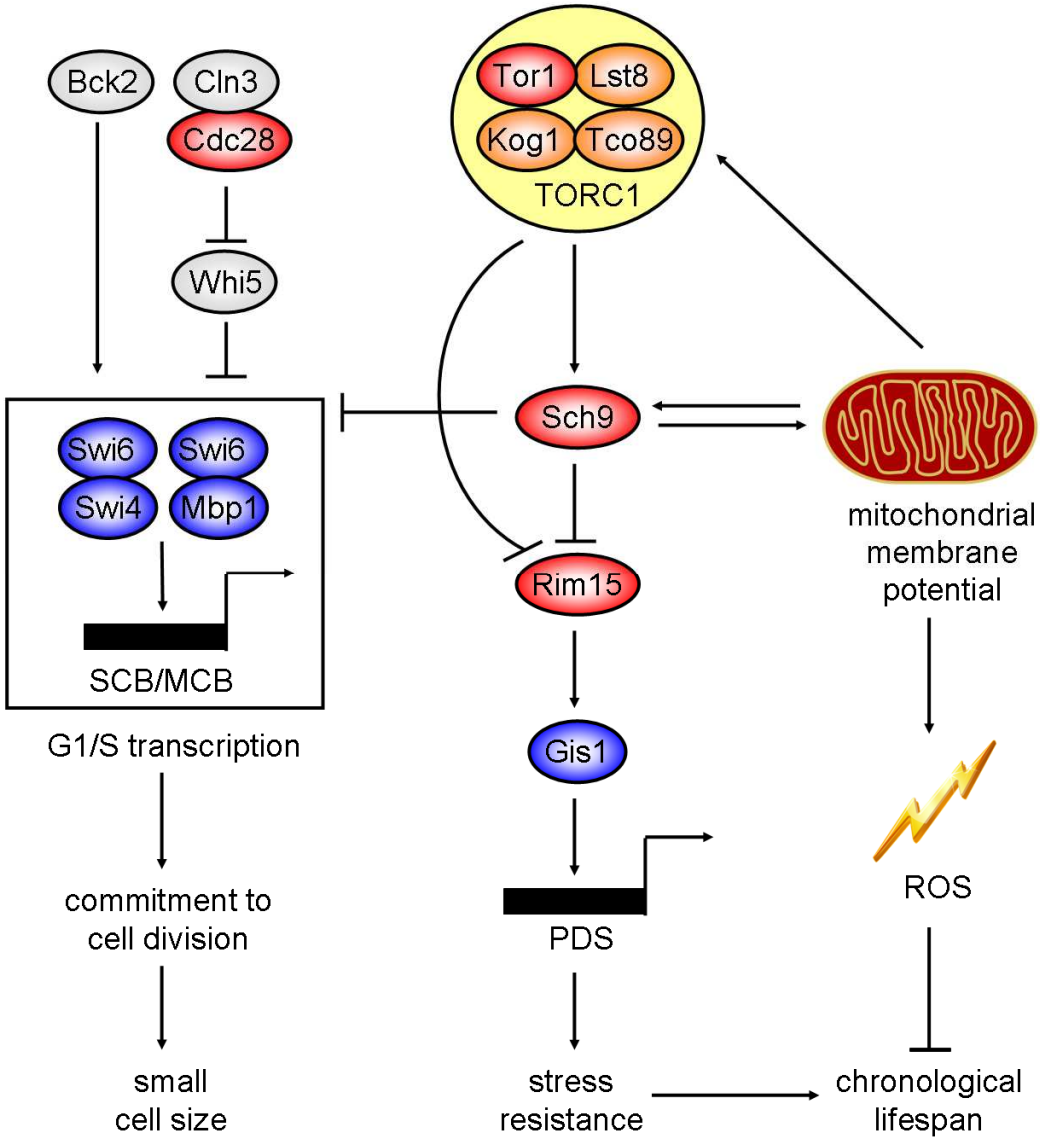
polymerase II – has been proposed to exit the nucleus bound to messenger ribonucleoprotein particles (mRNPs) and subsequently regulate translation (Rother and Strasser, 2007). Similarly, the mammalian SF2/ASF protein has been proposed to physically associate with a subset of mRNAs in the nucleus, undergo export to the cytoplasm as part of the mRNP, and enhance translation by direct recruitment of mTOR (Bushell, et al., 2008; Michlewski, et al., 2008).

This work also found that Sch9 became enriched at mitochondria under conditions that have previously been shown to produce ROS and induce apoptosis in yeast, including treatment with acetic acid (Ludovico, et al., 2001), treatment with H_20_2 (Madeo, et al., 1999), and hyper-activation of the Ras-PKA pathway (Breitenbach, et al., 2005; Gourlay and Ayscough, 2006; Phillips, et al., 2006). Interestingly, translocation of other proteins to mitochondria under similar conditions has been noted in several recent studies. The ribosome-associated protein Tma19, the Hsp70-family protein Ssa2, and the proteasome subunit Pre6 were reported to relocalize to mitochondria in response to treatment with acetic acid or H_2O_2 (Rinnerthaler, et al., 2006). Similarly, another study found that the kinases Pkc1 and Bck1 relocalized to mitochondria after treatment with farnesol, whose main effect is to generate ROS (Fairn, et al., 2007). In each of the above cases, the functional consequences of relocalization of these proteins to mitochondria were unclear. Other recent studies have linked aberrant trafficking of Ras2 to mitochondria with mitochondrial dysfunction and apoptosis (Leadsham, et al., 2009; Wang and Deschenes, 2006). These findings are intriguing given that mitochondrial function and mitochondrial proteins have been consistently implicated in apoptotic cell death in yeast (Aerts, et al., 2009; Braun, et al., 2006; Buttner, et al., 2008; Buttner, et al., 2007; Eisenberg, et al., 2007; Fannjiang, et al., 2004; Li, et al., 2006b; Ludovico, et al., 2002; Pereira, et al., 2008a; Pozniakovsky, et al., 2005; Ruckenstuhl, et al., 2009; Silva, et al., 2005; Wissing, et al., 2004).

Sch9 is a powerful regulator of lifespan, and its involvement in apoptosis would be unsurprising given evidence that apoptotic cell death occurs during the physiological aging process in yeast (Fabrizio, et al., 2004; Fabrizio and Longo, 2008; Herker, et al., 2004; Laun, et al., 2001). In fact, a recent study found that accumulation of extracellular acetic acid – a well-known trigger of yeast apoptosis – is actually the primary cause of aging in the standard yeast chronological aging assay, and that cells deleted for *SCH9* are resistant to acetic acid-induced cell death (Burtner, et al., 2009). It remains an open question as to whether Sch9 translocation to mitochondria reflects a functional role for Sch9 in an apoptotic cell death programme, but the

possibility is intriguing given recent reports implicating TORC1 signaling in yeast apoptosis (Almeida, et al., 2009).

Figure 3-5. Preliminary model showing connections between TORC1, Sch9, mitochondrial function, and lifespan. Solid arrows and T-bars represent stimulatory and inhibitory relationships, respectively. Transcription factors are shown in blue, kinases in red, and TORC1 components in orange. TORC1, target of rapamycin complex 1; SCB, Swi4/6 cell cycle box; MCB, MluI cell cycle box; PDS, post diauxic shift; ROS, reactive oxygen species.


Bck2
Cln3
Cdc28
Whi5
Swi6
Swi6
Swi4
Mbp1
SCB/MCB
G1/S transcription
commitment to
cell division
small
cell size
Tor1
Lst8
Kog1
Tco89
TORC1
Sch9
Rim15
Gis1
PDS
stress
resistance
mitochondrial
membrane
potential
ROS
chronological
lifespan

3.4 Material and Methods

3.4.1 Yeast media, strains, and plasmids

Yeast culture and genetics was performed according to standard techniques (Amberg, et al., 2005). Synthetic complete media (SC) is 0.2% amino acid mix, 0.5% ammonium sulfate, 0.17% yeast nitrogen base; where indicated, specific amino acids or uracil were omitted to select for plasmid maintenance. Rich media (XY) is 2% bactopeptone, 1% yeast extract, 0.01% adenine, 0.02% tryptophan. All chemicals were from Sigma-Aldrich (St. Louis MO) unless otherwise stated. The specific inhibitor of the analogue-sensitive *sch9*^{as} allele, 1-naphthylmethyl-PP1 (1NM-PP1), was from Toronto Research Chemicals (Toronto, Canada). Deletion strains were obtained from the genome-wide *MAT***a** deletion collection (EUROSCARF) or the genome-wide *MAT*$\boldsymbol{\alpha}$ deletion collection (Research Genetics) (Giaever, et al., 2002); strains bearing GFP-tagged proteins were obtained from the Yeast-GFP collection (Invitrogen) (Huh, et al., 2003). Yeast strains were constructed using standard PCR-based methods (Goldstein and McCusker, 1999; Longtine, et al., 1998). *rho*^{0} strains were generated from the corresponding *rho*$^{+}$ parental strain by treatment of log phase cells with 25 μg/mL ethidium bromide for 4 hours and plating for single colonies (Fox, et al., 1991). *rho*^{0} strains were verified for their inability to grow on glycerol media and for the absence of mtDNA nucleoids by staining with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Nunnari, et al., 2002). Results involving *rho*^{0} strains were verified with 3-6 independent *rho*^{0} isolates. In experiments involving chemical inhibitors of respiration, inhibitors were verified for their ability to completely block growth on a non-fermentable carbon source at the concentrations used. Strains and plasmids used in this work are listed in Table 3-1 and Table 3-2, respectively.

3.4.2 Halo assays

Saturated overnight yeast cultures were washed once in distilled water, diluted 1:200 in molten top agar at 42°C, poured onto solid medium, and allowed to recover for 2 hours before addition of filter discs.

3.4.3 Cell viability spot assays

Saturated overnight cultures were washed once, diluted 1:10 in distilled water, and serially diluted in 10-fold steps. Volumes of 4 μL or 6 μL were spotted onto synthetic- or rich-media plates, respectively.

3.4.4 Live-cell microscopy

DIC and fluorescence microscopy were performed with an Eclipse E600FN microscope (Nikon) and imaged on an Orca II CCD camera (Hamamatsu, Bridgewater, NJ). Metamorph Software (Universal Imaging, West Chester, PA) was used to capture and process images. To visualize cells, an aliquot of culture was pelleted by centrifugation for 5 seconds, supernatant removed, and cells resuspended in residual liquid mounted on a glass slide. Total time from incubation to visualization was < 3 min. To assess mitochondrial membrane potential, log phase cells were incubated with 1 μM MitoTracker Red CMXRos (Invitrogen) for ~5 seconds immediately before pelleting and visualization.

3.4.5 Determination of petite frequency

Strains were grown overnight in XY +3% glycerol to select for a starting population of respiratory-competent cells, then re-inoculated into SC +2% glucose + 200 nM 1-NM-PP1, and grown at 37°C for ~18 generations. Cells were plated on XY +2% glucose plates and incubated for 3 days to allow individual colonies to form. Colonies were overlaid with 2,3,5-triphenyltetrazolium chloride (TTC) as described (Bateman, et al., 2002; Ogur, et al., 1957), and respiring (red) and non-respiring (white) colonies were scored. Three independent replicates were performed for each strain, with >400 colonies scored for each replicate.

3.4.6 Cell size analysis

Saturated overnight cultures were diluted 300-fold into fresh medium and grown for 6-9 hours (>3 population doublings) at 30°C to a cell density of 0.3-3.0 x 10^7 cells/mL, a cell density range in which size distributions are generally constant. To obtain each size distribution, 100 μL culture was diluted into 10 mL Isoton II diluent (Beckman Coulter), sonicated gently for 10 seconds to disperse aggregated cells, and analyzed with a Coulter Channelizer Z2 (Beckman Coulter).

3.4.7 TCA lysis and immunoblotting

For immunoblotting, strains were grown to log phase (A_{600} = 0.3) in 10 mL SC-ura + 2% glucose and treated (or not) as indicated. At harvest, cultures were cooled rapidly by the addition of an equal volume of crushed ice, pelleted, washed once in ice-cold water, and 100 µL of 20% trichloroacetic acid TCA was added before flash-freezing in liquid nitrogen. Cells were disrupted by glass bead lysis in 20% TCA, lysates separated by 10% SDS-PAGE (100V, 2 hours), and resolved proteins transferred to nitrocellulose membranes (BioTrace NT). ^{HA}Sch9 was detected using anti-HA antibody 12CA5 [1:10,000 dilution in 5% milk powder/ Tris-buffered saline + 0.1% Tween-20 (TBS-T); overnight incubation at 4°C] followed by sheep anti-mouse IgG-HRP (Sigma) (1:10,000 dilution in 5% milk powder/TBS-T; 2 hour incubation at room temperature). Immunoreactive bands were visualized using Supersignal West Pico chemiluminescent detection (Pierce).

Table 3-1. Yeast strains.

strain	relevant genotype	background	*MAT*	source
BY4741 (yMT1448)	*his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	S288C	**a**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
BY4742 (yMT1449)	*his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	S288C	**α**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
BY4743 (yMT1450)	*his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0*	S288C	**a/α**	Brachmann, C.B. *et al*. (1998). *Yeast* **14**, 115-132.
yMT2594	*sch9::sch9$^{as(T492G)}$*	S288C	**a**	Jorgensen, P. *et al*. (2004). *Genes Dev* **18**, 2491-2505.
yMT2422	*sch9::kanR-pGAL1-SCH9*	S288C	**a**	Jorgensen, P. *et al*. (2004). *Genes Dev* **18**, 2491-2505.
yMT2290	*sch9Δ::natR*	S288C	**a**	Paul Jorgensen
yMT4237	*sch9::sch9$^{as(T492G)}$-natR*	S288C	**α**	this work
yMT4238	*sch9::sch9$^{as(T492G)}$-natR*	S288C	**a**	this work
yMT4240	*rpa190::RPA190^{GFP}-his5+*	S288C	**a**	Invitrogen
yMT4241	*sch9::sch9$^{as(T492G)}$-natR rpa190::RPA190^{GFP}-his5+*	S288C	**a**	this work
yMT4229	*gal1Δ::kanR*	S288C	**a**	EUROSCARF
yMT4239	*gal1Δ::kanR sch9::sch9$^{as(T492G)}$-natR*	S288C	**a**	this work
yMT2558	*sch9Δ::natR* carrying pMT3212 *<pSCH9-SCH9>*	S288C	**a**	Paul Jorgensen
yMT3106	*sch9Δ::natR* carrying pMT3572 *<pSCH9-^{GFP}SCH9>*	S288C	**a**	Paul Jorgensen
yMT4253	*pde2Δ::kanR sch9Δ::natR* carrying pMT3572 *<pSCH9-^{GFP}SCH9>*	S288C	**a**	this work

yMT3103	*sch9Δ::natR* carrying pMT3569 <*pSCH9-^{3HA}SCH9*>	S288C	**a**	Paul Jorgensen
yMT3989	*pep4Δ::kanR sch9Δ::natR* carrying pMT3569 <*pSCH9-^{3HA}SCH9*>	S288C	**a**	this work
yMT4243	*rho^{0} pep4Δ::kanR sch9Δ::natR* carrying pMT3569 <*pSCH9-^{3HA}SCH9*>	S288C	**a**	this work
yMT4242	*rho^{0} sch9Δ::natR* carrying pMT3572 <*pSCH9-^{GFP}SCH9*>	S288C	**a**	this work
yMT4244	*rho^{0}*	S288C	**α**	this work
yMT4249	*rho^{0} sch9::sch9$^{as(T492G)}$-natR*	S288C	**α**	this work
yMT2641	*whi5Δ::natR*	S288C	**a**	Joy Nishikawa
yMT4250	*rho^{0} whi5Δ::natR*	S288C	**a**	this work
yMT2643	*cln3Δ::LEU2 bck2Δ::kanR whi5Δ::natR*	S288C	**a**	Joy Nishikawa
yMT4245	*sch9::sch9$^{as(T492G)}$ cln3Δ::LEU2 bck2Δ::kanR whi5Δ::natR*	S288C	**a**	this work
yMT4248	*rho^{0} cln3Δ::LEU2 bck2Δ::kanR whi5Δ::natR*	S288C	**a**	this work
yMT4252	*sch9::sch9$^{as(T492G)}$ sfp1::sfp1^{hER}-his5+ whi5Δ::kanR cln3::URA3-pGAL1-CLN3-1*	S288C	**a**	this work
BS111 (yMT4246)	*ade2 can1 ura3 leu2::pGAL-CLN1^{3HA} cln2::TRP1 cln3::HIS3 gal1gal10::hisG*	W303	**α**	Schenider, B.L. *et al*. (2004). *Mol Cell Biol* **24**, 10802-10813.
yMT4247	*sch9::sch9$^{as(T492G)}$ ade2 can1 ura3 leu2::pGAL-CLN1^{3HA} cln2::TRP1 cln3::HIS3 gal1gal10::hisG*	W303	**α**	this work

All S288C strains are congenic with BY4741/BY4742 and thus carry *ura3Δ0*, *his3Δ1*, and *leu2Δ0*; note that *met15Δ0* and *lys2Δ0* were generally not followed in crosses and their status is unknown.

Table 3-2. Plasmids.

plasmid	relevant insert	yeast marker	vector type	source
pMT4496	*pGAL1-HAP4* in pBY011	*URA3*	*CEN*	Hu, Y. *et al*. (2007). *Genome Res* **17**, 536-543.
pRS316 (pMT273)	empty	*URA3*	*CEN*	Sikorski, R. S., and Hieter, P. (1989). *Genetics* **122**, 19-27.
pMT3212	*pSCH9-SCH9* in pRS316	*URA3*	*CEN*	Jorgensen, P. *et al*. (2004). *Genes Dev* **18**, 2491-2505.
pMT3569	*pSCH9-*^{3HA}*SCH9* in pRS316	*URA3*	*CEN*	Jorgensen, P. *et al*. (2004). *Genes Dev* **18**, 2491-2505.
pMT3572	*pSCH9-*^{GFP}*SCH9* in pRS316	*URA3*	*CEN*	Jorgensen, P. *et al*. (2004). *Genes Dev* **18**, 2491-2505.

Chapter 4 – Thesis summary and future directions

4.1 Thesis summary

The target of rapamycin (TOR) kinases regulate cell growth in accordance with external conditions. In this work, I have sought to expand the signaling network surrounding TORC1 in *Saccharomyces cerevisiae*.

Our laboratory's ongoing effort to systematically chart the yeast kinase and phosphatase interactions revealed many novel physical interactors of TORC1. In this work, I found evidence that the novel TORC1 interactors Mks1, Fmp48, and Nnk1 link TOR signaling to various facets of nitrogen metabolism and mitochondrial function. The uncharacterized kinase Nnk1 (Ykl1171w) modulated rapamycin sensitivity, interacted with the nitrogen catabolite repression (NCR) regulator Ure2, impacted Gln3 nuclear localization, and controlled transcription of Gln3 target genes. Nnk1 also interacted with the NAD^+-linked glutamate dehydrogenase Gdh2. In turn, Gdh2 modulated rapamycin sensitivity, was phosphorylated in Nnk1 immune complexes *in vitro*, and was relocalized to a discrete cytoplasmic focus in response to *NNK1* overexpression or respiratory growth. The uncharacterized kinase Fmp48 modulated rapamycin sensitivity, regulated respiratory function and mitochondrial morphology, and Fmp48-associated kinase activity was increased by rapamycin treatment. The retrograde response regulator Mks1 modulated rapamycin sensitivity, regulated respiratory function, and ectopic expression of *MKS1* caused severe growth inhibition with properties suggestive of reduced TORC1 signaling.

In a separate series of experiments – performed before the Sch9 kinase was known to be a direct substrate of TORC1 – I found evidence that Sch9 acts as both an upstream regulator and downstream sensor of mitochondrial function. Loss of Sch9 conferred a respiratory growth defect, a defect in mitochondrial DNA (mtDNA) transmission, lower mitochondrial membrane potential, and decreased levels of reactive oxygen species (ROS). Conversely, loss of mtDNA caused loss of Sch9 enrichment at the vacuolar membrane, loss of Sch9 phospho-isoforms, and small cell size suggestive of reduced Sch9 activity. The ability of a protonophore – but not other interventions that inhibit respiration – to recapitulate the above effects suggested that the proton gradient across the inner mitochondrial membrane may be the molecular signal to which Sch9 responds. Epistasis analysis suggested that reduced Sch9 activity may account for the small size of cells lacking mtDNA. The ability of loss of Sch9 to confer small cell size was unaffected by the absence of all known upstream regulators of the G1/S transcriptional machinery, or by fixing

the level of G1 cyclin by heterologous transcriptional control. I also found that Sch9 exhibits dynamic relocalization in response to stress. Live cell imaging of GFP-tagged Sch9 showed that some cellular stresses caused sequential enrichment of Sch9 in the nucleus, followed by the nuclear envelope, and eventually in cycloheximide-inhibitable cytoplasmic foci that may correspond to messenger ribonucleoprotein (mRNP) granules. Sch9 also became enriched at mitochondria under conditions that have previously been shown to produce ROS and induce apoptosis in yeast.

Taken together, this work reveals intimate connections between TORC1, nitrogen metabolism, and mitochondrial function. These findings are intriguing when viewed in the larger context of the evolutionarily conserved role of TOR signaling in regulating aging, cancer, and other human diseases.

4.2 Future directions

"[I]t is common knowledge that the human brain can keep track of only so many variables. It is also common experience that once the number of components in a system reaches a certain threshold, understanding the system without formal analytical tools requires geniuses, who are so rare even outside biology."

– Dr. Yuri Lazebnik (Lazebnik, 2002)

4.2.1 Nnk1

Although this work has sketched a tentative model linking TORC1, Nnk1, Ure2 and Gdh2, many questions remain with respect to Nnk1 regulation, its targets, and information flow in this system. Although the most conventional model would have TORC1 phosphorylate Nnk1, which in turn phosphorylates Gdh2 and Ure2, insufficient evidence exists to conclusively assign directionality to the TORC1-Nnk1, Nnk1-Gdh2, and Nnk1-Ure2 connections – more exotic models in which Gdh2 or Ure2 communicates cellular nitrogen status to Nnk1 and TORC1 are also conceivable.

To establish if Nnk1 lies downstream of TORC1, the effect of rapamycin on Nnk1 phosphorylation status *in vivo* and Nnk1 activity *in vitro* could be examined, as well as the ability of Tor1 immunoprecipitated from yeast to phosphorylate bacterially expressed Nnk1 *in vitro*. The possibility that Nnk1 lies upstream of TORC1 could be examined by assessing the effect of *NNK1* overexpression on TORC1 activity using phosphorylation of Sch9 as a read-out.

