THE ROLE OF PROTEIN PHOSPHATASES IN REGULATION OF DROSOPHILA S6 KINASE BY NUTRIENT SIGNALING PATHWAYS

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DEDICATION

To my family and friends for their encouragement and support, and to Melissa and Jellybean for all of their love

.

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The regulation of cellular growth is a highly complex, but fundamental, process and the inability of cells to initiate or repress cell growth in the presence of appropriate signals underlies many disease states including cancer and diabetes. Many studies have investigated the mechanisms by which cells activate growth; however, few have focused upon the means by which cells turn off growth in response to environmental signals. As many of the protein components of nutrient signaling pathways are proto-oncogenes, of equal importance is identifying and characterizing the components utilized to down-regulate growth, as mutations in these genes may also lead to genesis of disease. Protein phosphatase 2A (PP2A) is a major intracellular protein phosphatase implicated in

the regulation of numerous processes, including cellular growth. Elucidating the role of PP2A in nutrient signaling pathways will aid in the understanding of the biochemical processes that eukaryotes employ to properly respond to their environment.

I have cloned the *Drosophila* homolog of the Tap42/α4 gene (dTap), which retains 28% amino acid identity to the human α4 gene product. Utilizing a combination of RNA interference (RNAi) and nutrient starvation conditions, I have tested the sequestration model of dTap as a repressor of PP2A activity and shown that in S2 cells, dTap does not antagonize nutrient signaling pathways by repressing PP2A activity. Although dTap does not act as an inhibitor of PP2A activity towards dS6K, loss of dTap conferred rapamycin resistance to S2 cells and prolonged loss of dTap resulted in cell death.

I have also utilized RNAi to screen the gene products of the PP2A-like family of phosphatases and *Drosophila* PP2A regulatory subunits for effects upon nutrient signaling pathways. I have shown that the PP2A catalytic subunit is required for the dephosphorylation of *Drosophila* S6 kinase (dS6K) during periods of inhibition of growth. The ablation of two other gene products, the PP2A-B56-2 regulatory subunit and the dPP4 catalytic subunits, results in hypophosphorylation of dS6K during amino acid starvation, suggesting that the activities of these two gene products are required for maintenance of nutrient signaling pathways and proper growth in *Drosophila*.

TABLE OF CONTENTS

Chapter 1

Chapter 2

Chapter 3

PRIOR PUBLICATIONS

Silverstein, A.M., Davis., A.J., Bielinski., V.A., Esplin, E.D., Mahmood, N.A., and Mumby, M.C. Protein Phosphatase 2A. Handbook of Cell Signaling, Volume 2, Chapter 189. Elsevier Science (2003), 405-415.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4EBP – eIF4e-Binding Protein