It may also be informative to examine if Nnk1 kinase activity or any of the TORC1-Nnk1, Nnk1-Gdh2, or Nnk1-Ure2 physical interactions are modulated by rapamycin, starvation, or various nutrient and stress conditions. Construction of a kinase-dead and analogue-sensitive allele of Nnk1 would provide a powerful tool for future studies. (For Nnk1, the kinase-dead mutation corresponds to K478A, the *as1* mutation to T525G, the *as2* mutation to T525A, and the *as3* mutation to C602A.) A kinase-dead or analogue-sensitive allele of *NNK1* is needed as a control to provide evidence that *in vitro* phosphorylation of Gdh2 in Nnk1 immune complexes is not due to a co-precipitating kinase. It is also worth noting that an *nnk1Δ* strain had normal rapamycin sensitivity, no obvious growth defect on any carbon source examined, and a transcriptional response to rapamycin that was identical to wild-type (data not shown). These observations suggest that Nnk1 may act in concert with one or more other redundant kinases, which may be uncovered by screening for deletions that are synthetic lethal with *nnk1Δ*.

The functional connections between Nnk1 and its interactors Gdh2 and Ure2 also await further clarification. To provide further evidence that Gdh2 and Ure2 lie downstream of Nnk1, the effect of *NNK1* deletion and overexpression on their phosphorylation status *in vivo* could be examined, as well as the ability of Nnk1 immunoprecipitated from yeast to phosphorylate bacterially expressed Gdh2 and Ure2 *in vitro*. Nnk1, Gdh2, and Ure2 immunoprecipitated from yeast could be further analyzed by mass spectrometry to identify putative sites of *in vivo* phosphorylation, which could then be subject to mutagenesis. Definitively validating kinase substrates ultimately requires meeting a stringent standard of evidence (Berwick and Tavare, 2004). The effect of *NNK1* deletion and overexpression on Gdh2 activity could be assessed via biochemical assay of Gdh2 enzymatic activity [for example, see (Hemmings, 1980; Nunez de Castro, et al., 1970; Uno, et al., 1984)] or via metabolomic analysis. Initial attempts to measure the flux of key yeast nitrogen metabolites upon *NNK1* overexpression were confounded by the carbon source shift required to induce the *pGAL1-NNK1* allele, so an alternative strategy for regulation of *NNK1* which does not involve a nutrient shift will likely be needed.

Little is known about the focus to which $Gdh2^{GFP}$ relocalizes upon *NNK1* overexpression or during respiratory growth. To investigate the nature of Gdh2 foci, they could be colocalized with markers of previously described cytoplasmic foci, including the pre-autophagosomal structure (PAS) (Kawamata, et al., 2008), RNA processing bodies (P-bodies) (Brengues and Parker, 2007; Brengues, et al., 2005; Sheth and Parker, 2003), stress granules/EGP bodies

(Buchan, et al., 2008; Hoyle, et al., 2007), TAM bodies (Gill, et al., 2006), T-bodies (Malagon and Jensen, 2008), eIF2B foci (Campbell, et al., 2005) and many other metabolic enzymes shown to form foci upon growth of cells into stationary phase (Narayanaswamy, et al., 2009).

If Gdh2 foci are found to be distinct from these known structures, their nature could be probed by subjecting $Gdh2^{GFP}$ and $Gdh2^{HA}$ to mass spectrometric analysis under focus-inducing conditions in order to identify potential interaction partners. In another unbiased approach, a *pGAL1-NNK1* allele could be crossed into the genome-wide collection of ORF^{GFP} strains to screen for other proteins that relocalize to a focus upon *NNK1* overexpression. Alternatively, a *pGAL1-NNK1* $GDH2^{GFP}$ strain could be crossed to the genome-wide collection of single-gene-deletion strains to screen for factors required for $Gdh2^{GFP}$ focus formation.

4.2.2 Fmp48

Many aspects of Fmp48 regulation and function remain to be elucidated. Although a tentative model suggests that Fmp48 is negatively regulated by TORC1, a kinase-dead or analogue-sensitive allele of Fmp48 should be constructed as a control to provide evidence that the rapamycin-induced increase in Fmp48-associated kinase is not due to a co-precipitating kinase. In addition, the effect of rapamycin on Fmp48 phosphorylation status *in vivo* could be examined, as well as the ability of Tor1 immunoprecipitated from yeast to phosphorylate bacterially expressed Fmp48 *in vitro*. It may also be informative to examine if Fmp48 kinase activity or the TORC1-Fmp48 physical interaction is modulated by starvation, mitochondrial function, or various nutrient and stress conditions.

Although *FMP48* overexpression caused a growth defect on a non-fermentable carbon source and abnormal mitochondrial morphology, the exact nature of the mitochondrial defect conferred by *FMP48* overexpression is not known, nor is the molecular mechanism by which Fmp48 impinges upon mitochondrial function. The mitochondrial phenotype of cells overexpressing *FMP48* could be characterized in more detail – for instance, using assays for mitochondrial membrane potential, ROS levels, mitochondrial transcript levels, and mitochondrial translation rate. Insight into Fmp48's function may also be gained by exploring its other interactions in the KPI network (Figure 2-3A), which include interesting connections with Mks1 and Ksp1 (themselves new TORC1 interactors), the glycogen synthase complex, the nucleolar RENT complex, and the metabolic transcription factor Adr1.

4.2.3 Mks1

Much remains unknown about Mks1 and its relationship with TORC1 and mitochondrial function. Mks1 phosphorylation was known to be rapamycin-sensitive, and the physical interaction identified in this work suggests that Mks1 may be a direct target of TORC1 – a possibility that could be further explored by assessing the ability of Tor1 immunoprecipitated from yeast to phosphorylate bacterially expressed Mks1 *in vitro*. It may also be informative to examine if the TORC1-Mks1 physical interaction is modulated by rapamycin, starvation, mitochondrial function, or various nutrient and stress conditions. The terminal phenotype of *MKS1*-overexpressing cells could also be characterized in more detail to assess the mechanism of Mks1 toxicity.

The exact nature of the mitochondrial defect conferred by aberrant Mks1 levels is not known, nor is the molecular mechanism by which Mks1 impinges upon mitochondrial function. The mitochondrial phenotype of cells lacking or overexpressing *MKS1* could be characterized in more detail – for instance, using assays for mitochondrial membrane potential, ROS levels, mitochondrial transcript levels, and mitochondrial translation rate. Insight into Mks1's function may also be gained by exploring its other interactions in the KPI network (Figure 2-4B), which include interesting connections with Fmp48 (another TORC1 interactor with links to mitochondrial function), casein kinase 2, and Clb3-Cdc28.

4.2.4 Novel KPI network connections suggestive of new functions

The numerous interaction partners identified for TORC1/2 in the KPI network (Figure 2-1A) highlight many avenues for further investigation, some of which I mention in passing below.

The Ksp1 kinase interacts with multiple TORC1 components (Breitkreutz, et al., 2010) and has been found to be phosphorylated in a rapamycin-sensitive manner (Huber, et al., 2009). Ksp1 also interacts with the protein kinase A (PKA) catalytic subunit Tpk2 (Breitkreutz, et al., 2010), suggesting a possible mechanism for crosstalk with the PKA pathway, which is thought to act in parallel or downstream of TORC1 signaling (Schmelzle, et al., 2004). Indeed, loss of Tpk2 or the PKA regulatory subunit Bcy1 has been reported to alter the subcellular localization of Ksp1, while loss of Ksp1 has been found to alter the subcellular localization of Bcy1 (Bharucha, et al., 2008). Ksp1 also interacts with the Tra1 kinase (Breitkreutz, et al., 2010), suggesting that Ksp1 may represent a link between TORC1 and transcription. The Tra1 kinase, like Tpk2

(Pokholok, et al., 2006), is known to be physically associated with the transcription apparatus (Brown, et al., 2001; Grant, et al., 1998; Saleh, et al., 1998).

The Sky1 kinase interacts with Tor1 (Breitkreutz, et al., 2010) and has been found to be phosphorylated in a rapamycin-sensitive manner (Huber, et al., 2009). Sky1's other interactions suggest a role in mRNA splicing and export (Breitkreutz, et al., 2010), consistent with recent findings (Dagher and Fu, 2001; Gilbert, et al., 2001; Siebel, et al., 1999). I speculate that Sky1 may be a TOR effector that mediates the regulated splicing of ribosomal protein transcripts in response to starvation and environmental stress (Pleiss, et al., 2007).

The interaction of Bck1 kinase and Tor1/2 (Breitkreutz, et al., 2010) may underlie reported connections between TOR signaling and the cell wall integrity (CWI) MAPK cascade, which are complex (De Virgilio and Loewith, 2006a). TORC1 signaling is thought to negatively regulate the CWI pathway (Ai, et al., 2002; Araki, et al., 2005; Krause and Gray, 2002; Reinke, et al., 2004; Torres, et al., 2002), while TORC2 signaling is thought to positively regulate the CWI pathway in the control of actin organization (Bickle, et al., 1998; Helliwell, et al., 1998b; Loewith, et al., 2002; Schmidt, et al., 1997; Schmidt, et al., 1996).

The Npr1 kinase, known to be phosphorylated in a rapamycin-sensitive manner (Gander, et al., 2008; Schmidt, et al., 1998), interacts with components of at least 2 other important nutrient signaling pathways. Npr1 associates with the Snf1 kinase and its subunits Snf4 and Gal83 (Breitkreutz, et al., 2010), which have recently been implicated in nitrogen signaling downstream of TOR (Orlova, et al., 2006). Npr1 also associates with the Gcn2 kinase (Breitkreutz, et al., 2010), which phosphorylates eIF2α to inhibit translation in response to amino acid starvation (Hinnebusch, 2005). Under favourable nutrients conditions, Gcn2 is known to be phosphorylated and inhibited in a rapamycin-sensitive manner (Cherkasova and Hinnebusch, 2003). Thus, Npr1 may represent a TOR effector that mediates crosstalk with carbon metabolism and translational regulation.

Many other novel TOR connections are also worth noting. The Ime2 kinase interacts with TORC1 components (Breitkreutz, et al., 2010), suggesting a link between TOR signaling and meiosis. The latter would be consistent with the finding that TORC1 inhibition by rapamycin causes nuclear accumulation and stabilization of the Ime1 transcription factor (Colomina, et al., 2003). The Rck1 kinase interacts with Tor1 and its other interactions suggest a role in

transcription (Breitkreutz, et al., 2010). Loss of the related Rck2 kinase has been reported to confer rapamycin sensitivity (Li, et al., 2008). The interaction of Tor2 and the Mih1 phosphatase (Breitkreutz, et al., 2010) suggests a potential role for TOR at the G2/M transition. TOR signaling has been reported to regulate mitotic onset in *S. cerevisiae* (Nakashima, et al., 2008) and *S. pombe* (Petersen and Nurse, 2007), albeit in opposite directions. Finally, the interaction of the Rio2 kinase with TORC2 components hints at a potential role for TORC2 signaling in ribosome biogenesis. Rio2's physical interactions in the KPI network (Breitkreutz, et al., 2010) and other functional studies (Geerlings, et al., 2003) implicate Rio2 in rRNA processing and ribosome assembly.

4.2.5 Sch9

Loss of Sch9 conferred several mitochondrial phenotypes, including a thermosensitive growth defect on a non-fermentable carbon source, increased petite frequency, reduced mitochondrial membrane potential, and lower ROS levels. Other recent studies have further noted that loss of Sch9 leads to increased oxygen consumption, increased mitochondrial translation rates, and increased steady-state levels of electron transport chain proteins (Lavoie and Whiteway, 2008; Pan and Shadel, 2009). The mechanism by which Sch9 impacts mitochondrial function is unknown. Given that Sch9 and Fmp48 both appear to regulate mitochondrial function downstream of TORC1, it may be informative to explore potential interactions between these two kinases. Interestingly, there are many phospho-proteins in yeast mitochondria (Reinders, et al., 2007) that could represent Sch9 or Fmp48 targets. This knowledge could be integrated with the findings of a recent study that defined the consensus phosphorylation site motifs of 61 yeast kinases – including Nnk1, Fmp48, and Sch9 (Mok, et al., 2010) – to further refine a list of potential substrates.

Loss of mtDNA caused loss of Sch9 phosphorylation, loss of Sch9 vacuolar membrane enrichment, and small cell size. However, this effect could not be recapitulated by inhibition of respiration, so the specific molecular signal sensed by TOR/Sch9 is unclear. Loss of mtDNA leads to loss of genes encoding components of the electron transport chain and the F_0 portion of the ATP synthase, forcing *rho*^{0} cells to maintain a reduced mitochondrial membrane potential via an alternate mechanism involving the adenine nucleotide translocator (ANT) (Chen and Clark-Walker, 1999; Dupont, et al., 1985; Giraud and Velours, 1997; Kominsky, et al., 2002; Lefebvre-

Legendre, et al., 2003). To investigate if this lower mitochondrial membrane potential is the molecular signal sensed by TOR/Sch9, one could examine cells doubly deleted for both a nuclear-encoded component of the electron transport chain and a nuclear-encoded component of the F_0 portion of the ATP synthase – such cells must generate their mitochondrial membrane potential in the same manner as *rho*^{0} cells and would be expected to exhibit reduced Sch9 activity if the above hypothesis is correct. To directly test the model that decreased TOR/Sch9 activity accounts for the small size of *rho*^{0} cells, a recently described TOR-independent *SCH9*^{2D3E} phospho-mimic allele (Urban, et al., 2007) could be assessed for its ability to block the decreased cell size conferred by loss of mtDNA.

Certain stress conditions – including treatment with 7% ethanol or resuspension in Isoton II diluent – caused sequential enrichment of Sch9 in the nucleus, followed by the nuclear envelope, followed by cycloheximide-inhibitable cytoplasmic foci that I speculate may correspond to mRNP granules. To help establish the identity of these Sch9 foci, they could be colocalized with markers of previously described cytoplasmic foci, including RNA processing bodies (P-bodies) (Brengues and Parker, 2007; Brengues, et al., 2005; Sheth and Parker, 2003), stress granules/EGP bodies (Buchan, et al., 2008; Hoyle, et al., 2007), TAM bodies (Gill, et al., 2006), T-bodies (Malagon and Jensen, 2008), and eIF2B foci (Campbell, et al., 2005). If Sch9 foci are found to correspond to mRNP granules, deletion analysis could be used to test whether Sch9 regulates granule formation, or whether specific granule components are required for recruitment of Sch9 to these structures.

Finally, on a more general note, it seems likely that many other Sch9 substrates remain to be discovered. In order to further expand the signaling network surrounding Sch9, the analogue-sensitive *sch9*^{as} allele could be crossed into the genome-wide collection of *ORF*^{GFP} strains to screen for proteins that change their localization upon inhibition of Sch9 kinase activity – an analogous strategy using rapamycin has proven useful in identifying TOR effectors (Marion, et al., 2004; Shin, et al., 2009).

4.2.6 Perspective

In a commentary published in *Cancer Cell* in 2002, Dr. Yuri Lazebnik compared a dysfunctional cell to a broken transistor radio, and performed an interesting thought experiment, asking: “Could biologists figure out how to fix a broken radio?” The answer nicely encapsulates

much of the essence of cell biological research in the first decade of the 21st century. I quote it at length below:

> *"Conceptually, a radio functions similarly to a signal transduction pathway in that both convert a signal from one form into another (a radio converts electromagnetic waves into sound waves). My [broken] radio has about a hundred various components, such as resistors, capacitors, and transistors, which is comparable to the number of molecules in a reasonably complex signal transduction pathway. I started to contemplate how biologists would determine why my radio does not work and how they would attempt to repair it. Because a majority of biologists pay little attention to physics, I had to assume that all we would know about the radio is that it is a box that is supposed to play music.*
>
> *How would we begin? First, we would secure funds to obtain a large supply of identical functioning radios in order to dissect and compare them to the one that is broken. We would eventually find how to open the radios and will find objects of various shape, color, and size. We would describe and classify them into families according to their appearance. We would describe a family of square metal objects, a family of round brightly colored objects with two legs, round-shaped objects with three legs and so on. Because the objects would vary in color, we would investigate whether changing the colors affects the radio's performance. Although changing the colors would have only attenuating effects (the music is still playing but a trained ear of some can discern some distortion) this approach will produce many publications and result in a lively debate.*
>
> *A more successful approach will be to remove components one at a time or to use a variation of the method, in which a radio is shot at a close range with metal particles. In the latter case radios that malfunction (have a "phenotype") are selected to identify the component whose damage causes the phenotype. Although removing some components will have only an attenuating effect, a lucky postdoc will accidentally find a wire whose deficiency will stop the music completely. The jubilant fellow will name the wire Serendipitously Recovered Component (Src) and then find that Src is required because it is the only link between a long extendable object and the rest of the radio. The object will be appropriately named the Most Important Component (Mic) of the radio. A series of studies will definitively establish that Mic should be made of metal and the longer the object is the better, which would provide an evolutionary explanation for the finding that the object is extendable.*
>
> *However, a persistent graduate student from another laboratory will discover another object that is required for the radio to work. To the delight of the discoverer, and the incredulity of the flourishing Mic field, the object will be made of graphite and changing its length will not affect the quality of the sound significantly. Moreover, the graduate student would convincingly demonstrate that Mic is not required for the radio to work, and will suitably name his object the Really Important Component (Ric). The heated controversy, as to whether Mic or Ric is more important, will be fueled by the accumulating evidence that some*

> *radios require Mic while other, apparently identical ones, need Ric. The fight will continue until a smart postdoctoral fellow will discover a switch, whose state determines whether Mic or Ric is required for playing music. Naturally, the switch will become the Undoubtedly Most Important Component (U-Mic). Inspired by these findings, an army of biologists will apply the knockout approach to investigate the role of each and every component. Another army will crush the radios into small pieces to identify components that are on each of the pieces, thus providing evidence for interaction between these components. The idea that one can investigate a component by cutting its connections to other components one at a time or in a combination ("alanine scan mutagenesis") will produce a wealth of information on the role of the connections.*
>
> *Eventually, all components will be cataloged, connections between them will be described, and the consequences of removing each component or their combinations will be documented. This will be the time when the question, previously obscured by the excitement of productive research, would have to be asked: Can the information that we accumulated help us to repair the radio?"*
>
> – Dr. Yuri Lazebnik (Lazebnik, 2002)

The above description wryly captures the somewhat messy way in which cell biology proceeds. It also serves to illustrate that genomic and proteomic analysis often furnishes us with a wealth of components and connections, but relatively little true understanding of how to control cell behaviour. Indeed, all too often, high-throughput biology has the potential to inundate us with *inventories*, while leaving us starved for *insights* (Aitchison and Galitski, 2003).

And yet, much has changed since these words were written in 2002. Systems biology – with its conceptual shift towards the global analysis of networks, processes and systems – was an emerging field in 2002 (Kitano, 2002), but has since matured, with much of the pioneering work being done in the budding yeast *Saccharomyces cerevisiae* (Snyder and Gallagher, 2009). "Solving" the cell undoubtedly remains a huge challenge, but the landscape has shifted in significant ways. Recent years have witnessed the characterization of genome-scale regulatory networks at the level of the transcriptome and proteome, driven in large part by technological advances in mass spectrometry-based proteomics (Choudhary and Mann, 2010), genetic interaction mapping (Dixon, et al., 2009), next-generation sequencing (Park, 2009; Wang, et al., 2009), chemical biology (Lehar, et al., 2008; Smith, et al., 2010), and visualization tools for the resulting interaction data (Gehlenborg, et al., 2010).

In particular, there is now increasing emphasis on understanding information flow within biological systems (Brent and Bruck, 2006; Nurse, 2008) and how signal transduction is

regulated by temporal and spatial factors (Kholodenko, et al., 2010; Scott and Pawson, 2009). Indeed, many argue that signaling networks, rather than individual genes and proteins, should be the objects of study and the targets for therapeutic intervention in disease (Jorgensen and Linding, 2010; Russell and Aloy, 2008; Zanzoni, et al., 2009). Recent studies have begun to address the dynamic behaviour and systems-level properties of biological networks, including cooperativity and the role of scaffolds (Brent, 2009; Gibson, 2009; Zeke, et al., 2009), feedback loops and bistability (Burrill and Silver, 2010; Ferrell, et al., 2009), and robustness in the face of genetic and environmental perturbation (Barkai and Shilo, 2007; Kitano, 2007). The development of techniques for single-cell analysis has opened up an entirely new domain of inquiry (Bennett and Hasty, 2009; Locke and Elowitz, 2009; Muzzey and van Oudenaarden, 2009; Spiller, et al., 2010; Wang and Bodovitz, 2010), and enabled studies of the stochastic nature of transcription and translation, cell-to-cell variability, and biological noise (Eldar and Elowitz, 2010; Kaufmann and van Oudenaarden, 2007; Raj and van Oudenaarden, 2008; Wilkinson, 2009). Indeed, the emerging field of synthetic biology has already achieved modest but significant success in engineering synthetic cell circuits, oscillators, and other simple biological devices (Bashor, et al., 2010; Haynes and Silver, 2009; Kiel, et al., 2010; Lim, 2010; Mukherji and van Oudenaarden, 2009).

Taken together, these advances give us cause for cautious optimism. We may yet transform biology from a discipline that is qualitative and descriptive, to a mature science that is quantitative and predictive – and thereby usher in a day when we *will* be able to fix the radio.

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Appendix 1: A method for rapid regulation of protein activity via fusion to hormone-binding domains

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Rapid regulation of protein activity in fission yeast

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Abstract

Background

The fission yeast *Schizosaccharomyces pombe* is widely-used as a model organism for the study of a broad range of eukaryotic cellular processes such as cell cycle, genome stability and cell morphology. Despite the availability of extensive set of genetic, molecular biological, biochemical and cell biological tools for analysis of protein function in fission yeast, studies are often hampered by the lack of an effective method allowing for the rapid regulation of protein level or protein activity.

Results

In order to be able to regulate protein function, we have made use of a previous finding that the hormone binding domain of steroid receptors can be used as a regulatory cassette to subject the activity of heterologous proteins to hormonal regulation. The approach is based on fusing the protein of interest to the hormone binding domain (HBD) of the estrogen receptor (ER). The HBD tag will attract the Hsp90 complex, which can render the fusion protein inactive. Upon addition of estradiol the protein is quickly released from the Hsp90 complex and thereby activated. We have tagged and characterised the induction of activity of four different HBD-tagged proteins. Here we show that the tag provided the means to effectively regulate the activity of two of these proteins.

Conclusion

The estradiol-regulatable hormone binding domain provides a means to regulate the function of some, though not all, fission yeast proteins. This system may result in very quick and reversible activation of the protein of interest. Therefore it will be a powerful tool and it will open experimental approaches in fission yeast that have previously not been possible. Since fission yeast is a widely-used model organism, this will be valuable in many areas of research.

Background

Regulating protein function or protein level is often useful in order to investigate diverse biological processes. The fission yeast *Schizosaccharomyces pombe* is a popular model organism. It is genetically tractable and a wide variety of methods have been developed to facilitate molecular genetic manipulations in *S. pombe*.

It is usually more advantageous to regulate the activity of the target protein than the protein level, because this results in faster regulation of the protein's activity at wild type protein levels. The most commonly used approach to regulate the activity of the protein of interest is the isolation of conditional mutants, which have been vital tools in many areas of research. Indeed, one of the many advantages of fission yeast as a model system is that it is haploid, which makes it easier to isolate and work with conditional mutants. Most conditional mutants are temperature sensitive. However, not all genes can be mutated such that the corresponding protein becomes temperature sensitive. Furthermore, a temperature shift in itself might stress the cells. Temperature-sensitive proteins often have considerable residual activity at the restrictive temperature such that they rescue the temperature-sensitive mutant when overexpressed. Another common problem is that many temperature-sensitive proteins are not fully active at the permissive temperature. Therefore, temperature shifts of temperature-sensitive mutants are frequently far from the ideal "on" and "off" states that might be desired when regulating protein function. The reversibility of the inactivation varies greatly from mutant to mutant. Upon shift back to the permissive temperature, some temperature-sensitive proteins regain their activity, thus allowing block-and-release experiments. However, many other temperature sensitive proteins do not regain their activities after a period of temperature shift or are degraded at the restrictive temperature. Temperature-sensitive mutants have been particularly useful to explore the functions of essential proteins. However, it is difficult to identify temperature-sensitive mutants of non-essential genes, unless their function is known so that appropriate screens can be designed.

Regardless of the many advantages associated with the use of conditional mutants, they are not always available or applicable. A commonly used alternative is regulating the level of the protein of interest, either by regulating transcription or by regulating protein degradation (see below).

Numerous plasmids have been designed for regulated expression of genes [1], but there are no good tight and rapidly inducible promoters for use in fission yeast. The nmt1 (*n*o *m*essage in *t*hiamine) promoter was the first regulatable promoter to be described in fission yeast [2] and it remains the most commonly used one. This promoter is strong, but mutated versions with reduced strengths are available [3]. The promoter is repressed by thiamine (vitamin B1). The main drawback with the *nmt* promoter is that induction of protein expression is rather slow and it takes several generations to achieve full activation, presumably because the cellular vitamin pools have to be depleted first. Furthermore, thiamine confers over 100-fold repression of nmt1-driven transcription, but the promoter is still somewhat leaky and many cloned genes are expressed to near wild-type levels even in the presence of thiamine, such that they can complement chromosomal mutations. Shut-off experiments, where expression of the protein of interest is turned off by the addition of thiamine, are particularly inefficient for stable proteins, since not only is the promoter leaky, but the protein of interest also has to be diluted out as the cells grow.

There are several other and less widely used regulatable promoters that to some extent can be used in fission yeast. Although they confer regulated expression, there are also severe drawbacks to their use, as detailed below. The tetracycline regulatable promoter is a derivative of the Cauliflower Mosaic Virus (CaMV) promoter, fused to a tetracycline binding site [4]. The use of this promoter requires not only cloning the gene of interest behind the CaMV promoter but also manipulating the parent strain such that it expresses the Tet repressor. The *fbp1* promoter is repressed by glucose but it can only be used in liquid cultures [5]. The invertase promoter is also repressed by glucose and is activated by sucrose within an hour of medium shift. However, the glucose produced by invertase activity leads to repression of the promoter within a short time, so this promoter can only be used for short periods of expression [6]. Since regulation of the latter two promoters requires changing the carbon source, their use implies dramatically changing the growth conditions during the course of the experiment.

Only recently has a uracil-regulateable promoter been described that allows rapid activation and inactivation of transcription [7]. This system is expected to become a useful tool to regulate protein expression, but it should be noted that it might not always be sufficient to regulate transcription levels to achieve efficient regulation of protein levels.

The above regulatable systems all employ heterologous promoters. The expression levels from these promoters might or might not correspond to that from the native promoter of the gene of interest. The degron method, that circumvents this drawback, is based on regulated degradation of the target protein and has been used successfully in fission yeast [8-10]. However, it depends on a temperature shift to 37°C and the degron tag must be on the N-terminus of the target protein. Depending on the stability of the protein of interest, additional measures might also need to be taken to inactivate the protein. One improvement to the method in fission yeast was to combine the degron with an existing temperature sensitive mutation [8,9]. Another strategy that was employed in budding yeast is overexpression of the ubiquitin ligase Ubr1 [10,11]. This approach however cannot be used in fission yeast to improve degron-directed degradation [10]

In summary, despite having a selection of approaches to regulate protein levels, fission yeast researchers often find it difficult to achieve the desired expression level of their favourite proteins.

Here we describe the application of a system that is based on regulated protein function [12,13] without the need for a temperature shift, as opposed to regulated transcription or protein degradation. We have tested the system on four proteins and were able to regulate the activity of two of them.

Results

The principle

The approach we have used is based on the normal regulatory activity of the hormone binding domain (HBD) of vertebrate steroid receptors. The Hsp90 molecular chaperone binds the HBD in the absence of estrogen hormones. Upon addition of estradiol a hormone-induced conformational change in the HBD results in the dissociation of Hsp90 [14].

The HBD can also confer sensitivity to estradiol to the activity of heterologous proteins [13]. Fusing a heterologous protein of interest with the hormone-binding domain of the estrogen receptor (ER) renders it inactive presumably because it is bound by the Hsp90 (Fig. 1). Within a few minutes of addition of estradiol the hormone-induced conformational change in the HBD results in dissociation of the Hsp90 and activation of the chimeric protein (Fig. 1) [12,13]. The

mechanism of inhibition by Hsp90 is thought to be by steric interference [14] but regulation of the intracellular localization of the chimeric protein has also been reported [15].

In the following sections, we shall refer to the fusion protein as "*active*" or "*inactive*" in quotation marks, reflecting the presence or absence of estradiol, respectively. This indicates the protein activity expected based on the model described above and shown in Figure 1, rather than that observed experimentally.

Cdc13-des2-HBD

Cdc13 is the mitotic B-type cyclin in fission yeast. Cdc13 protein levels are stringently regulated through the cell cycle. The protein starts accumulating at the G1-S transition until, in late G2, the high level required for entry into and progression through mitosis is reached [16]. Cdc13 is then degraded via the APC (anaphase promoting complex) at the end of mitosis [17,18]. We wished to be able to regulate the Cdc13 levels independently of the cell cycle stage, i.e. allowing regulation that would be independent of APC activity. Therefore we employed a non-degradable mutant form of Cdc13, Cdc13-des2, which lacks the recognition sequence that targets the protein for ubiquitylation by the APC [18].

We fused sequences encoding the ER hormone binding domain to the 3' end of the *cdc13-des2* ORF. It had been previously shown that fission yeast cells expressing Cdc13-des2 from the medium strength nmt41 promoter are inviable when the promoter is induced [18] (Fig. 2A), but the cells are viable when the promoter is repressed. To ensure more physiological levels of Cdc13, we used the weak nmt81 promoter to regulate the expression of the Cdc13-des2-HBD fusion protein.

Expression of Cdc13-des2 or Cdc13-des2-ERHBD from the nmt81 promoter was not lethal even when the promoter was induced (Fig. 2A). However, when estradiol was added (fusion protein "active"), the cells expressing ERHBD-tagged Cdc13-des2 grew very poorly as shown by a spot test of serially diluted cells (Fig. 2A, compare "active" to "inactive"). These observations suggest that the fusion protein is indeed activated in the presence of estradiol.

Fission yeast cells expressing Cdc13-des2 from the medium strength nmt41 promoter delay at the anaphase-telophase transition [18]. To measure more accurately the activity of the Cdc13-des2-HBD fusion protein, we counted anaphase indices in the presence and absence of estradiol

and/or thiamine (Fig. 2B). Expression of Cdc13-des2 from the weak nmt81 promoter leads to a marginal increase of anaphase index, whereas expression from the medium strength nmt41 promoter brings about a pronounced anaphase delay (Fig. 2B, white bars). Interestingly, addition of estradiol to cells expressing Cdc13-des2-HBD (fusion protein "active") (Fig. 2B, shaded bar) results in an anaphase delay comparable to that in cells expressing the protein without the HBD tag from the medium strength nmt41 promoter. The anaphase index significantly increases by an hour after hormone addition, indicating a quick response, and remains high for at least one generation time (data not shown). At later timepoints cut cells were observed both with and without the ERHBD tag (data not shown). These data strongly suggest that estradiol indeed activates the Cdc13-des2-HBD fusion protein.

It is noteworthy that expression of Cdc13-des2-HBD produces a higher anaphase index and, at later timepoints after hormone addition, more cut and septated cells than expression of Cdc13-des2 from the same promoter. One possible explanation is that the expression level of Cdc13 and/or the copy number of the plasmid is affected by the presence of estradiol. However, western blot analysis of Cdc13 levels shows no increase of Cdc13 level by the presence of the hormone, nor does the tag increase the amount of the protein (Fig. 2C). We do not observe an increased amount of the endogenous Cdc13 either (Fig. 2C), which would be expected if the HBD tag was cleaved off. If there is a difference, it is that the tagged protein is present in somewhat lower amounts then the untagged protein. We considered the possibility that the HBD tag itself is responsible for the mitotic defects but we deem this most unlikely. The differences between the effects of expressing Cdc13-des2 with and without the HBD tag are quantitative, not qualitative, indicating that the tag itself does not confer a novel function on the fusion protein. Consistently, in the absence of estradiol the cells carrying the tagged construct grow like wild type cells (Fig. 2B). It is likely that a sudden increase of Cdc13 levels upon hormone addition disturbs the localization and/or function of Cdc13 and thus aggravates the effects of overexpressing a non-degradable Cdc13.

A major limitation with the use of the nmt promoter is the high background expression level even in the presence of thiamine. We wished to evaluate the effectiveness of inhibiting Cdc13-des2-HBD protein function with the HBD tag in the absence of estradiol versus repressing expression of Cdc13-des2-HBD from the nmt81 promoter in pREP82 by addition of thiamine to the growth medium. To this end we compared the anaphase indices of cells where we inhibited

Cdc13-des2-HBD protein activity by not adding estradiol (but maintained full expression from the nmt81 promoter) to that of cells where transcription from the nmt81 promoter was repressed by addition of thiamine (while the fusion protein was "active"). In the latter case (transcriptional regulation), repression of the promoter still allowed enough Cdc13-des2-HDB expression to produce an anaphase delay (see shaded bar, left panel on Fig. 2B). In contrast, when the cells expressing the fusion protein were grown in the absence of estradiol (fusion protein "inactive"), the anaphase index corresponds to that of wild type cells that do not carry the *nmt.cdc13-des* construct indicating that the fusion protein is indeed inactive (see "HBD-E", right panel on Fig. 2B). We conclude that negatively regulating Cdc13-des2 protein activity using the HBD tag results in lower background activity than regulating transcription with the nmt promoter.

Psf2-HBD

GINS is a tetrameric complex essential for the initiation and elongation steps of DNA replication [19,20]. The four subunits of GINS are essential for cell viability in budding yeast [19,20] therefore analysis of GINS function requires the isolation of conditional mutant alleles. In fission yeast temperature-sensitive alleles of the Psf2 and Psf3 subunits have been isolated and it was shown that Psf2 and Psf3 are required for DNA replication [21,22]. We explored whether the HBD could confer conditionality on the Psf2 subunit of fission yeast GINS. We fused sequences encoding the ERHDB to the 3' ends of the *psf2*$^{+}$ gene in the chromosome using the PCR-mediated gene targeting method [23]. Haploid cells expressing Psf2-ERHBD were viable when grown in the presence of estradiol in the growth medium but were inviable on medium lacking estradiol (Fig. 3A).

To determine whether the lethality was indeed due to a defect in DNA replication, a strain expressing Psf2-ERHBD was arrested in G1 by nitrogen starvation and released from the block in the presence or absence of estradiol. Cells released from the starvation block in the absence of estradiol ("inactive") only show some evidence of DNA replication at 5 h (Fig. 3B left panel), consistent with a role of Psf2 in DNA replication. Cells released from the starvation block in the presence of estradiol (fusion protein "active") carry out DNA replication 3–4 h after release (Fig 3B right panel) confirming that the fusion protein is indeed active. These data demonstrate that the ERHBD tag confers conditionality on Psf2 and the fusion protein can be activated by

estradiol. Similar results were obtained with Psf1-ERHBD (data not shown, manuscript in preparation), the activity of which is also regulated by estradiol.

A similar experiment had been performed by Gomez et al [21] using the temperature sensitive *psf2* allele, where the cells were arrested by nitrogen starvation and then released from the block at the restrictive temperature. It is interesting to note that the *psf2-HBD* allele arrests more tightly than the available $psf2^{ts}$ allele (compare fig. 5 in [21] to fig. 3B in the current paper). The mechanism of leakage at the late time-points in *psf2-HBD* is not known. Possible mechanism include release of some fusion protein from the Hsp90 complex even in the absence of estradiol or the fusion protein might be cleaved such that wild type Psf2 is produced.

We addressed the possibility that the presence of the tag might affect the stability of Psf2 and performed western blot analysis of extracts prepared from cells grown in the presence and absence of estradiol. Neither the N starvation-refeed procedure we used to synchronize the cells, nor the presence or absence of estradiol significantly affect the level of Psf2 (Fig. 3D).

To explore the reversibility of the arrest caused by inactivation of Psf2 -HBDby the absence of estradiol, cells were initially released from the N starvation block for 4 h in the absence of estradiol (fusion protein "inactive"). As shown above, the cells remain arrested with a 1C DNA content during this time (Fig 3C). After the 4-hour incubation in the absence of hormone, estradiol was added to the culture (fusion protein "active"). The cells carry out substantial DNA replication within 1 h, and replication is largely complete by 6 h (Fig. 3C) suggesting that the estradiol block is rapidly reversible.

Limitations

HO-HBD

HO is an endonuclease that initiates mating-type switching by generating a double-strand break in the DNA in budding yeast [24,25]. Since the double-strand break occurs at a specific site, its fate can be conveniently investigated at the molecular level. Therefore, HO activity is often exploited to investigate checkpoint and repair pathways. However, such studies in fission yeast are hampered by the poor regulatability of the expression of HO. Its expression from the nmt promoter leads to a gradual accumulation of double strand breaks, which are processed as they arise. Thus, a mixed population of cells is investigated at any one time during the course of such

an experiment, making it difficult to interpret the results. We therefore fused the ERHBD to the C-terminus of the HO endonuclease to test whether regulation of HO protein function by estradiol would provide a better tool to create double strand breaks in a controlled manner. We found that the HO-HBD fusion protein retains only a little endonuclease activity as compared to untagged HO, even in the presence of estradiol ("active") [see Additional files 1 and 2]. Similar result was obtained with an N terminally tagged HBD-HO fusion protein (Yari Fontebasso and Johanne Murray, personal communication).

Wee1-HBD

There are several protocols to synchronise *S. pombe* cells in different parts of the cell cycle. Induced synchronisation is often preferred over selection synchronisation because it is experimentally easier, especially for large cultures, and gives a high level of synchrony. However, induced synchronisation is usually dependent on temperature shifts which are sometimes not desirable. We sought to use Wee1 to generate synchronous cultures without involving a temperature shift. Wee1 is a protein kinase that inhibits entry into mitosis by phosphorylating Cdc2 [26,27]. Overexpressing Wee1 leads to a reversible G2 arrest. However, the currently available expression systems allow too strong expression even when *wee1* is repressed, since fission yeast cells delay in G2 and become elongated even when one extra copy of *wee1* is introduced into the cells. Therefore, long term overexpression can only be achieved if the overproduced Wee1 protein is inactive. We attempted to inactivate Wee1 by fusing it to the HBD. We fused the HBD to the C-terminus of Wee1, where the catalytic domain is located. We found that the Wee1-HBD fusion protein retains its activity in the absence of estradiol ("inactive") [see Additional files 1 and 3].

Discussion

Here we show that the estradiol-regulatable hormone-binding domain provides a means to regulate efficiently and quickly the function of some fission yeast proteins, namely Cdc13-des2 and Psf2. In contrast, the HO-HBD fusion protein retains little activity even in the absence of estradiol ("active"), while the Wee1-HBD fusion protein was active even in the absence of estradiol ("inactive").

The Hsp90 complex was highly conserved through evolution. Therefore we expected that the HBD tag might confer sensitivity to estradiol to proteins in fission yeast. Analysis of each HBD-tagged protein requires an individual assay, therefore a large-scale analysis of the regulatability of fission yeast proteins is not feasible. Since here we show that the activities of some fission yeast proteins fused to the HBD are indeed regulated by estradiol, we speculate that the mechanism of regulation is probably through binding to Hsp90, as it is in other organisms.

In those cases when the ERHBD tag confers regulatability, the rate of activation and the tightness of the "off" state favourably compare with those obtainable with currently available expression systems. Fast activation of the fusion proteins is reflected in the rapid increase of the anaphase index and swift entry into S phase after activation of Cdc13-des2-HBD and Psf2-HBD, respectively. In the absence of estradiol (fusion protein "inactive") a tight inactivation is observed in both cases; cells expressing Cdc13-des2-HBD do not delay in anaphase and *psf2-HBD* cells remain arrested with unreplicated DNA for at least one generation time.

Switch-off experiments require removal of estradiol by extensive washing, which in itself stresses the cells and might be undesirable in a physiological experiment. However, as inactivation is tight and it does not require potentially time-consuming protein degradation once estradiol is removed, we expect that the system will be usable to switch off protein function within the time-scale of one cell cycle.

Since the initial discovery that HBD-tagged heterologous proteins are subject to hormonal regulation [13] a large number of proteins from various organisms has been tagged [28]. It is difficult to predict whether a fusion protein will be regulated by the hormone. In general, the effectiveness of the system may be determined by how the Hsp90 complex is positioned relative to the key functional domains of the tagged protein. We have fused the HBD close to the kinase domain of Wee1, expecting it to be inactivated by such a fusion. Apparently, the kinase domain might not be accessible to the Hsp90 complex, since the fusion protein is active in the absence of estradiol. However, Wee1 binds Hsp90 and this interaction protects it from degradation by the proteasome [29-31]. Although it is not clear which motifs or structural elements in protein kinases are recognized by the Hsp90 chaperone, the kinase domain is a possible candidate site of interaction. Thus, estradiol might not be able to induce a conformational change that is sufficient

to override the interaction between Wee1 and Hsp90. Few endogenous Hsp90 substrates are known in fission yeast. Regulating such substrates with the HBD tag will obviously be difficult.

It is noteworthy that the proteins that were regulated by the HBD fusion and estradiol were proteins that depend on complex formation with other proteins for their function. Components of protein complexes might be more sensitive to regulation by steric interference, because complex formation may be affected. This conclusion is in line with the general trend observed in a large number of HBD fusion proteins [28]. It appears that proteins that must interact with other proteins or DNA to carry out their function, such as transcription factors or recombinases, have been successfully regulated by fusion to the HBD and estradiol presumably because their function can be inhibited by steric interference [12,13,28]. Simultaneous regulation of several components of a complex through this approach might be even more effective. On the other hand, enzymes such as β-galactosidase, galactokinase or URA3, that have small molecules as substrates, were not inactivated by steric interference by Hsp90 [13].

The classic model of steroid hormone receptor (SHR) action dictates that SHR-s are sequestered by chaperones in the cytoplasm and are released upon hormone addition. Indeed, Hsp90 is mainly cytoplasmic, but at least in some cell types it is also nuclear, especially after certain stresses [32-34]. In fission yeast Hsp90 is mainly cytoplasmic, but it is not excluded from the nucleus [35]. Localisation signals on the target protein might not be concealed by interaction with the chaperone, so different localisation signals might compete to determine the localisation of the "inactive" fusion protein. Thus, the intracellular localisation of a fusion protein in the absence of hormone is difficult to predict. After hormone addition, the localisation signals on the tagged protein are expected to determine the localisation of the fusion protein.

Conclusion

The estradiol-regulatable hormone-binding domain provides a means to regulate efficiently and quickly the function of at least some fission yeast proteins. In some cases the system provides lower background protein activity and better kinetics of regulation than currently available regulatable expression systems. Since fission yeast is a useful model organism in a number of areas of biological research, this tool will greatly facilitate research in these fields.

Methods

General fission yeast methods

General fission yeast methods and growth media were as described before [36]. Estradiol (Sigma E2758) was made as a 10 mM stock in ethanol and used at a final concentration of 125–500 nM. Nourseothricin (ClonNAT) was obtained from Werner Bioagents. Cells were grown in EMM medium with supplements as required. Thiamine was made as a 10 mg/ml stock in water and used at a final concentration of 5 μg/ml in EMM. To derepress the *nmt* promoter, the cultures were washed three times with water and reinoculated at appropriate cell density in EMM.

Plasmid and strain constructions

The strains used in this work are listed in Table 1.

pFA6-ERHBD-kanMX6

A C terminal tagging vector in the pFA6a-kanMX6 series [23] was constructed by replacing the GFP region with the HBD in pFA6a-GFP(S65T)-kanMX6 [23,37]. HBD was amplified from pHCA/GAL4(848).ER (D. Picard) as a PacI-AscI fragment using the following primers:

AAAA **TTA ATT AA**C TCT GCT GGA GAC ATG AGA GCT GCC

AAAA **GG CGC GCC**TCA GAC TGT GGC AGG GAA ACC CTC TGC

and inserted into PacI AscI digested pFA6a-GFP(S65T)-kanMX6.

Cdc13-des2-HBD, Wee1-HBD and HO-HBD

The *cdc13-des2, wee1* and *HO* genes were amplified by PCR with Nde1 site introduced at START and Pac1 site introduced upstream of STOP using the primers shown in Table 2 (the sequences for the introduced sites are underlined): The PCR products were cut with Nde1 and Pac1.

HBD was isolated as a Pac1 – Asc1 fragment from pFA6-ERHBD-kanMX6. The pREP82 plasmid was cut with Sma1 and Asc1 linker was inserted. The Nde1 and Pac1 cut *cdc13-des2*, *wee1* and *HO* PCR products were ligated with the HBD into Nde1 Asc1 cut pREP82-Asc1.

Psf2-HBD

In order to tag the *psf2*$^{+}$ gene in the chromosome, the PCR-mediated gene targeting method for fission yeast [23] was used with plasmids pFA6a-HBD-kanMX6 and pFA6a-HBD-natMX6 as templates, the latter being constructed by transferring the PacI-AscI HBD fragment from the former into pFA6a-GST-natMX6 [38]. The primers used for amplification are shown below. Sequences with identity to the template plasmid are underlined.

PSF2-CTAG-5 5'-TGGAAATTAACGAAATACGTCCTATATTTCGAGAG GTGATGGACAGAATGCGCAAAATTGTTCAAGTTTCCCAAGAAGAACGGATCCCCGG GTTAATTAA-3'

PSF2-CTAG-3 5'-ATTTCACTACTACAAAGTTGGTATTCATAAACACTT CGTAGGATTCATTATCATTATTTTTAAAGTACATCATCCACACGGAATTCGAGCTCGT TTAAAC-3'

The resulting PCR products (5–10 μg) were transformed into S. pombe h^{-N} and h^{+S} strains as described and transformants selected with either 100 μg/ml G418 or 100 μg/ml nourseothricin [23,38]. Transformants were then screened by PCR to confirm that the gene was successfully tagged. The sequences of the PCR primers used for this can be obtained from the authors on request.

Flow cytometry

Was performed using SYTOX Green as described previously [8].

Immunoblots

Cell extracts were made by the TCA protein extraction method [39]. Detection was performed using the ECF or ECL kits (Amersham Biosciences).

Authors' contributions

CAB made and characterized the HO-ERHBD construct under the guidance of BG. IG tagged the *psf2* gene in the chromosome and performed the initial analysis of the tagged strain under the direction of SAM. CCP characterized the *psf-ERHBD* strain under the direction of SK. JS constructed the pFA6-ERHBD-kanMX6 plasmid under the guidance of MT. HCS constructed the *nmt82.cdc13-des2-ERHBD* plasmid and performed the initial characterization of the

construct under the guidance of BG. EB, SK, SAM and MT contributed to writing the manuscript and designing experiments. BG constructed and characterized the *nmt82.wee1-ERHBD* plasmid, completed the characterization of *nmt82.cdc13-des2-ERHBD* and wrote the manuscript.

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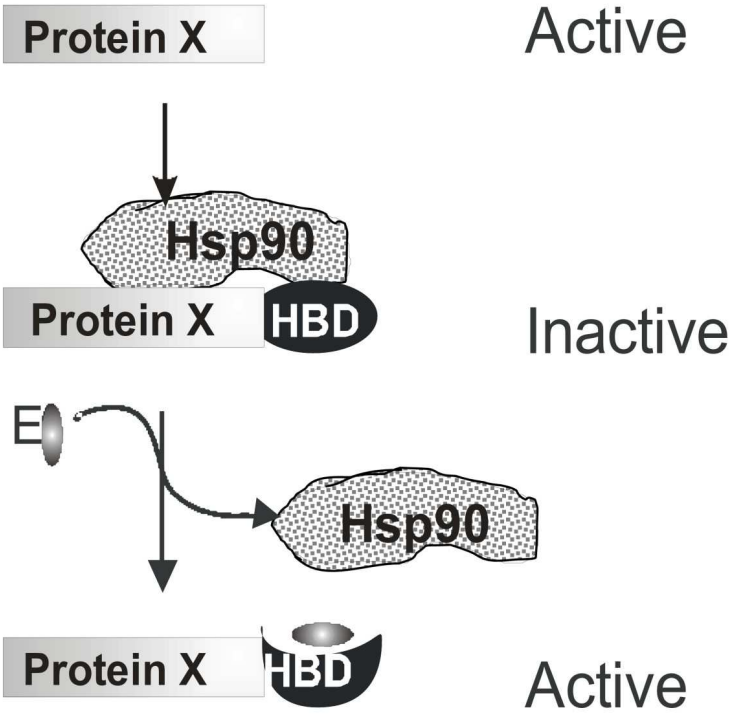
Figure Legends

Figure 1. The principle of regulating protein function by estradiol. See text for details.

Figure 2. The activity of Cdc13-des2-HBD is regulated by estradiol. **A**, Cells transformed with plasmids carrying the nmt.cdc13-des2 and nmt.cdc13-des2-HBD constructs were serially diluted (4X) and plated onto minimal plates with and without thiamine and estradiol as indicated. The cells carrying the ERHBD tagged cdc13-des2 growing poorly in the presence of estradiol are highlighted with a white rectangle. **B**, Expression of Cdc13-des-HBD results in anaphase delay in the presence of estradiol. The nmt promoter was induced for 20 h before addition of estradiol for 1 h. Anaphase index is shown before and 1 h after addition of estradiol. Bars show anaphase indices in the presence (left panel) and absence (right panel) of thiamine. Anaphase index observed in wild type cells is shown for comparison. The bar representing the tagged construct in the presence of estradiol is shaded. **C**, Cdc13 levels are not increased by the presence of the tag or estradiol. Cells carrying the nmt81.cdc13-des2 and nmt81.cdc13-des2-HBD plasmids were grown in minimal medium in the presence of thiamine, then thiamine was washed out to induce the nmt promoter. Estradiol was added to half of the cultures after 20 h induction. Samples for protein extracts were taken at the indicated times. TCA extracts were made and western blot analysis was performed using the SP4 anti-Cdc13 antibody [40] and the anti-PSTAIRE (Santa Cruz) antibody to detect Cdc2 which serves as loading control.

Figure 3. Psf2-HBD is inactivated in the absence of estradiol. **A**, *psf2-HBD* and wild type cells were serially diluted and spotted onto YE plates containing estradiol at the indicated concentrations. **B**, Strain P1520 (*psf2-HBD:kanMX*6) was grown in EMM plus 125 mM estradiol to log phase then shifted to EMM-N (+estradiol) for 16 at 25°C. Cells were released from the block by transferring to EMM+N in the absence (left) or presence (right) of estradiol. **C**, As in A, except that cells were released into EMM+N in the absence of estradiol, and 125 nM estradiol was added at 4 h. **D**, Cell extracts were made using the TCA method from cells incubated with and without estradiol as indicated. The extracts were run on protein gels and Psf2 was detected using an antibody against the YFP tag.

Figure 1.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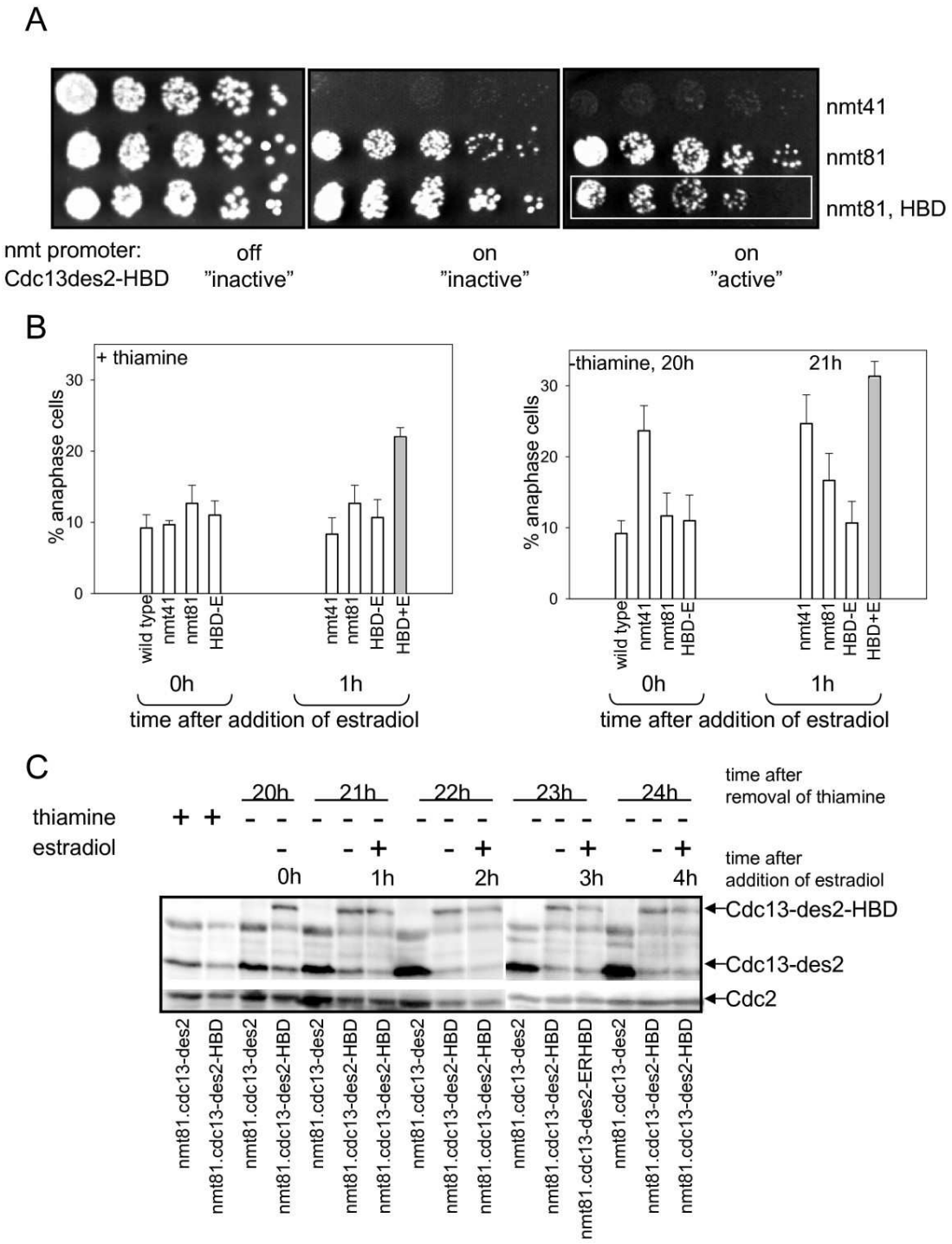
Hsp90
Protein X
HBD
Inactive
E
Hsp90
Protein X
HBD
Active

Figure 2.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nmt41
nmt81
nmt81, HBD
nmt promoter:
Cdc13des2-HBD
off
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on
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on
”active”
B
+ thiamine
% anaphase cells
wild type
nmt41
nmt81
HBD-E
HBD+E
0h
1h
time after addition of estradiol
-thiamine, 20h
21h
C
time after
removal of thiamine
thiamine
estradiol
20h
21h
22h
23h
24h
time after
addition of estradiol
Cdc13-des2-HBD
Cdc13-des2
Cdc2
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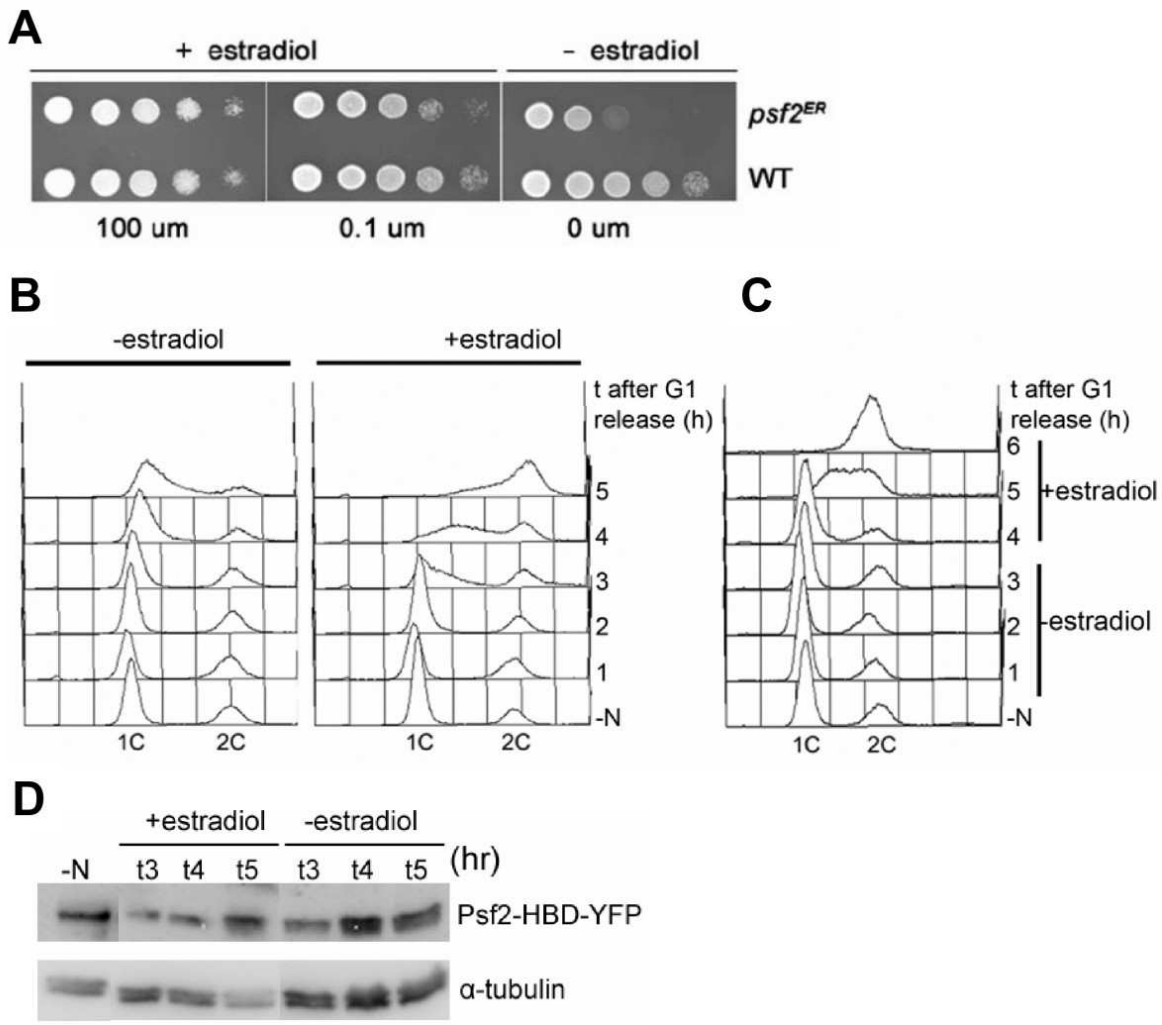
nmt81.cdc13-des2-HBD
nmt81.cdc13-des2-ERHBD

Figure 3.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psf2ER
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4
3
2
1
-N
1C
2C
C
t after G1 release (h)
6
5
4
3
2
1
-N
+estradiol
-estradiol
1C
2C
D
+estradiol
-estradiol
-N
t3
t4
t5
t3
t4
t5
(hr)
Psf2-HBD-YFP
α-tubulin

Tables.

Table 1

Strains used in this study

Strain	Carrying the plasmid
ade6-M210 leu1-32 h-	cdc13-des2-pREP41
	cdc13-des2-pREP81
ade6-M210 ura4-D18 h+	cdc13-des2-ERHBD-pREP81
	HO-ERHBD-pREP81
	wee1-ERHBD-pREP81
psf2-ERHBD:kanMX6 h+	
psf2-ERHBD:YFP:kanMX6 h-	

Table 2

Primers used for plasmid construction

	Primers	Template
cdc13-des2-HBD:	TCCTC**CATATG**ACTACCCGT	pREP81-cdc13-des2
	A CAC TAA A**TT AAT T**AA CCA TTC	
wee1-HBD:	GGAATTC**CATATG**AGCTCTTCTTCTAATAC	Genomic
	C C**TT AAT TAA**AAC ATT CAC CTG CCA ATC TT	
HO-HBD:	GGAATTC**CATATG**CTTTCTGAAAACACGAC	Genomic
	C C**TT AAT TAA**GCA GAT GCG CGC ACC TGC GT	

Appendix 2: Role of the TORC1 effectors Sch9 and Sfp1 in ribosome biogenesis and cell size control

Jorgensen, P., Rupes, I., Sharom, J.R., Schneper, L., Broach, J.R., Tyers, M. (2004). A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes Dev* **18**, 2491-2505.

A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size

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Abstract

Cell-size homeostasis entails a fundamental balance between growth and division. The budding yeast *Saccharomyces cerevisiae* establishes this balance by enforcing growth to a critical cell size prior to cell cycle commitment (Start) in late G1 phase. Nutrients modulate the critical size threshold, such that cells are large in rich medium and small in poor medium. Here, we show that two potent negative regulators of Start, Sfp1 and Sch9, are activators of the ribosomal protein (*RP*) and ribosome biogenesis (*Ribi*) regulons, the transcriptional programs that dictate ribosome synthesis rate in accord with environmental and intracellular conditions. Sfp1 and Sch9 are required for carbon-source modulation of cell size and are regulated at the level of nuclear localization and abundance, respectively. Sfp1 nuclear concentration responds rapidly to nutrient and stress conditions and is regulated by the Ras/PKA and TOR signaling pathways. In turn, Sfp1 influences the nuclear localization of Fhl1 and Ifh1, which bind to *RP* gene promoters. Starvation or the absence of Sfp1 causes Fhl1 and Ifh1 to localize to nucleolar regions, concomitant with reduced *RP* gene transcription. These findings suggest that nutrient signals set the critical cell-size threshold via Sfp1 and Sch9-mediated control of ribosome biosynthetic rates.

[*Keywords*: Ribosome, nutrients, nucleolus, size, Start]

Cell-size homeostasis requires that proliferating cells coordinate growth and the cell cycle, such that each division is matched by a doubling of mass. Cell growth and the cell cycle are, however, separate processes that can be uncoupled, either by endogenous developmental mechanisms, for instance, during oogenesis and the rapid cleavage cycles following fertilization, or within the laboratory. In eukaryotic cells, doubling time is usually limited, not by the time required to duplicate and divide the genome, but rather by the time required to double cell mass. Consistently, cell cycle progression is typically dependent on cell growth. In contrast, mass accumulation continues unabated during most cell cycle arrests (Saucedo and Edgar 2002).

In the unicellular budding yeast, growth and the cell cycle are coupled at Start, the short interval during late G1 phase after which cells are committed to division (Hartwell et al. 1974). Passage through Start has several requirements as follows: (1) growth to a critical cell size (Johnston et al. 1977), (2) nutrient sufficiency (Hartwell et al. 1974), (3) attainment of a critical translation rate (Hartwell and Unger 1977; Moore 1988), and for haploids, (4) absence of mating pheromone (Hartwell et al. 1974). The first three conditions are likely to be interrelated. The critical size requirement and minimum translation rate explain why slowing growth rate increases the length of G1 phase, whereas the time required to transit the rest of the cell cycle is relatively constant (Hartwell and Unger 1977). The critical-size threshold maintains uniform cell size over many generations, and under minimal nutrient conditions forces cells to accumulate the energy stores required to complete the division cycle. At constant nutrient levels, the critical-size requirement couples growth and division only in daughter cells, as mother cells by definition have already attained critical size.

Start depends on activation of the SBF and MBF transcription factor complexes that bind the promoters of G1/S-regulated genes (Nasmyth 1996). SBF and MBF are composed of related DNA-binding proteins Swi4 and Mbp1, respectively, which interact with a common regulatory subunit Swi6 to drive expression of a massive suite of ~200 genes. Among these, the key transcripts are the G1 cyclins *CLN1* and *CLN2* and the B-type cyclins *CLB5* and *CLB6*. Cln1 and Cln2 activate Cdc28, the cyclin-dependent kinase (CDK) that controls all cell cycle transitions in budding yeast. Cln1/2–Cdc28 phosphorylation events trigger bud emergence and inactivate Sic1 and Cdh1, two key inhibitors of B-type cyclin–Cdc28 activity. Once unleashed, Clb5/6–Cdc28 complexes initiate DNA replication (Nasmyth 1996). Start can therefore be viewed as the short

interval required to accumulate sufficient Cln1/2–Cdc28 activity to phosphorylate Cdh1 and Sic1, and perhaps other substrates.

The connection between cell size and SBF/MBF activation remains enigmatic. It has recently been discovered that SBF, and likely MBF, activation occurs upon dissociation of the Start-repressor Whi5 (Jorgensen et al. 2002; Costanzo et al. 2004; de Bruin et al. 2004). Loss of the interaction between SBF/MBF and Whi5, which correlates with movement of Whi5 to the cytoplasm, is driven by phosphorylation of Whi5 by Cln3–Cdc28 (Costanzo et al. 2004; de Bruin et al. 2004). Cln3 appears to act in parallel to Bck2, a poorly understood activator of SBF/MBF, as cells lacking *CLN3* and *BCK2* are inviable due to permanent G1 arrest (Wijnen and Futcher 1999). Remarkably, deletion of *WHI5* bypasses this arrest (Costanzo et al. 2004; de Bruin et al. 2004). Because Start activation is highly sensitive to Cln3 dosage, it has been presumed that a critical concentration of Cln3–Cdc28 activity triggers Start. Although Cln3 protein and Cln3–Cdc28 kinase activity do not increase during G1 phase (Tyers et al. 1993; McInerny et al. 1997), a size-dependent increase in Cln3 nuclear abundance may help activate Start (Futcher 1996; H. Wang et al. 2004).

Nutrients modulate the critical cell-size threshold in proportion to the proliferation rate (Johnston et al. 1979; Lorincz and Carter 1979; Tyson et al. 1979). The effects of nutrients on critical cell size are conveyed rapidly, as cells are not committed to Start at a given size threshold until just before the threshold is reached (Lorincz and Carter 1979). Thus, shifting cells from poor to rich medium temporarily increases the fraction of unbudded G1-phase cells as these cells grow to the new threshold (Johnston et al. 1979). Nutrient effects are mediated in part by the Ras/PKA pathway; decreased Ras/PKA signaling, as in a *cdc25-1* strain, decreases critical cell size, whereas hyperactive Ras/PKA signaling, as in a $RAS2^{Val19}$ strain, increases critical cell size (Baroni et al. 1989). Strains that exhibit constitutive PKA activity do not adjust cell size in response to carbon source quality (Tokiwa et al. 1994).

Ribosome biogenesis is a chief occupation of growing cells, accounting for >50% of total transcription in yeast and mammalian cells (Warner 1999; Moss and Stefanovsky 2002). In yeast, the rate of ribosome synthesis is dictated by the rate of transcription of the RNA and protein subunits of the ribosome (Warner 1999). rRNA and *RP* gene transcription is exquisitely sensitive to the growth potential of the cell and is rapidly repressed in response to a wide variety

of internal and external stresses (Ju and Warner 1994; Warner 1999; Gasch et al. 2000). The 137 *RP* genes, referred to here as the *RP* regulon, are tightly coregulated. The vast majority of genes in the *RP* regulon have promoter-binding sites for Rap1, whereas a few have sites for Abf1 (Warner 1999). As Rap1 silences telomeric repeats but activates glycolytic and *RP* genes (Warner 1999), its activity depends on context, presumably generated by combinations of associated co-factors. The *RP*-specific transcription factors that cooperate with Rap1 have not yet been identified.

A large number (>200) of other genes, which we term the *Ribi* regulon, show nearly identical transcriptional responses as *RP* genes to environmental or genetic perturbations (Gasch et al. 2000; Hughes et al. 2000; Wade et al. 2001; Jorgensen et al. 2002; Miyoshi et al. 2003). The promoters of these coexpressed genes are strongly enriched for the presence of two motifs, termed RRPE and PAC, and therefore appear to constitute a distinct regulon (Gasch et al. 2000; Hughes et al. 2000; Wade et al. 2001; Jorgensen et al. 2002). Most of these genes encode proteins involved in ribosome biogenesis, a process involving more than 100 accessory factors that assemble and modify rRNA and RPs in the nucleolus (Hughes et al. 2000; Wade et al. 2001; Fatica and Tollervey 2002; Jorgensen et al. 2002). A number of additional functional categories are present in this regulon, including subunits of RNA Polymerase I and III, enzymes involved in ribo-nucleotide metabolism, tRNA synthetases, and translation factors (Gasch et al. 2000; Wade et al. 2001; Jorgensen et al. 2002). The *Ribi* regulon thus consists of non-RP genes that boost translational capacity. Two central nutrient-signaling conduits, the Ras/PKA and TOR signaling pathways, can activate rRNA, *RP*, and *Ribi* transcription (Klein and Struhl 1994; Neuman-Silberberg et al. 1995; Cardenas et al. 1999; Hardwick et al. 1999; Powers and Walter 1999; Y. Wang et al. 2004). Of these, the control of rRNA synthesis is best understood, as it is known to depend largely on phosphorylation of the critical initiation factor TIF-1A/Rrn3 (Grummt 2003). Both Rap1-binding sites and RRPE elements can render gene transcription sensitive to Ras/PKA signaling, although the presumed kinase targets have not been identified (Klein and Struhl 1994; Neuman-Silberberg et al. 1995; Y. Wang et al. 2004).

Systematic determination of cell-size distributions for all yeast deletion strains has recently identified many new potential Start regulators (Jorgensen et al. 2002; Zhang et al. 2002). Many of the genes that encode potential Start repressors are implicated in ribosome biogenesis, suggesting not only a link between these two seemingly disparate systems, but also that cell-size

mutants may identify new regulators of ribosome synthesis (Jorgensen et al. 2002). Beginning with the most potent size regulators, Sfp1 and Sch9, we have elaborated a dynamic transcriptional network that dictates the ribosome synthesis rate and the critical cell-size threshold.

Results

Sfp1 and Sch9 strongly influence cell size

Of all nonessential genes, deletions in either *SFP1* or *SCH9* caused the greatest decrease in cell size (Whi phenotype). These deletion strains also proliferate slowly and accumulate cells in G1 phase, suggesting *SFP1* and *SCH9* are important for mass accumulation (Fig. 1A) (Jorgensen et al. 2002). Sfp1 is a putative transcription factor with a split zinc-finger domain at its C terminus that is essential for function (Fingerman et al. 2003). Sch9 is an AGC family kinase that possesses a C2 lipid-binding domain and is the closest yeast homolog of the metazoan pro-survival kinase Akt/PKB (Fabrizio et al. 2001; Jorgensen et al. 2002). Sch9 kinase activity is required for its function (data not shown). To conditionally overexpress Sch9, we constructed a strain bearing a galactose-inducible allele integrated at the endogenous locus (*GAL1–SCH9*). Like *SFP1*, overexpression of *SCH9* caused a large cell phenotype, but unlike *SFP*1, *SCH9* overexpression was not toxic (Fig. 1A). To enable conditional control of Sch9 activity at endogenous levels without confounding nutrient source shifts, we engineered an analog-sensitive (as) allele of *SCH9* at the endogenous locus ($sch9^{as}$). Substitution of a bulky amino acid with either alanine or glycine in the ATP-binding pocket of kinases often permits highly specific inhibition by analogs of the cell-permeable kinase inhibitor PP1, such as C3-1′-naphthyl-methyl PP1 (1NM-PP1) (Bishop et al. 2000). Unexpectedly, the integrated $sch9^{as}$ allele separated the proliferation and size functions of *SCH9*, conferring a nearly wild-type proliferation rate, but a strong Whi phenotype (Fig. 1B). $Sch9^{as}$ was almost fully inhibited in vivo by 100 nM 1NM-PP1, as determined by effects on doubling time and cell size (Fig. 1B). At this concentration, 1NM-PP1 had no effect on wild-type cells (data not shown) (Bishop et al. 2000).

Sfp1 and Sch9 activate the RP and Ribi regulons

We have previously shown that Sfp1 activates the *RP* and *Ribi* regulons (Jorgensen et al. 2002; Fingerman et al. 2003). Further expression profiles of *GAL1–SFP1* and *sfp1*Δ/Δ strains

elaborated the *Ribi* regulon to 236 genes (Fig. 1C; Supplementary Table 1). We next asked whether Sch9 also controls these genes. Addition of 100 nM 1NM-PP1 resulted in a rapid repression (<30min) of the *RP* regulon (~2.5-fold) and the *Ribi* regulon (~1.5-fold) in an $sch9^{as}$ strain, followed by a slight but reproducible recovery of both regulons (Fig. 1C). These transcriptional effects preceded effects of 1NM-PP1 on proliferation rate and cell size (Fig. 1B). Northern analysis confirmed that representative members of the *RP* (*RPP0, RPL11B*) and *Ribi* (*NOP1, NSR1*) regulons were induced upon expression of *GAL1–SCH9* (Fig. 1D). Sfp1 and Sch9 are thus both required for maximal *RP* and *Ribi* expression. However, inhibition of $sch9^{as}$ also induced 38 genes not altered in *sfp1*Δ/Δ cells (Fig. 1E), almost all of which were strongly induced as cultures deplete glucose and enter stationary phase (DeRisi et al. 1997). Many stationary phase genes are also repressed by Ras/PKA, consistent with the genetic placement of Sch9 parallel to Ras/PKA (Toda et al. 1988).

Sfp1 and Sch9 are negative regulators of Start

A number of observations suggested that Sfp1 and Sch9 might regulate the critical cell size threshold at Start. First, *sfp1*Δ and *sch9*Δ cells are much smaller than other deletion mutants with the same proliferation rate (Jorgensen et al. 2002). Second, the hypomorphic alleles $sfp1^{MYC13}$ (Jorgensen et al. 2002) and $sch9^{as}$ caused marked decreases in cell size with little increase in doubling time, similar to mutations in canonical Start regulators (Fig. 1A,B). Third, increasing the doubling time of wild-type cells to that of *sfp1*Δ or *sch9*Δ cells by treatment with sublethal doses of cycloheximide reduced the size of new-born daughter cells, but not average cell size (Fig. 2A). We directly tested the role of Sfp1 and Sch9 at Start by synchronous release of G1-phase daughter cells obtained by centrifugal elutriation. The slow rate at which daughter cells lacking Sfp1 or Sch9 activity accumulated volume underscored the role of these gene products in cell growth (Fig. 2B). All aspects of Start, including the activation of SBF- and MBF-dependent transcription, bud emergence, and DNA replication initiation were accelerated with respect to cell size in strains that lacked Sfp1 or Sch9 activity (Fig. 2C–G). For example, whereas wild-type cells expressed the MBF-regulated gene *RNR1* at a size of ~30 fL, cells that lack Sfp1 or Sch9 activity do so at sizes of ~16 fL and ~21 fL, respectively. These results indicate that Sfp1 and Sch9 lie upstream of the G1/S transcriptional machinery. In contrast, treatment of wild-type cells with a sublethal dose of cycloheximide that slowed growth to the same extent as loss of Sch9

actually delayed Start, as measured by all of the above parameters (Fig. 2B–D,H). We note that the absence of Sfp1, and to a lesser extent Sch9, caused considerable heterogeneity in traversal through Start, as shown by the extended window of G1/S transcription and the slow accumulation of cells with buds and G2 DNA content. This effect may arise from a reduced translation rate, as cycloheximide-treated wild-type G1 phase cultures behaved in a similar manner. The heterogeneity in Start progression is not, however, caused by heterogenous cell growth rate (Fig. 2D).

Strains lacking SFP1 or SCH9 are impervious to carbon source effects on cell size

If Sfp1 and Sch9 mediate the effects of nutrients on the critical cell size threshold, strains lacking these gene products should be largely impervious to nutrient modulation of cell size. The size distributions of *sfp1*Δ/Δ and *sch9*Δ/Δ diploid cells were, in fact, barely reduced on poor carbon sources, unlike wild-type and *whi5*Δ/Δ cells (Fig. 3A,B). *sfp1*Δ/Δ cells appeared to be at near minimal cell size, as overexpression of the hyperactive *CLN3-1* allele had little impact on the mean size of these cells (Fig. 3B). Importantly, *sfp1*Δ and *sch9*Δ strains proliferated much more slowly than wild type on all carbon sources examined (data not shown), unlike strains that lack the large ribosomal subunit *RPL11B* gene, which is only rate-limiting for growth when cells are in glucose medium (Zhao et al. 2003).

In contrast to *sfp1*Δ and *sch9*Δ cells, the size of *cln3*Δ *bck2*Δ *whi5*Δ cells was fully responsive to carbon source (Fig. 3C,D). Introduction of *sfp1*Δ into the *cln3*Δ *bck2*Δ *whi5*Δ background rendered cells very small, but not as small as the *sfp1*Δ mutation alone (Fig. 3C,D). Because *sfp1*Δ is not fully epistatic for cell size to the triple *cln3*Δ *bck2*Δ *whi5*Δ mutant or to any of the single mutants (data not shown), Whi5, Cln3, and Bck2 must still play a role in *sfp1*Δ cells. These results argue that nutrient signaling, via Sfp1 and Sch9, influences the activation of SBF and MBF by a mechanism independent of known upstream regulators.

Sch9 abundance and phosphorylation are altered by nutrient signals

As the *RP* and *Ribi* regulons respond dynamically to the environment, we examined Sfp1 and Sch9 abundance and localization under different nutrient and stress conditions using functional fusions to epitope tags and GFP variants. In log phase, ^{GFP}Sch9 localized throughout the cell, but was unexpectedly enriched at the vacuolar membrane (Fig. 4A). We note that a C-terminal

Sch9^{GFP} fusion protein was not functional (data not shown). ^{GFP}Sch9 localization was dynamic, as the vacuolar membrane signal largely disappeared following carbon starvation (Fig. 4B,C). ^{GFP}Sch9 localization was not altered by different carbon sources or by rapamycin (data not shown). The abundance of ^{HA3}Sch9 was, however, dependent on TOR activity as all forms, and in particular ^{HA3}Sch9 phospho-isoforms, decreased upon rapamycin treatment (Fig. 4D,E). Under steady-state proliferation on different carbon sources, ^{HA3}Sch9 abundance correlated with cell growth rate, *RP/Ribi* transcription, and cell size (Fig. 4D) (Gasch et al. 2000).

Sfp1 nuclear concentration is regulated by multiple nutrient and stress signals

As expected for a transcription factor, Sfp1^{YFP} was localized predominantly to the nucleus in growing cells (Fig. 5A). Strikingly, Sfp1^{YFP} rapidly (~5 min) relocalized to the cytoplasm upon exposure to a broad range of stresses. Cytoplasmic relocalization was also observed with a ^{GFP}Sfp1 fusion protein (data not shown). Quantitation of the Sfp1^{YFP} nuclear:cytoplasmic (N:C) ratio revealed that under some conditions (carbon starvation, oxidative stress), Sfp1^{YFP} became uniformly distributed (i.e., an N:C ratio of unity), whereas in others (rapamycin, tunicamycin, nitrogen starvation), a greater fraction of Sfp1^{YFP} was retained in the nucleus (Fig. 5A,B). Neither the abundance nor the electrophoretic mobility of Sfp1^{MYC13} was altered upon carbon starvation or rapamycin treatment (Fig. 5C). Sfp1^{YFP} relocalization correlated temporally with *RP/Ribi* repression as carbon starvation, nitrogen starvation, rapamycin, and H_20_2 treatment all provoked rapid Sfp1^{YFP} export and *RP/Ribi* gene repression (Fig. 5B,D). Because most *RP* and *Ribi* transcripts are highly unstable, their abundance precisely mirrors transcription rate (Warner 1999; Grigull et al. 2004). An exception to this rapid response was secretory pathway stress induced by tunicamycin, which is known to gradually inhibit *RP* gene expression by a PKC-dependent pathway (Li et al. 2000). Correspondingly, tunicamycin caused a gradual decline in both nuclear Sfp1^{YFP} and *RP/Ribi* transcription (Fig. 5B) (Miyoshi et al. 2003). In log phase proliferation on different carbon sources, the extent of Sfp1^{YFP} nuclear localization was also proportional to cell-growth rate, *RP/Ribi* expression, and cell size (Fig. 5E) (Gasch et al. 2000).

Importantly, Sfp1 localization responded quickly to improved nutrient conditions. Addition of glucose to stationary phase cells rapidly (~5 min) increased the nuclear concentration of Sfp1^{YFP} (Fig. 5F). This nuclear import of Sfp1^{YFP} occurred even in the presence of a lethal concentration of cycloheximide, and so did not require protein synthesis (Fig. 5F). In fact, cycloheximide

treatment of cells in suboptimal raffinose medium rapidly increased the nuclear concentration of Sfp1^{YFP}, but had no effect on Ste12^{YFP}, an unrelated transcription factor (Fig. 5G; data not shown). Sfp1 may thus effect the compensatory feedback loop that induces *RP* transcription within 15 min of cycloheximide treatment (Cardenas et al. 1999).

Sfp1 localization also depended on TOR and Ras/PKA activity. The rapamycin-resistant *TOR1-1* allele blocked Sfp1^{YFP} nuclear depletion in response to rapamycin (Fig. 5H). However, the partial nuclear retention of Sfp1^{YFP} after rapamycin treatment of wild-type cells argues that repression of the TOR pathway does not suffice to explain the complete relocalization upon carbon starvation (Fig. 5B). A *tpk1^{wimp}* strain, which has weak constitutive PKA activity (Cameron et al. 1988), had reduced nuclear Sfp1^{CFP} (Fig. 5I), whereas overexpression of a hyperactive *RAS2^{Val19}* allele stimulated nuclear accumulation of Sfp1^{CFP} (Fig. 5J) and *RP/Ribi* induction with the same kinetics (Y. Wang et al. 2004). However, because a *tpk1^{wimp}* strain still relocalized Sfp1^{CFP} upon carbon starvation, diminished Ras/PKA signaling was also not sufficient to account for the totality of Sfp1^{CFP} export (Fig. 5I). Likewise, a high level of Ras/PKA activity caused by either of the hyperactive *GAL10–RAS2^{Val19}* or *GPA2^{Ala273}* alleles could not drive Sfp1 into the nucleus of carbon-starved cells (data not shown). Deletion of Snf1, an AMP-activated kinase that signals many stress responses, also had no effect on Sfp1 relocalization upon carbon starvation (data not shown). Multiple signals must therefore regulate nuclear localization of Sfp1. We note that Sfp1 cannot completely account for the pronounced effect of TOR and Ras/PKA signaling on *RP* transcription, as loss of Sfp1 activity causes an approximately threefold repression of *RP* genes as opposed to the >10-fold decreases caused by loss of TOR or PKA activity (Fig. 1C) (Neuman-Silberberg et al. 1995; Powers and Walter 1999).

Sfp1 and Sch9 act in parallel

Consistent with the above Sfp1 localization results, *sfp1*Δ strains are sensitive to cycloheximide (Fingerman et al. 2003) and to decreases in Ras/PKA and TOR signaling (Fig. 6A), arguing that these pathways regulate additional activators of *RP* and *Ribi* expression. The incomplete overlap in expression profiles (Fig. 1E), lack of genetic epistasis (Fig. 6B), and the inviability of the *sfp1*Δ *sch9*Δ double mutant indicate that Sfp1 and Sch9 act in parallel pathways (Jorgensen et al. 2002). Consistently, inhibition of *sch9^{as}* did not affect the localization of Sfp1^{CFP} (data not

shown). However, because both *sfp1*Δ and *sch9*Δ strains have substantial growth defects, the inviability of the double mutant might simply reflect nonspecific additive growth defects. To precisely modulate Sfp1 function, we constructed an $sfp1^{ER}$ allele in which the genomic *SFP1* sequence is fused to the ligand-binding domain of the estrogen receptor (ER). In the absence of the natural ligand β-estradiol, the ER domain is sequestered in an inactive complex with Hsp90 (Mattioni et al. 1994). The activity of $Sfp1^{ER}$ in vivo exhibits a smooth dose dependence on β-estradiol concentration (Fig. 6C). To quantitatively assess genetic synergy, an $sfp1^{ER}$ $sch9^{as}$ double-mutant strain was challenged with increasing concentrations of β-estradiol and 1NM-PP1, in effect applying overlapping gradients of Sfp1 and Sch9 activity. Severe proliferation defects were observed at combined concentrations of β-estradiol and 1NM-PP1 that individually caused little defect, consistent with a parallel role for each in *RP/Ribi* transcription (Fig. 6D). As Sfp1 and Sch9 both appear to be downstream of TOR signaling (Figs. 4D, 5A,H), their combined action may account for the TOR requirement in *RP/Ribi* gene expression.

Sfp1 appears to act at RP promoters via Fhl1 and Ifh1

Genome-wide chromatin immunoprecipitation (ChIP) analysis of many potential transcriptional regulators in yeast, including Sfp1, has been recently reported (Lee et al. 2002). Of the 211 intergenic regions most enriched in Sfp1 complexes at $p < 0.02$, 42 corresponded to *RP* genes, a proportion not due to chance ($p < 10^{-14}$). But, the less than twofold promoter enrichment in the reported ChIP data for Sfp1 (Lee et al. 2002), and our own ChIP analysis (data not shown), suggests that the Sfp1–*RP* promoter interaction is at the limit of detection. Sfp1–*Ribi* promoter interactions have not yet been detected by ChIP (Lee et al. 2002; Fingerman et al. 2003; P. Jorgensen and M. Tyers, unpubl.). To determine how Sfp1 and Sch9 might regulate *RP* promoters, we examined two novel transcription factors that also bind to these promoters, the forkhead/FHA domain protein Fhl1 and the zinc-finger protein Rgm1 (Lee et al. 2002). Fhl1 and a genetically interacting factor, Ifh1, are required for an unknown aspect of ribosome synthesis, suggesting that they may be activators of *RP* transcription (Hermann-Le Denmat et al. 1994; Cherel and Thuriaux 1995).

We detected several strong genetic interactions within this network of putative *RP* gene regulators. In light of the severe proliferation defects of *sfp1*Δ and *fhl1*Δ strains, we were surprised to find that a *sfp1*Δ *fhl1*Δ double-mutant strain proliferated as well as a *fhl1*Δ strain; in

contrast, *sch9*Δ caused additive defects with *fhl1*Δ (Fig. 7A). However, deletion of *FHL1* did not cause an obvious cell size phenotype (Fig. 7B). The *sfp1*Δ size defect was epistatic to *fhl1*Δ as the *sfp1*Δ *fhl1*Δ double mutant was as small as the *sfp1*Δ single mutant (Fig. 7B). The control of Sfp1 over cell size may thus be distinct from its control over the *RP* regulon. Conversely, the colony size of an *sch9*Δ, but not an *sfp1*Δ strain was severely compromised by an allele of IFH1 (*ifh1*^{ER}) and by *rgm1*Δ, neither of which alone caused noticeable defects (Fig. 7C; data not shown).

These genetic interactions suggested that Sfp1 might influence *RP* transcription via Fh11 and Ifh1. To investigate Fhl1 and Ifh1 regulation, we created various C-terminal epitope tag or GFP variant fusions by integration of fusion cassettes at the genomic *FHL1* and *IFH1* loci, all of which provided full function in vivo. We first recapitulated the association of Fhl1^{HA3} with *RP* promoters by ChIP analysis; as controls, Fhl1 did not locate to another highly active promoter regulated by Rap1 (*PGK1*) or to *Ribi* promoters (Fig. 7D). We then determined that Ifh1^{MYC13} also bound specifically to *RP* promoters (Fig. 7D). The association of Ifh1^{MYC13} and Fhl1^{HA3} with *RP* promoters was reduced in *sfp1*Δ cells by approximately fourfold and approximately twofold, respectively (Fig. 7E). Neither the *sfp1*Δ mutation nor carbon starvation detectably altered Ifh1^{MYC13} or Fhl1^{HA3} abundance or electrophoretic mobility (data not shown).

Sfp1 dramatically influenced the localization of Ifh1 and Fhl1. In wild-type cells in glucose medium, Ifh1^{CFP} and Fhl1^{YFP} were predominantly nuclear, but excluded from the nucleolus (Fig. 8A,B). In *sfp1*Δ strains, however, a high percentage of cells showed striking enrichment of Ifh1^{CFP} and Fhl1^{YFP} in a subnuclear focus that corresponded to the nucleolus, as shown by colocalization with Bud21^{YFP} or Bud21^{CFP} (Fig. 8A,B). These foci were not observed in strains lacking Sch9 activity or after treatment with a sublethal concentration of cycloheximide (Fig. 8C; data not shown), indicating that reduced growth or translation rate was not sufficient to induce nucleolar relocalization. We then examined whether Ifh1^{CFP} and Fhl1^{YFP} changed localization in response to a nutrient stress that causes Sfp1 to exit the nucleus. Upon carbon starvation, Ifh1^{CFP} rapidly infiltrated the nucleolus of most cells (Fig. 8D,E). Likewise, in most cells, almost all Fhl1^{YFP} relocalized near the nucleolus after carbon-source depletion (Fig. 8F,G). As controls, two unrelated transcription factors, Ste12^{YFP} and Rst1^{YFP}, did not form foci in *sfp1*Δ cells or upon carbon starvation (data not shown). Fhl1^{YFP} and Ifh1^{CFP} nucleolar localization thus inversely correlated with the nuclear concentration of Sfp1. Surprisingly, in spite of the

transcriptional repression of *RP* genes and Ifh1 and Fhl1 relocalization to the nucleolus, Ifh1 and Fhl1 were readily detected at *RP* promoters under conditions of carbon source limitation (Fig. 8H). The activity of Fhl1/Ifh1 may therefore be dictated by the nuclear environment of promoter regions.

Discussion

An unexpected but fundamental connection has emerged between two ostensibly disparate systems, ribosome biogenesis and Start. Phenotypic identification of potential Start repressors isolated not only 15 ribosome biogenesis factors but, more significantly, upstream activators of the mRNA transcriptional program that sets the rate of ribosome synthesis, namely Sfp1 and Sch9 (Jorgensen et al. 2002). We have elaborated these initial connections to uncover a dynamic transcription factor network that responds to nutrient signals in part through altered localization of critical regulators. Other uncharacterized potential Start repressors may well participate in this emerging network that controls *RP* and *Ribi* expression (Jorgensen et al. 2002).

A dynamic transcriptional network at RP promoters

Sfp1 is a primary element in *RP/Ribi* regulation. Given that the *Ribi* regulon responds more rapidly than the *RP* regulon in response to Sfp1 (Jorgensen et al. 2002), it is puzzling that Sfp1 binds weakly to *RP* promoters, but apparently not to *Ribi* promoters (Lee et al. 2002; Fingerman et al. 2003). Nevertheless, Sfp1 activates *Ribi* transcription via the RRPE element (Fingerman et al. 2003). Consistent with a primary regulatory role, Sfp1 nuclear concentration responds within minutes to environmental conditions and appears to dictate the nuclear localization of Fhl1 and Ifh1, two novel regulators of RP promoters (Fig. 9A). Additional activators of the *RP* and *Ribi* regulons include the Sch9, TOR, and Ras/PKA pathways, all of which respond to nutrient signals. Sfp1 is downstream of TOR and Ras/PKA kinases, but the observed genetic redundancies indicate that these signaling pathways must modify multiple elements of the *RP* promoter network. Sfp1, Fhl1, and Rgm1 are likely to bind directly to *RP* promoter elements, such as the T-rich sequence or more recently described *cis* elements (Warner 1999; Pilpel et al. 2001; Beer and Tavazoie 2004), thereby placing the *RP* regulon under complex combinatorial control (Fig. 9A). As Rap1-binding sites mediate nearly all transcriptional activation at *RP* promoters (Warner 1999), it seems likely that most other components will subserve Rap1, perhaps by switching Rap1 between activation and repression modes. In support of this model,

overexpression of the N terminus of *IFH1* disrupts telomeric and mating-type silencing, both of which require Rap1 (Singer et al. 1998).

The dynamic nature of the *RP/Ribi* control network is manifest at several levels. First, the ability of the two regulons to respond quickly to environmental conditions rests on adept transcriptional responses coupled with mRNA instability (Warner 1999; Grigull et al. 2004). Second, Sfp1 relocalization is remarkably responsive to the environment. As it is never completely excluded from the nucleus, the *RP* and *Ribi* promoters may be quite sensitive to the nuclear concentration of Sfp1, as might befit weak interactions of Sfp1 with *RP* promoters. Third, Fhl1 and Ifh1 are unexpectedly subject to nucleolar relocalization. Ifh1 and/or Fhl1 may potentially have dedicated nucleolar roles, such as repression of RNA Polymerase I and III, and thereby constitute an unsuspected line of communication between *RP* and rRNA transcription (Fig. 9B). In an alternate, but not mutually exclusive model, the nucleolar focus of Ifh1 and Fhl1 in *sfp1*Δ and carbon-starved cells may signify a cluster of repressed *RP* genes, as Ifh1 and Fhl1 continue to bind to *RP* promoters in these cells (Fig. 9B). Intriguingly, physical interactions have been found between Fhl1 and the rDNA-binding factor Hmo1 and between Ifh1 and the nucleolar protein Utp22 (data not shown), suggesting that Fhl1/Ifh1 may tether inactive *RP* genes to nucleolar structures. Spatial control at the level of gene compartmentalization is evident in nucleolar tRNA gene clusters in rapidly growing cells and in Rap1-dependent clustering of silenced loci at the nuclear periphery (Gasser 2001; Thompson et al. 2003). Furthermore, the relocalization of transcription factors appears to be a common feature in ribosome biogenesis as the limiting regulatory factor for rRNA transcription, Rrn3, as well as RNA PolI subunits, are released from the nucleolus upon rapamycin treatment (Tsang et al. 2003; Mayer et al. 2004).

Sch9 relays nutrient signals

An unexpected level of spatio-dynamic control has emerged with the localization of Sch9 to the vacuole. Despite its discovery as a high-copy suppressor of the Ras/PKA pathway in yeast (Toda et al. 1988), and its recently discovered roles in cell longevity and size (Fabrizio et al. 2001; Jorgensen et al. 2002), the presumed components that lie upstream and downstream of Sch9 have proven elusive. Budding yeast expresses two bona fide orthologs of the PDK1 kinase that activates Akt/PKB in metazoans, called Pkh1 and Pkh2, although these kinases are activated by sphingolipids, not phosphati-dylinositol trisphosphate (Casamayor et al. 1999). Pkh1 and Pkh2

activate Ypk1 and Ykr2, the yeast orthologs of the metazoan kinase SGK (Casamayor et al. 1999). As its sequence contains the predicted activating phosphorylation sites in the activation loop and hydrophobic motif, Sch9 may also be a Pkh1/2 substrate. As the vacuole is an important reservoir of amino acids, phosphate, and other metabolites, Sch9 may communicate the status of these internal nutrient pools to *RP/Ribi* transcription and Start. Elaboration of the Sch9 pathway in yeast may provide insights into the regulation of cell size by Akt/PKB in metazoans.

Ribosome biogenesis and nutrient modulation of critical cell size

On the basis of the above observations, we propose a refined model for nutrient modulation of the critical cell-size threshold at Start. Sfp1, Sch9, and Ras/PKA function in a nonlinear network that dictates both critical cell size and expression of the *Ribi* and *RP* regulons. Critical cell size at Start is decreased when any of these components is crippled, whereas either constitutive activation (Ras/PKA) or inactivation (Sfp1, Sch9) renders cell size impervious to carbon source control (Baroni et al. 1989; Tokiwa et al. 1994). Each component of this trio is needed for proper *RP* and *Ribi* gene expression (Klein and Struhl 1994; Neuman-Silberberg et al. 1995; Y. Wang et al. 2004). Like Ras/PKA signaling, we have found that Sfp1 and Sch9 are sensitive to nutrient conditions, at the level of localization and abundance, respectively. In addition, strains deleted for numerous genes implicated in the actual events of ribosome biogenesis, as well as ribosome structural genes, are similarly, if less dramatically, uncoupled for growth and division (Jorgensen et al. 2002). Finally, the cell size at which SBF/MBF activation, budding, and DNA replication initiation occur is diminished in cells proliferating in poor nutrients (Johnston et al. 1979; Lorincz and Carter 1979; Tyson et al. 1979; Stuart and Wittenberg 1995; Flick et al. 1998). All of these observations can be unified by a model in which nutrient control of the critical cell-size threshold at Start is communicated by rates of ribosome production (Fig. 9C).

In this model, nutrient status influences the Start machinery via proximal events in ribosome biogenesis, rather than by downstream changes in protein synthetic rate. This configuration would effectively anticipate future changes in translation rate and adjust the cell-size threshold accordingly. In support of this idea, upon shift of cells from ethanol to glucose medium, rates of rRNA transcription reach maximal levels within a few minutes, whereas protein synthetic rate does not achieve a maximum until nearly an hour after the shift (Kief and Warner 1981).

Concordantly, upon nutrient shifts, cells adjust their critical cell-size threshold very rapidly (Lorincz and Carter 1979).

This model begs the question of how ribosome biogenesis might impinge on the Start machinery. As ribosome biogenesis factors implicated in size control lie along the entire pre-60S assembly pathway, the cell may monitor flux through this branch (Fatica and Tollervey 2002). Notably, inhibition of ribosome biogenesis by secretory defects also appears to proceed via the 60S branch (Zhao et al. 2003). Dissection of the ribosome biogenesis signal is complicated by redundancy among the many components, the existence of feedback loops, and its essential role in the cell. As G1/S transcription is greatly accelerated in cells lacking *SFP1* or *SCH9*, it is possible that SBF/MBF are a target of the presumed signal. Other less-direct models in which Ras/PKA, Sch9, and Sfp1 impinge on a second common target might also be considered. For instance, decreasing the overall rate of *RP* and *Ribi* transcription in nutrient-limited cells might free up RNA Polymerase II and other core transcriptional regulators for recruitment by SBF/MBF, and presumably all other promoters (Thomas 2000).

Two mechanisms to explain nutrient modulation of critical cell size have been proposed previously, both of which invoke changes in the abundance of G1 cyclins (Baroni et al. 1994; Tokiwa et al. 1994; Polymenis and Schmidt 1997; Flick et al. 1998). However, such models do not account for all of the data presented here and elsewhere. Carbon source control of Start occurs in part via transcriptional regulation of *CLN1*, because *cln1*Δ strains fail to appropriately increase critical cell size when shifted from poor to glucose medium; moreover, glucose and cAMP repress *CLN1* even relative to other G1/S transcripts like *CLN2* (Tokiwa et al. 1994; Flick et al. 1998). However, in these studies, critical cell size at Start was inferred indirectly by bud emergence, rather than by measurement of SBF/MBF-dependent transcription. Because SBF/MBF activation is highly dependent on *CLN3*, but independent of *CLN1* and *CLN2* (Tyers et al. 1993; Dirick et al. 1995; Stuart and Wittenberg 1995), Cln1 likely controls the length of the Start interval rather than the timing of Start entry.

A second model postulates that Cln3 couples the critical cell size threshold to nutrients by virtue of Cln3 translational control and protein instability (Polymenis and Schmidt 1997; Hall et al. 1998). It is clear that carbon source controls critical cell size at the level of SBF/MBF activation, an effect that in principle might be transmitted through Cln3 (Stuart and Wittenberg 1995). As

shown here, however, carbon source and *SFP1* strongly impact cell size even in cells that lack the known upstream regulators of SBF/MBF, thereby obviating the Cln3-based model. Furthermore, the observation that in poor nutrients cells have very low levels of Cln3 and translational capacity yet pass Start at a small size, is also at odds with this model (Tokiwa 1995; Hall et al. 1998). That is, nutrient upshifts delay Start, despite increases in Cln3 abundance (Johnston et al. 1977; Lorincz and Carter 1979; Tokiwa et al. 1994). From this perspective, nutrient modulation of the critical cell-size threshold is quite remarkable, as not only must the yeast cell growing in poor nutrients pass Start with less translational capacity, but it must do so with much less Cln3. That is, in poor nutrients, less Cln–Cdc28 activity seems to be required to pass Start. Consistently, Start-defective *cdc28-4* mutants at a semipermissive temperature cycle threefold faster in pyruvate medium than in glucose medium (Shuster 1982).

To synposize, a sharp distinction must be drawn between the critical cell-size threshold, which is altered by nutrients and ploidy, and the mechanism by which cells gage their size, perhaps through translation rate. A sizing role for translation rate is suggested by the increased critical size caused by sublethal doses of cycloheximide. Furthermore, even cells beyond the critical cell-size threshold require a minimum rate of protein synthesis before budding can occur (Moore 1988). Given these observations, the critical cell-size threshold and the critical translation-rate requirement may be one and the same. As argued above, the mechanisms that set this threshold may be entirely distinct from the processes that measure size.

Ribosome biogenesis and cell cycle control

Several recent findings suggest that primordial links between ribosome biogenesis and the cell cycle may couple growth and division in metazoan systems (Saucedo and Edgar 2002; Ruggero and Pandolfi 2003). As in yeast, disruption of ribosome biogenesis, but not the translational machinery, in flies causes a small cell-size phenotype (Montagne et al. 1999; Thomas 2000). In mice, blocking the synthesis of new ribosomes allows hepatocytes to grow, but not enter the cell cycle (Volarevic et al. 2000). Similarly, in human cells, overexpression of a dominant-negative version of a conserved ribosome biogenesis factor called Bop1 causes G1-phase arrest in a p53-dependent manner (Pestov et al. 2001). Many additional connections between p53 and the nucleolus have been unearthed (Ruggero and Pandolfi 2003). For example, the critical activator of p53, the Arf tumor suppressor, is a nucleolar protein and an inhibitor of ribosomal RNA

processing (Sugimoto et al. 2003). It has recently been proposed that all stresses that stabilize p53 do so by disrupting the nucleolus (Rubbi and Milner 2003).

As in yeast, mechanisms exist in mammalian cells to ensure that ribosomal content can be doubled with each cell cycle. Delineation of the signals that emanate from ribosome biogenesis to the cell cycle machinery in yeast should thus illuminate analogous processes in metazoans as well as the evolutionary history of size control processes. Like Sfp1, Myc is a direct modulator of *RP* transcription and cell size (Eisenman 2001), whereas Sch9 and Akt are highly related kinases that regulate ribosome synthesis and cell size (Saucedo and Edgar 2002). Understanding these connections has been lent new urgency by the discovery of numerous links between ribosome biogenesis and cancer (Ruggero and Pandolfi 2003), including the recent demonstration that *RP* genes are haploinsufficient tumor suppressors in zebrafish (Amsterdam et al. 2004).

Materials and methods

Yeast strains, plasmids, and medium

Extensive descriptions of all methods are provided as Supplemental Material. Yeast culture and size analysis was as described previously (Jorgensen et al. 2002). All experiments were performed with log phase cells at $O.D._{600} < 0.5$ and cell concentration $<3 \times 10^7$ cells/mL to minimize repression of the *Ribi* and *RP* regulons by inadvertent nutrient depletion (Ju and Warner 1994; DeRisi et al. 1997). Strains used in this study are listed in Supplementary Table 2 and were typically generated by genomic integration of standard C-terminal tagging cassettes, including GFP variant cassettes obtained from the Yeast Resource Center (University of Washington, Seattle). Centrifugal elutriation, Northern analysis, genome-wide expression profiles, chromatin immunoprecipitation, and as allele construction were carried out as described (Tyers et al. 1993; Bishop et al. 2000; Jorgensen et al. 2002; Costanzo et al. 2004).

Quantitative microscopy

Live-cell microscopy was performed with an Eclipse E600FN microscope (Nikon) and an Orca II CCD camera (Hamamatsu). Cultures in synthetic medium were rapidly concentrated and immediately visualized to mitigate starvation effects. For quantitation of $Sfp1^{YFP}$ or $Sfp1^{CFP}$

nuclear:cytoplasmic ratio, five serial sections were taken of each cell field. Sec63^{CFP} or Sec63^{YFP} was used to delineate the nuclear envelope. Metamorph (Universal Imaging) was used to capture and quantitate the fluorescence signal from an identically sized region from the nucleus and the cytoplasm. To induce carbon starvation, cells were washed once and resuspended in synthetic medium with 0.02% glucose. For nitrogen starvation, cells were resuspended in synthetic glucose medium with 1/50th the standard concentration of amino acids and ammonium sulfate. Ifh1^{CFP} and Fhl1^{YFP} signals were visualized in the same manner, except that nucleolar regions were demarcated with Bud21^{YFP} or Bud21^{CFP}. Because allowing cells to remain concentrated for even short periods of time caused redistribution of Sfp1, Fhl1, and Ifh1, all results were confirmed with directly mounted dilute cultures.

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Figure Legends

Figure 1. Sch9 and Sfp1 regulate cell size and *RP* and *Ribi* regulon transcription. (*A*) Galactose-regulated alleles of *SFP*1 and *SCH9*. (*B*) Inactivation of an analog-sensitive (as) allele of *SCH9* by 1NM-PP1. A total of 100 nM 1NM-PP1 or DMSO solvent was added at 0 min to log phase, glucose cultures of *sch9*^{as} and *sch9*Δ. Cell-size distributions were measured after 6 h and compared with reference distributions. Without 1NM-PP1, the *sch9*^{as} allele is hypomorphic, with nearly wild-type doubling time (t_d), but a strong Whi phenotype (t_d: 96 min, median cell size: 30 fL, vs. 87 min, 42 fL for wild type and 153 min, 28 fL for *sch9*Δ). (*C*) Sch9 and Sfp1 activate the *RP* and *Ribi* regulons. Expression profiles for *GAL1–SFP1* and *sfp1*Δ/Δ were repetitions of experiments described previously (Jorgensen et al. 2002). Expression profiles were determined for *sch9*^{as} and wild-type cultures harvested at indicated times after addition of 100 nM 1NM-PP1; expression ratios are relative to untreated cells from the same culture. The presence of RAP1, PAC, RRPE, or PAC + RRPE (P+R) promoter elements is indicated (Pilpel et al. 2001; Jorgensen et al. 2002). (*D*) Induction of representative genes in the *Ribi* (*NOP1, NSR1*) and *RP* (*RPPO, RPL11B*) regulons upon restoration of *GAL1–SCH9* or *GAL1–SFP1* expression. An *ACT1* loading control and *GAL7, SFP1*, and *SCH9* induction controls are shown. (*E*) Comparison of gene sets regulated by Sfp1 and Sch9. Expression profiles of *sfp1*Δ/Δ and inactivated *sch9*^{as} (1NM-PP1, 90 min) were plotted against one another. The number of genes in each functional group is indicated.

Figure 2. Sfp1 and Sch9 are negative regulators of Start. (*A*) Size distributions of *sfp1*Δ and *sch9*Δ strains. Doubling times were as follows: *sfp1*Δ 220 ± 13 min, *sch9*Δ 153 ± 3 min, wild type (WT) 89 ± 2 min, wild type (WT) + 200 nM cycloheximide 149 ± 4 min. (*B–H*) Determination of critical cell size. Small G1-phase daughter cells (>97% unbudded) were isolated from late log-phase cultures (3–4 × 10^7 cells/mL, raffinose medium with no drugs) of wild-type, *sfp1*Δ and *sch9*^{as} strains by centrifugal elutriation and reinoculated in glucose medium. A total of 100 nM 1NM-PP1 was added to the *sch9*^{as} culture upon reinoculation; 200 nM cycloheximide was added to a wild-type culture upon reinoculation. (*B*) Daughter cell size at various times after reinoculation for each culture. (*C*) Bud index as a function of cell size for each culture. (*D*) Cell-size distributions at the >25% budded time point for each culture. Passage through Start was monitored by cell size, bud index, and expression of SBF (*CLN2*)- and MBF

(*RNR1*)-specific transcripts for wild-type cells (*E*), *sch9*^{as} cells in 100 nM 1NM-PP1 (*F*), *sfp1*Δ cells (*G*), and wild-type cells in 200 nM cycloheximide (*H*). The time point at which cultures were >25% budded is highlighted in pink. All data was reproduced in duplicate experiments. 1NM-PP1 (100 nM) had no effect on the critical cell size of a wild-type strain (data not shown).

Figure 3. Cells lacking *SFP1* or *SCH9* fail to adjust cell size in response to carbon source. (*A*) Representative size distributions of log-phase cultures of the indicated strains in synthetic glucose (black), raffinose (blue), or glycerol (orange) medium. (*B*) Mean cell sizes of the indicated cultures (n = 6, range: 10–180 fL). Error bars extend one S.E. in each direction. The *sfp1*Δ/Δ *GAL1–CLN3-1* (*3-1*) strain was propagated in synthetic galactose medium. (*C*) Nutrient and *SFP1*-dependent control of size in cells that lack known upstream regulators of SBF/MBF. Wild-type and *cln3*Δ *bck2*Δ *whi5*Δ strains were in rich glucose, raffinose, or glycerol medium. The *sfp1*Δ and *sfp1*Δ *cln3*Δ *bck2*Δ *whi5*Δ strains were in rich glucose medium. (*D*) Mean cell sizes of the indicated cultures ($n \geq 2$, range: 10–180 fL). Error bars extend one S.E. in each direction.

Figure 4. Sch9 abundance and localization is modulated by nutrients. (*A*) Enrichment of ^{GFP}Sch9 at the vacuolar membrane (vm). (*B*) Depletion of ^{GFP}Sch9 from the vm after carbon starvation (37 min). (*C*) Kinetics of ^{GFP}Sch9 depletion from the vm. (Closed squares) +Glucose; (open diamonds) -glucose. Error bars extend one S.E. in each direction. (*D*) ^{HA3}Sch9 abundance and phosphorylation status are altered by nutrient conditions. Cells that expressed ^{HA3}*SCH9* from the *SCH*9 promoter were propagated in rich glucose, raffinose, or glycerol medium. Cells propagated in glucose medium were treated with rapamycin (rap, 200 ng/mL) or carbon starved (-C). ^{HA3}Sch9 was visualized by immunoblot with anti-HA antibody. ^{GFP}Sch9 served as a no-tag control. Asterisk indicates nonspecific cross-reactive species. (*E*) Sch9 is a phosphoprotein. ^{HA3}Sch9 expressed at endogenous levels was immunoprecipitated with anti-HA antibody and either mock treated (M), treated with alkaline phosphatase (P), or treated with alkaline phosphatase in the presence of phosphatase inhibitors (P+I) and detected by anti-HA immunoblotting.

Figure 5. Sfp1 nuclear concentration responds rapidly to environmental stimuli and is regulated by the Ras/PKA and TOR signaling pathways. (*A*) Sfp1 localization is regulated by nutrient signals. An *SFP1*^{YFP} *SEC63*^{CFP} strain was visualized; cell and nuclear membranes were

demarcated by Sec63^{CFP}. Carbon starvation (-C) was for 15 min, and rapamycin treatment (rap, 200 ng/mL) for 20 min. (*B*) Sfp1 exits the nucleus in response to various stress conditions. An *SFP1*^{YFP} *SEC63*^{CFP} strain was depleted for carbon or nitrogen or treated with H_20_2 (0.30 mM), rapamycin (200 ng/mL), or tunicamycin (2 µg/mL). The Sfp1^{YFP} nuclear:cytoplasmic ratio (N:C) of each cell at each time point was plotted (dashes, 30–100 cells), as was the average ratio (thick line). (*C*) Sfp1 abundance and electrophoretic mobility are not altered by carbon starvation (-C) or rapamycin (rap, 200 ng/mL) treatment. Cells were harvested after the indicated time (min). Sfp1^{MYC13} in cell lysates was visualized by immunoblot with anti-MYC antibody. Fhl1^{MYC13} served as a specificity control. (*D*) Sfp1 relocalization correlates withre-pression of the *RP* and *Ribi* regulons. Genome-wide expression profiles comparing mRNA abundance before and after carbon starvation (-C) were obtained de novo. Expression profiles for rapamycin addition (rap), oxidative stress (H_20_2), and nitrogen starvation (-N) were derived from published data (Hardwick et al. 1999; Gasch et al. 2000). Scale indicates fold change. (*E*) Nuclear localization of Sfp1 in different carbon sources. The average Sfp1^{YFP} N:C ratio was determined under steady-state proliferation in glucose, raffinose, and glycerol medium. Error bars extend one S.D. in each direction. (*F*) Sfp1 re-enters the nucleus rapidly in response to glucose. Stationary phase *SFP1*^{YFP} *SEC63*^{CFP} cells were re-fed with glucose in the presence or absence of cycloheximide (chx, 10 µM) and Sfp1^{YFP} N:C ratios determined. (*G*) Sfp1 may effect a feedback response to ribosome shortage. Sfp1^{YFP} N:C ratio was measured in *SFP1*^{YFP} *SEC63*^{CFP} cells proliferating in raffinose medium before and after addition of chx (10 µM). (*H*) The rapamycin-resistant allele *TOR1-1* blocks Sfp1 relocalization in response to rapamycin but not nitrogen starvation. A *TOR1-1* *SFP1*^{YFP} strain was treated with rapamycin (red dotted line, 200 ng/mL) or starved for nitrogen (black, offset for visualization) for 30 min. (*I*) Compromised Ras activity lowers the nuclear concentration of Sfp1. The N:C ratio of Sfp1^{CFP} was quantitated before and after 60 min of carbon starvation in a *tpk1*^{wimp} strain (red dotted line, *SFP1*^{CFP} *tpk1*^{wimp} *bcy1*Δ *tpk2*Δ *tpk3*Δ) and in a control strain (black line, *SFP1*^{CFP}, offset for visualization). At t = 0 min, the difference between the wild-type and wimp strain was significant (Student's t-test, $p = 4.4 \times 10^{-11}$). Due to higher cell autofluorescence, Sfp1^{CFP} N:C ratios are less than Sfp1^{YFP} N:C ratios. (*J*) Hyperactive Ras signaling drives Sfp1 into the nucleus. A *GAL10–RAS2*^{V19} *SFP1*^{CFP} strain (red dotted line) and a control *SFP1*^{CFP} strain (black, offset for visualization), proliferating in synthetic raffinose medium, were induced with galactose at t = 0, and visualized after 30 and 60 min. Both strains were deleted for *GAL1* and are incapable of metabolizing galactose.

Figure 6. Sfp1 and Sch9 function in parallel pathways. (*A*) *sfp1*Δ cells are sensitive to cycloheximide and to decreases in TOR or PKA pathway activity. (*Left*) Filter disks containing 3 nmole cycloheximide or 15 μg rapamycin were incubated on lawns of the indicated strains for 2 d. (*Right*) Synthetic proliferation defects between *ras2*Δ and *sfp1*Δ. Spore clones were scored after 4 d. (*B*) Sch9 does not control cell size strictly via Sfp1. Size distributions of an *sfp1*Δ/Δ strain in the presence or absence of a functionally null heterozygous *GAL1–SCH9* (*G9*/+) allele are shown. Mean cell sizes of the indicated strains in either rich glucose or galactose medium are indicated to the *right* ($n = 4$). (*C*) An $sfp1^{ER}$ allele is modulated by β-estradiol (E2). Size distributions of log phase $sfp1^{ER}$ cultures with or without 250 nM E2 (*left*) or in the presence of various E2 concentrations (*right*). (*D*) Progressively compromised *SCH9* and *SFP1* activity reveals synergistic proliferation defects. An $sch9^{as}$ $sfp1^{ER}$ strain was inoculated into varying concentrations of 1NM-PP1 and E2 in synthetic glucose medium. Doubling times (t_d) were determined for each culture and the increase relative to cultures proliferating in 6.25 nM 1NM-PP1 and 125 nM E2 (concentrations at which $Sch9^{as}$ and $Sfp1^{ER}$ were fully active) was calculated. (*Top*) The increase in doubling time (Δt_d) resulting from individually increasing 1NM-PP1 or decreasing E2 was used to calculate the predicted additive effects. Actual increases in doubling time (*middle*) and the difference (*bottom*) are plotted.

Figure 7. Sfp1 influences Fhl1 and Ifh1 interactions with *RP* promoters. (*A*) *FHL1* deletion is epistatic to *SFP1* deletion for colony size. Spore clones were imaged after 7 d (*top*) and 5 d (*bottom*). (*B*) *FHL1* deletion is not epistatic to *SFP1* deletion for cell size. Strains recently derived from a tetratype tetrad dissection (as in *A*) were sized in log phase in rich glucose medium. These representative distributions were highly reproducible. (*C*) Synthetic proliferation defects between *sch9*Δ and *rgm1*Δ and an allele of *IFH1*. Spore clones were imaged after 3 and 2 d, respectively. (*D*) Fhl1 and Ifh1 bind specifically to *RP* promoters. Real-time PCR was used to quantitate the efficiency with which the promoter regions of the indicated genes were captured in $Fhl1^{HA3}$ (F7 anti-HA antibody) or $Ifh1^{MYC13}$ (9E10 anti-MYC antibody) complexes. Binding to *Ribi* promoters (*URA7, RPA190, NSR1*) and control promoters (*PGK1, ACT1*) was not observed. (*E*) $Ifh1^{MYC13}$ and $Fhl1^{HA3}$ bind poorly to *RP* promoters in *sfp1*Δ cells. The *sfp1*Δ/wild-type ratio of ChIP efficiency for $Ifh1^{MYC13}$ and $Fhl1^{HA3}$ was calculated for two individual experiments. Error bars extend one S.E. in each direction.

Figure 8. Ifh1 and Fhl1 localize to the nucleolus in cells lacking *SFP1* and upon carbon starvation. (*A*) An *sfp1*Δ *IFH1*^{CFP} *BUD21*^{YFP} strain and a control *IFH1*^{CFP} *BUD21*^{YFP} strain were visualized in glucose medium. Blue arrows indicate cells with nucleolar foci of Ifh1^{CFP}. (*B*) An *sfp1*Δ *FHL1*^{YFP} *BUD21*^{CFP} strain and a control *FHL1*^{YFP} *BUD21*^{CFP} strain were visualized in glucose medium. Blue arrows indicate cells with nucleolar foci of Fhl1^{YFP}. Wild-type and *sfp1*Δ cells are not presented to scale to improve the visualization of Fhl1^{YFP} in the small nuclei and nucleoli of *sfp1*Δ cells. (*C*) Quantitation of Ifh1^{CFP} and Fhl1^{YFP} nucleolar foci. CFP and YFP fluorescence was visualized in a single plane in the indicated strains proliferating in glucose medium. If a clear nucleolar (Bud21$^{Y/CFP}$) crescent or dot was evident in this plane, it was determined whether or not an adjacent or overlapping focus of Ifh1^{CFP} or Fhl1^{YFP} was present. (chx) Cells in 400 nM cycloheximide. For each condition, >165 in focus nucleoli were scored. (*D*) Ifh1^{CFP} often relocalizes to the nucleolus upon carbon starvation. Carbon starvation was for 25 min (-C). Blue arrows indicate cells with nucleolar foci of Ifh1^{CFP}. (*E*) Ifh1^{CFP} nucleolar relocalization is rapid and sustained. At the indicated times after carbon starvation, Ifh1^{CFP} and Bud21^{YFP} fluorescence was visualized in five planes and the percentage of cells with nucleolar Ifh1^{CFP} fluorescence determined. In total, 290 cells were analyzed. (*F*) Fhl1 relocalizes to a perinucleolar focus upon carbon starvation. Carbon starvation was for 55 min (-C). Blue arrows indicate cells with nucleolar foci of Fhl1^{YFP}. (*G*) Fhl1^{YFP} perinucleolar relocalization upon carbon starvation is rapid and sustained. Cells were resuspended in synthetic medium with (black squares) or without (gray diamonds) glucose. Images were processed as in *C*. At least 240 in-focus nucleoli were examined for each time course. (*H*) Carbon starvation does not dissociate Ifh1 or Fhl1 from *RP* promoters. ChIP efficiency was determined in glucose medium and after 30 min of carbon starvation. Average ratio of -/+ glucose ChIP efficiency was determined for two individual experiments. Error bars extend one S.E. in each direction.

Figure 9. (*A*) Initial model for the transcription system at *RP* promoters. Sfp1 localization is controlled by various signals and in turn influences the localization of Ifh1 and Fhl1. As suggested by synthetic lethal interactions, Sch9 modulates *RP* transcription by a pathway parallel to Sfp1/Fhl1/Ifh1/Rgm1, perhaps by phosphorylating Rap1. Sfp1, Fhl1, and Rgm1 likely bind to the promoter directly via still uncharacterized sequence elements (gray box) (Warner 1999; Pilpel et al. 2001; Beer and Tavazoie 2004). Ifh1 and Fhl1 may activate *RP* transcription by switching Rap1 (or Abf1) between intrinsic transcriptional activation (A) and repressive (R)

functions. (*B*) Summary of transcription factor localization correlated with active and inactive (carbon starvation) *RP* transcription. Two possible nonmutually exclusive models explain the nucleolar relocalization of Ifh1 and Fhl1 upon carbon starvation. (*Left*) In the first model, unbound Ifh1 and Fhl1 are sequestered in or near the nucleolus (nucl), where they may have additional functions, such as repressing rRNA transcription. In the second model, Ifh1 and Fhl1 and repressed *RP* promoters are drawn into the nucleolar region upon carbon starvation. (*C*) A model of Start entry. Ribosome synthesis rates and ploidy establish the critical cell size threshold, which represses the SBF and MBF transcription factor complexes by an unknown mechanism. Cell size or a parameter that correlates with size, such as translation rate, signals to Cln3 and/or Bck2 to activate SBF and MBF. The model is complicated by the effects of ribosome synthesis on translation and by increased Cln3 abundance in rich nutrient conditions (dashed line).

Figure 1.

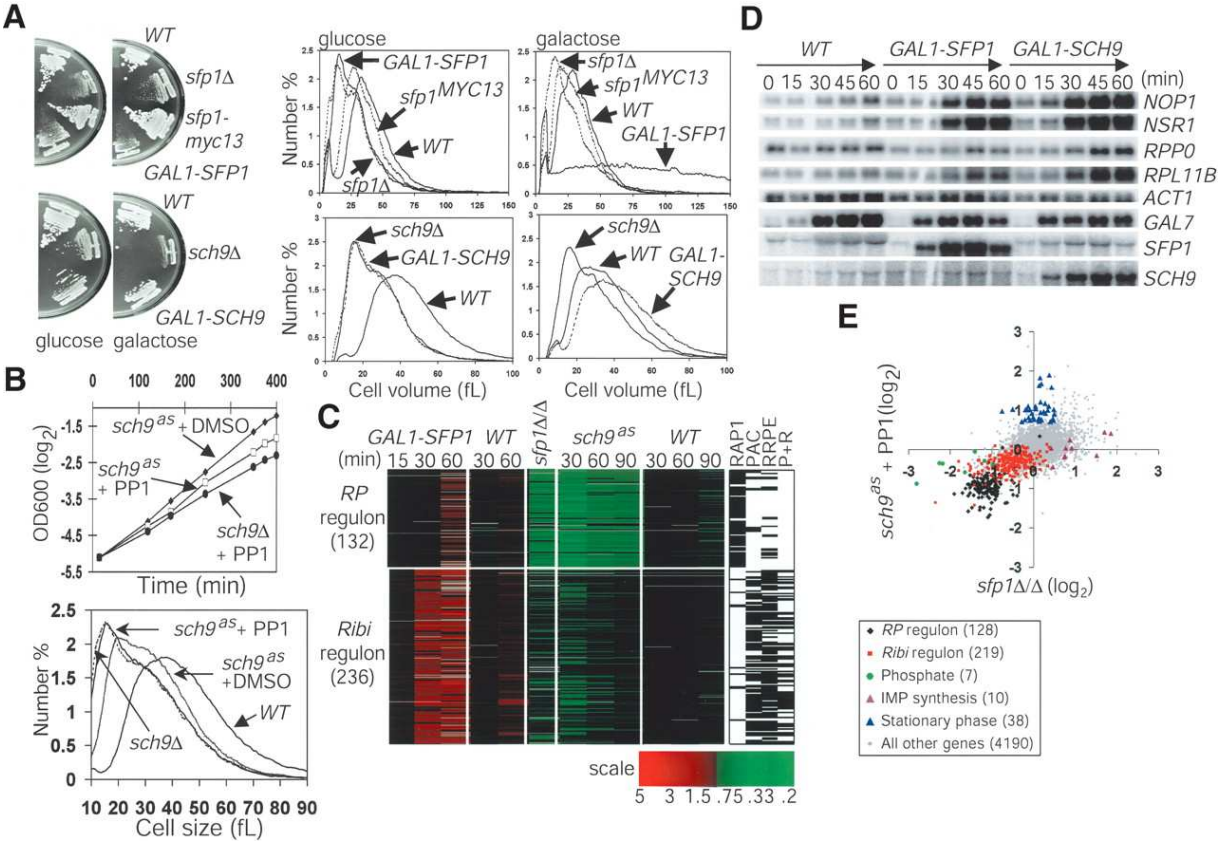
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Figure 2.

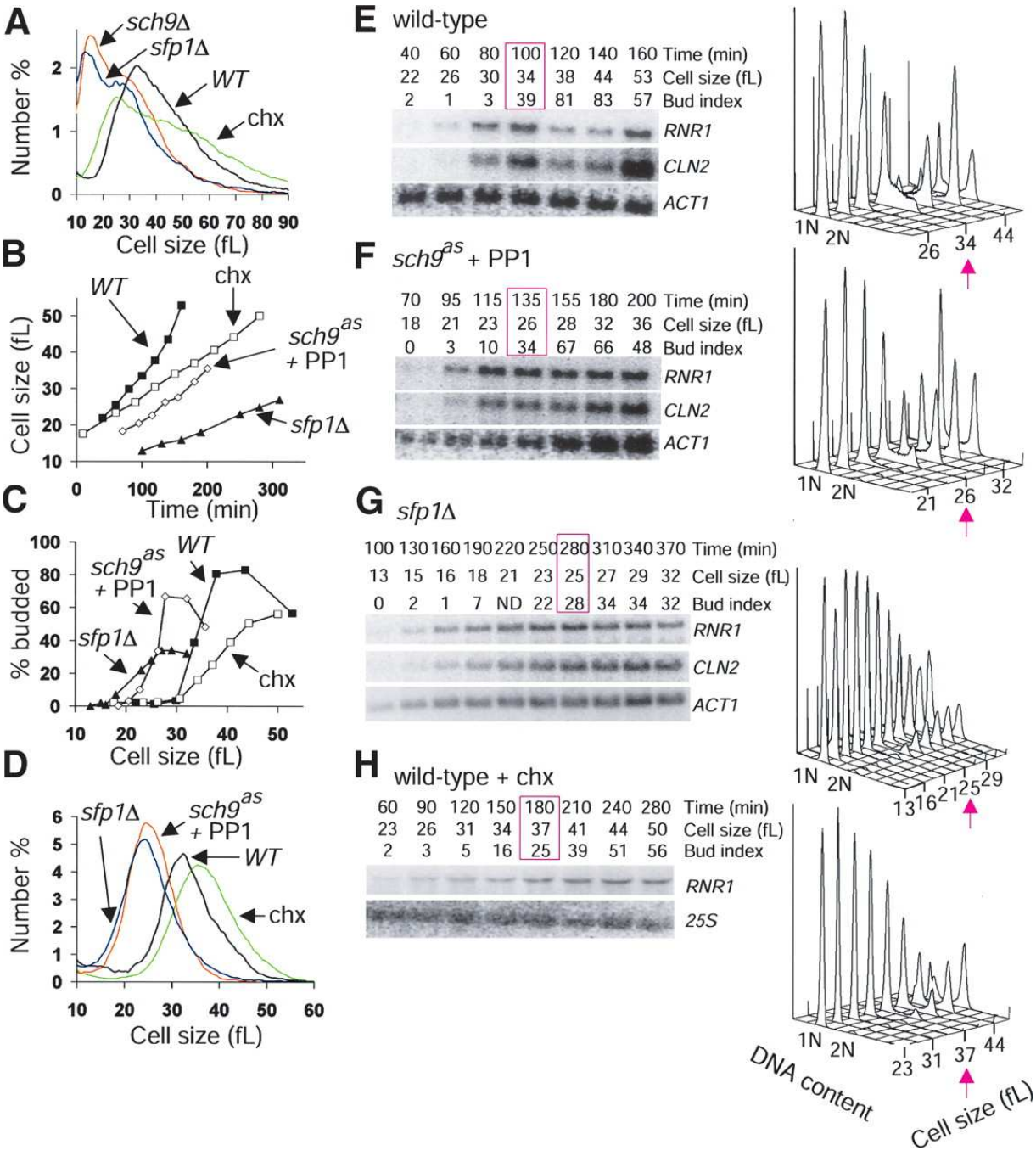
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Figure 3.

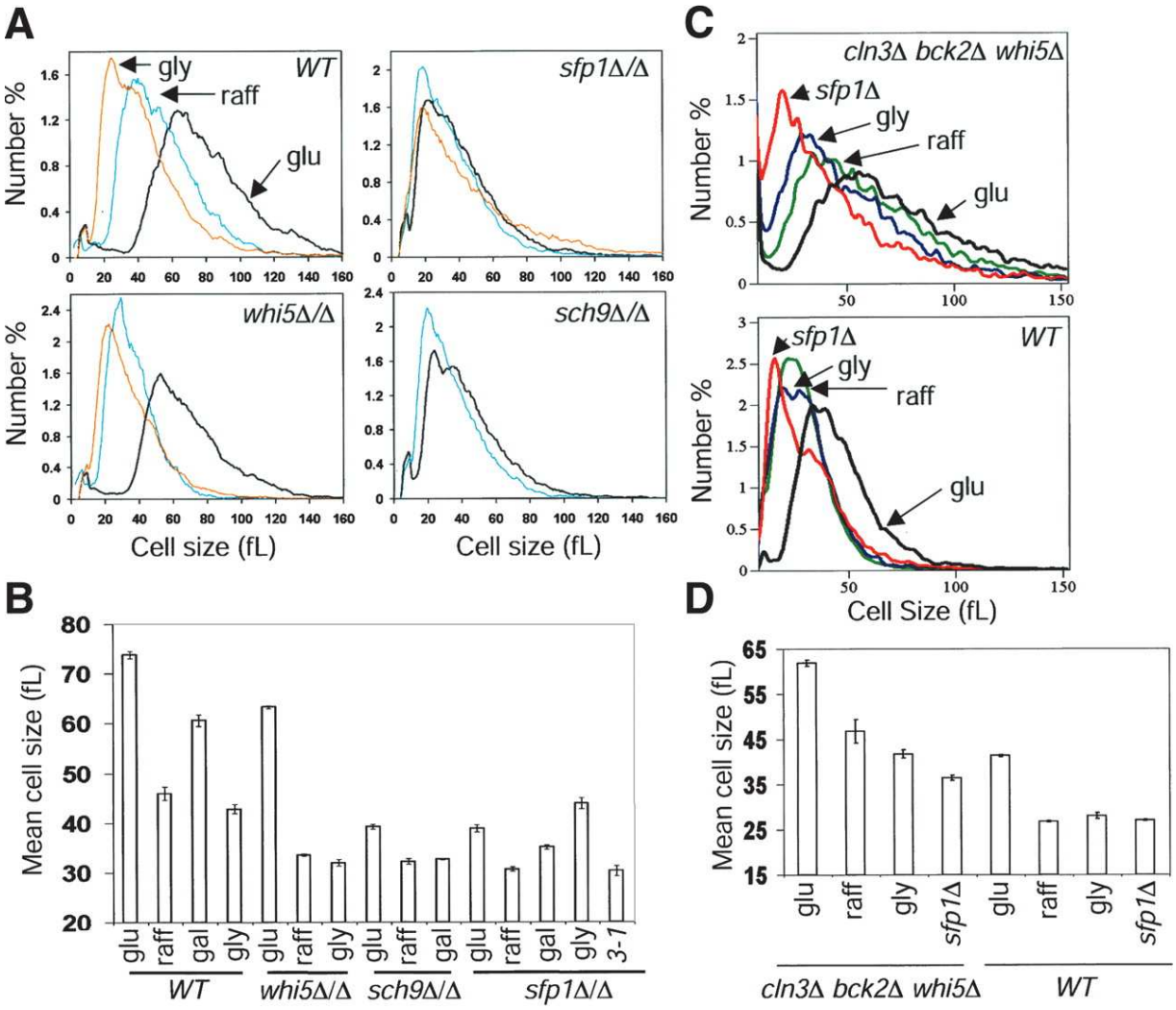
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Figure 4.

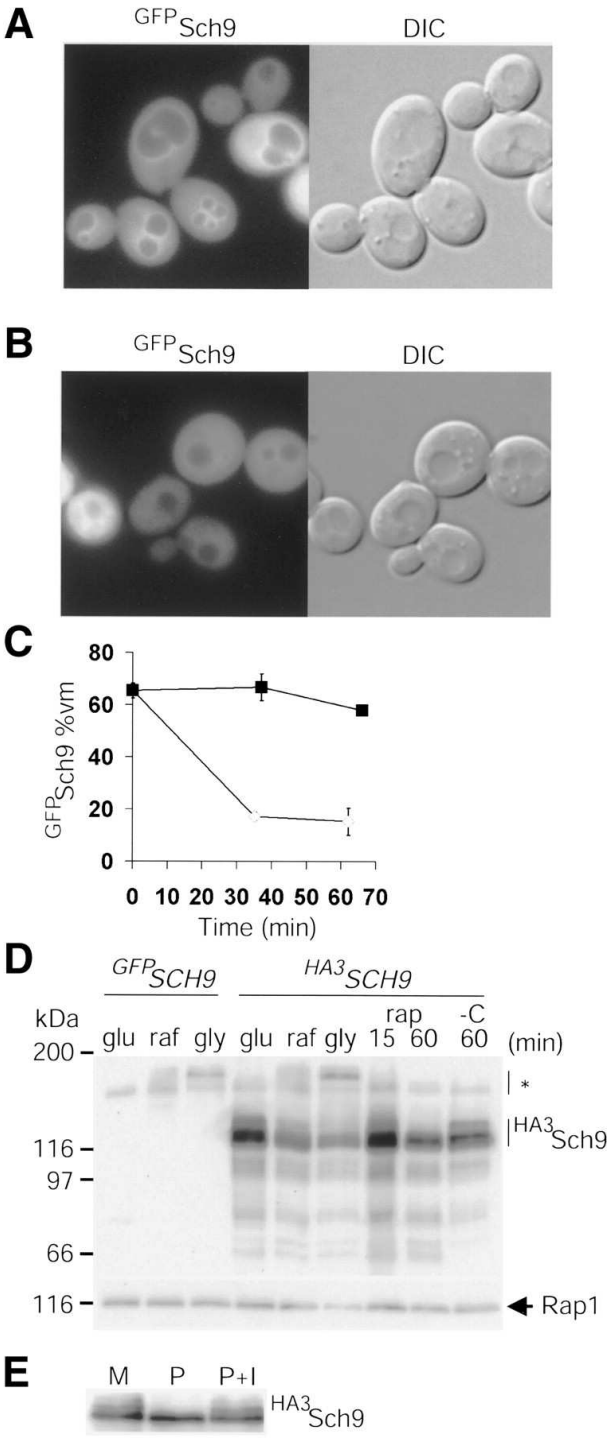
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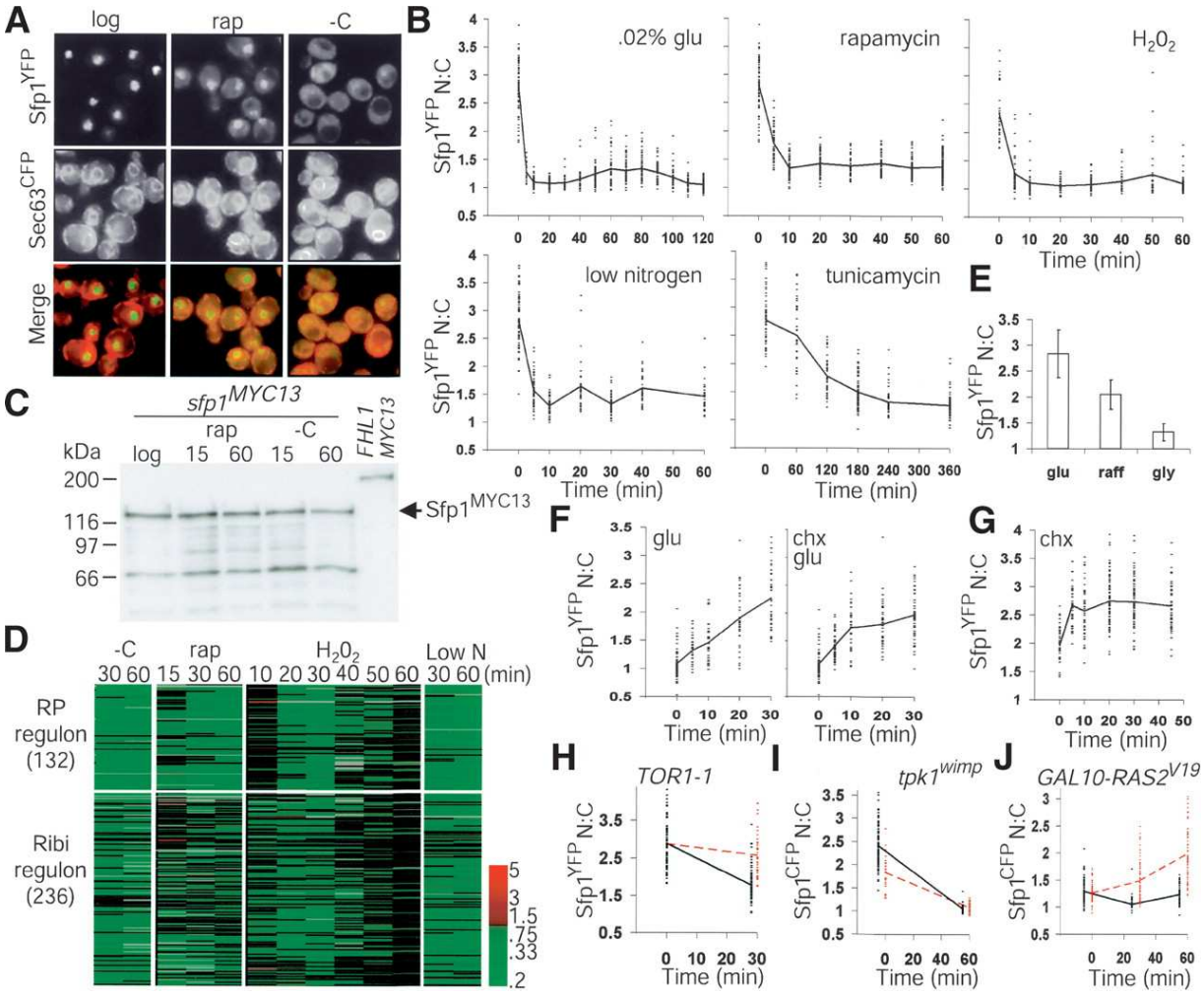
Figure 5.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H2O2
low nitrogen
tunicamycin
Sfp1YFP N:C
Time (min)
C
sfp1MYC13
rap
-C
kDa
log 15 60 15 60
FHL1 MYC13
200
116
97
66
Sfp1MYC13
D
-C
rap
H2O2
Low N
30 60 15 30 60 10 20 30 40 50 60 30 60 (min)
RP regulon (132)
Ribi regulon (236)
5
3
1.5
.75
.33
.2
E
glu
raff
gly
F
glu
chx glu
G
chx
H
TOR1-1
I
tpk1wimp
Sfp1CFP N:C
J
GAL10-RAS2V19

Figure 6.

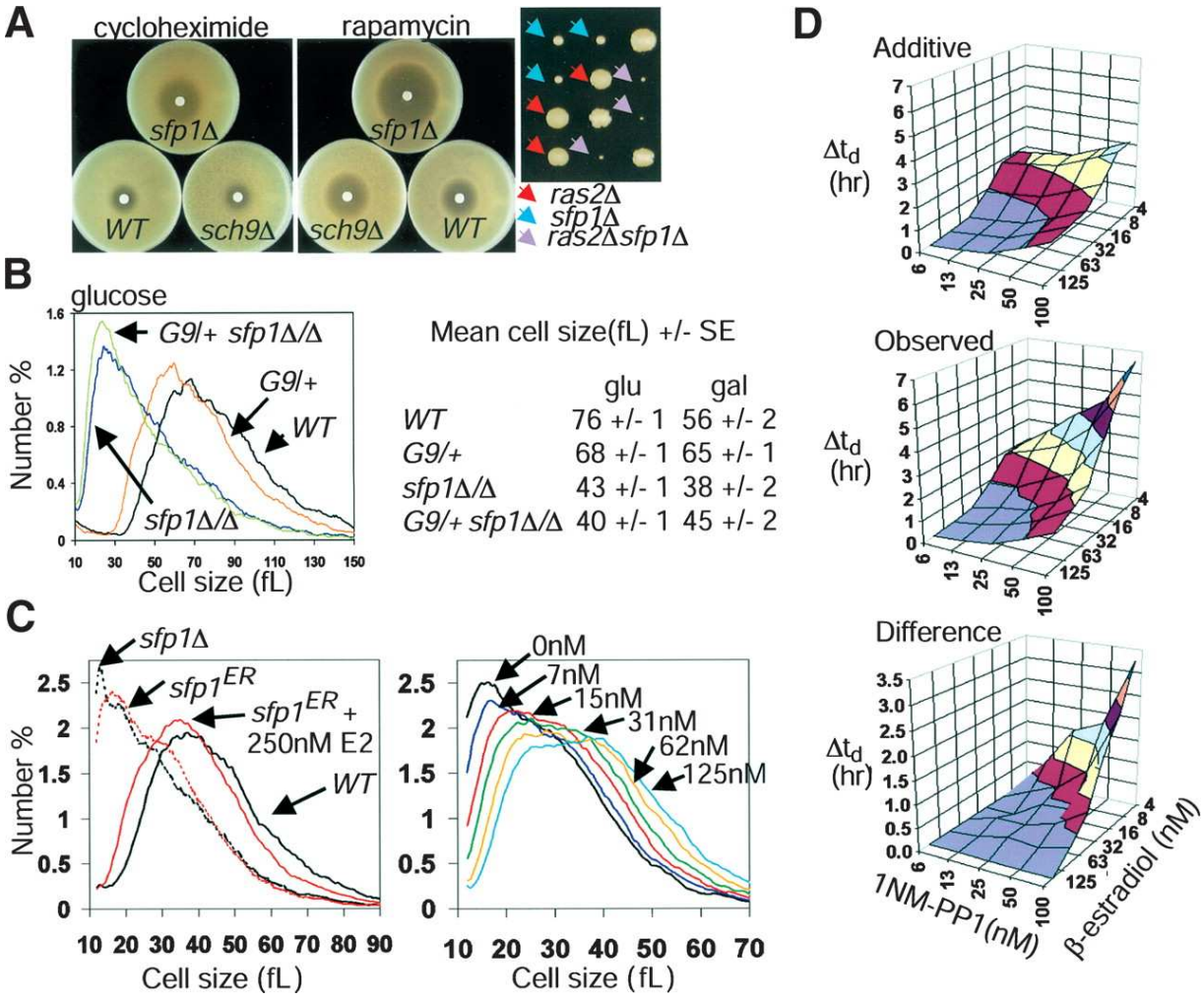

A
cycloheximide
rapamycin
sfp1Δ
sfp1Δ
WT
sch9Δ
sch9Δ
WT
ras2Δ
sfp1Δ
ras2Δsfp1Δ
B
glucose
G9/+ sfp1Δ/Δ
G9/+
WT
sfp1Δ/Δ
Number %
Cell size (fL)
Mean cell size(fL) +/- SE
glu gal
WT 76 +/- 1 56 +/- 2
G9/+ 68 +/- 1 65 +/- 1
sfp1Δ/Δ 43 +/- 1 38 +/- 2
G9/+ sfp1Δ/Δ 40 +/- 1 45 +/- 2
C
sfp1Δ
sfp1ER
sfp1ER + 250nM E2
WT
Number %
Cell size (fL)
0nM
7nM
15nM
31nM
62nM
125nM
Cell size (fL)
D
Additive
Observed
Difference
Δtd (hr)
7NM-PP1(nM)
β-estradiol (nM)

Figure 7.

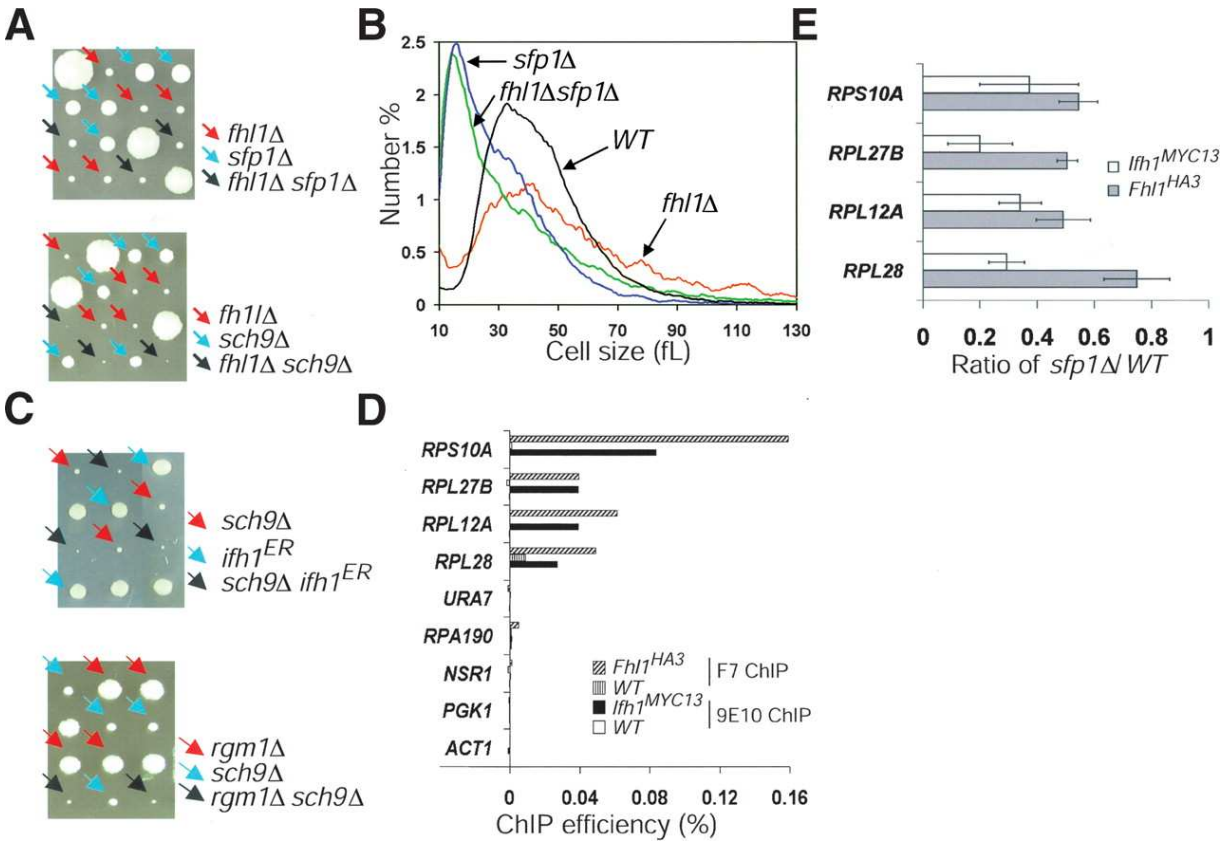

A
fhl1Δ
sfp1Δ
fhl1Δ sfp1Δ
fh1lΔ
sch9Δ
fhl1Δ sch9Δ
B
sfp1Δ
fhl1Δsfp1Δ
WT
fhl1Δ
Number %
Cell size (fL)
E
RPS10A
RPL27B
RPL12A
RPL28
Ifh1MYC13
Fhl1HA3
Ratio of sfp1Δ/ WT
C
sch9Δ
ifh1ER
sch9Δ ifh1ER
rgm1Δ
sch9Δ
rgm1Δ sch9Δ
D
RPS10A
RPL27B
RPL12A
RPL28
URA7
RPA190
NSR1
PGK1
ACT1
Fhl1HA3
WT
Ifh1MYC13
WT
F7 ChIP
9E10 ChIP
ChIP efficiency (%)

Figure 8.

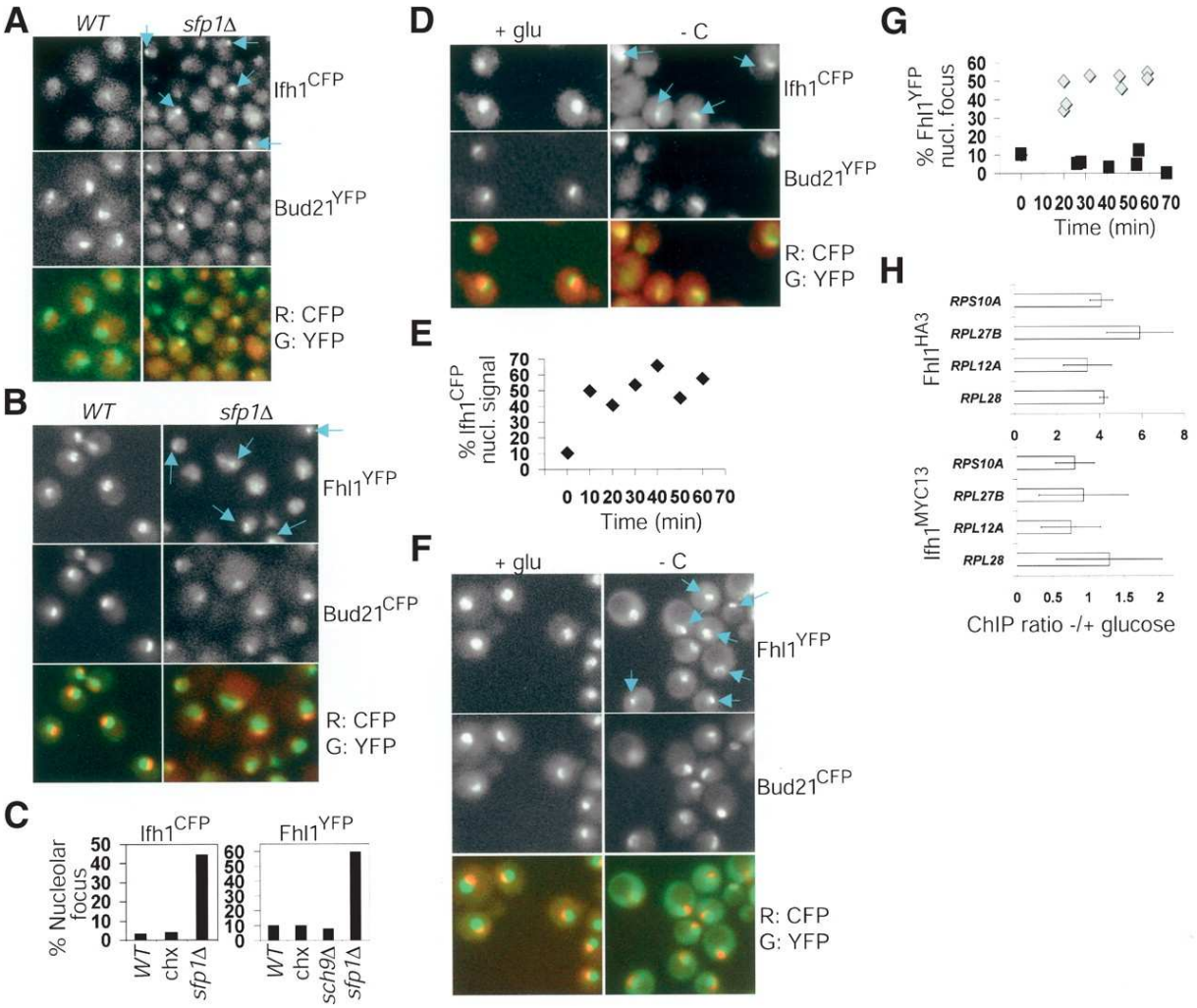
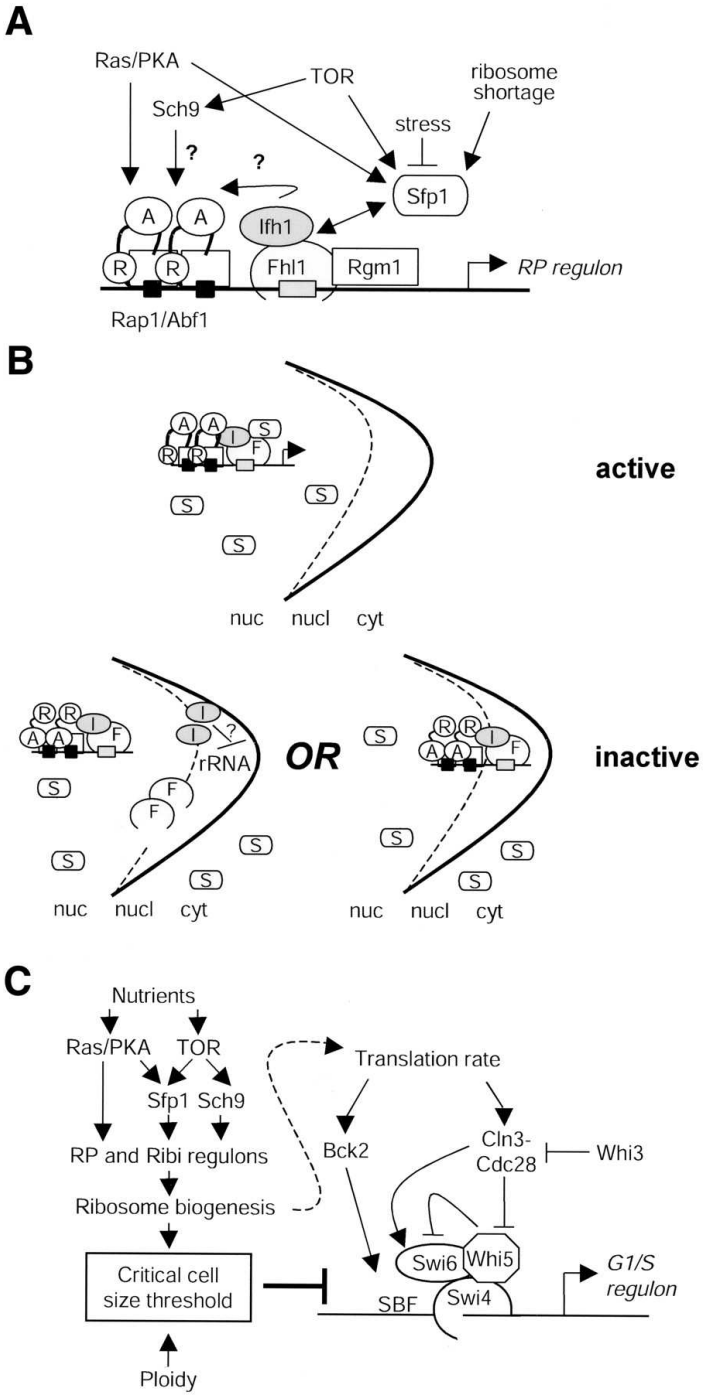

A
WT
sfp1Δ
Ifh1CFP
Bud21YFP
R: CFP
G: YFP
B
WT
sfp1Δ
Fhl1YFP
Bud21CFP
R: CFP
G: YFP
C
Ifh1CFP
FhI1YFP
% Nucleolar focus
WT
chx
sfp1Δ
sch9Δ
D
+ glu
- C
Ifh1CFP
Bud21YFP
R: CFP
G: YFP
E
% Ifh1CFP nucl. signal
Time (min)
F
+ glu
- C
Fhl1YFP
Bud21CFP
R: CFP
G: YFP
G
% Fhl1YFP nucl. focus
Time (min)
H
Fhl1HA3
RPS10A
RPL27B
RPL12A
RPL28
Ifh1MYC13
ChIP ratio -/+ glucose

Figure 9.


A
Ras/PKA
TOR
ribosome shortage
Sch9
stress
?
?
Sfp1
A
A
Ifh1
R
R
Fhl1
Rgm1
RP regulon
Rap1/Abf1
B
active
nuc
nucl
cyt
rRNA
OR
inactive
C
Nutrients
Ras/PKA
TOR
Translation rate
Sfp1 Sch9
Bck2
Cln3-
Cdc28
Whi3
RP and Ribi regulons
Ribosome biogenesis
Swi6
Whi5
G1/S
regulon
Critical cell
size threshold
SBF
Swi4
Ploidy