α4 – Alpha 4

Ab - Antibody

bp – Base pairs

BCR – B cell receptor

CL-A – Calyculin A

cDNA – Complementary deoxyribonucleic acid

DNA – Deoxyribonucleic Acid

dTap – *Drosophila* Type-2A Associated Protein

dsRNA – Double-stranded ribonucleic acid

dS6K – *Drosophila* S6 Kinase

dTOR – *Drosophila* Target of Rapamycin

ECL – Enhanced chemiluminescence

eIF4E – Eukaryotic translation initiation factor 4E

FBS – Fetal bovine serum

FKBP12 – FK506-binding protein 12

FRB – FKBP12/rapamycin binding domain

GAP – GTPase activating protein

GTP – Guanosine triphosphate

HEAT – Huntington-Elongation Factor 1A-protein phosphatase 2A A-TOR

HRP – Horseradish peroxidase

IR – Insulin Receptor

kDa – kilodaltons

Met-tRNAi – Initiator methionyl-tRNA

Min – minutes

mRNA – Messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin

nm - nanometer

OA – Okadaic Acid

p70-S6K – Mammalian S6 Kinase

P-S6K – Phospho-S6 Kinase

PCR – Polymerase Chain Reaction

PDK1 – Phosphoinositide-dependent kinase 1

PH – Pleckstrin homology

PI3K – Phosphatidylinositol 3-kinase

PIKK – Phosphatidylinositol 3-kinase-related kinase

PIP_3 – Phosphatidylinositol 3,4,5-trisphosphate

PKA – Protein Kinase A

PKB – Protein Kinase B

PP1 – Protein Phosphatase 1

PP2A – Protein Phosphatase 2A

PP4 – Protein Phosphatase 4

PP6 – Protein Phosphatase 6

PTEN – Phosphatase and Tensin homolog deleted on chromosome Ten

Rap – Rapamycin

Raptor – Regulatory Associated Protein of mTOR

Rheb – Ras homologue enriched in brain

RNA – Ribonucleic Acid

RNAi – RNA interference

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

S2 – Schneider's *Drosophila* S2 cell line

SDM – Schneider's *Drosophila* Medium

SDS – Sodium Dodecyl Sulfate

TSC – Tuber Sclerosis Complex

TOR – Target of Rapamycin

tRNA – Transfer Ribonucleic Acid

ul – Microliter

ug – Microgram

uM – Micromolar

Chapter 1

Introduction

A. Nutrient Signaling in Eukaryotes

1. Regulation of Translation by Amino Acids

The synthesis of proteins from amino acid precursors is one the most fundamental processes in cellular growth and the signaling pathways involved in governing translational responses to environmental stimuli are highly regulated, yet dynamic. Cellular growth and proliferation are closely linked; impairment of the biogenesis of proteins leads to a reduction in proliferation (Kawasome, Papst et al. 1998) and a block in the cell cycle (Morice, Wiederrecht et al. 1993). Protein synthesis consumes a vast amount of metabolic energy, therefore it is vital for cells to down-regulate this process at appropriate times (i.e., the absence of essential amino acids). Accordingly, global protein synthesis is greatly decreased in cells deprived of amino acids.

Amino acids regulate signaling pathways controlling two critical steps in the initiation of translation (Kimball and Jefferson 2004). Translational elongation is dependent upon the availability aminoacyl-transfer RNAs (tRNAs), the immediate precursors of protein synthesis. An increase in the amount of uncharged tRNAs prevents binding of initiator methionyl-tRNA to the 40S ribosome subunit, inhibiting formation of the 43S ribosome. The 43S ribosome consists of the met-tRNAi, eukaryotic initiation factor (eIF) 2, and GTP (Kapp and Lorsch 2004). Amino acid deprivation results in the phosphorylation of the eIF2 α-subunit by the protein kinase mGCN2 (Scorsone, Panniers et al. 1987).

mGCN2 kinase activity increases in the absence of amino acids (Harding, Novoa et al. 2000), possibly though direct interactions with uncharged transfer RNAs (tRNAs). Phosphorylated eIF2α inhibits the activity of the guanine nucleotide exchange factor eIF2B, preventing formation of the 40S pre-initiation complex (Clemens, Galpine et al. 1987).

The second rate-limiting step of protein synthesis involves binding of messenger RNA (mRNA) to the 43S pre-initiation complex. Amino acids modulate the activity of the eIF4E-binding proteins 4EBP-1 and 4EBP-2 (Hara, Yonezawa et al. 1998), antagonists of the eIF4F initiation complex. The phosphorylation state of 4EBPs is regulated through a signal transduction pathway involving the mTOR (mammalian Target of Rapamycin) and phosphatidylinositol 3-kinase (PI3K). mTOR activity is dependent upon the presence of free amino acids, whereas PI3K is activated by growth factor receptors (Harris and Lawrence 2003). In the presence of amino acids or insulin, the 4EBPs are phosphorylated on several sites (Brunn, Hudson et al. 1997; Hara, Yonezawa et al. 1998), allowing the eIF4E-mRNA complex to bind the 43S pre-initiation complex. During starvation, 4EBPs are dephosphorylated and sequester eIF4E from the initiation complex, disabling protein translation.

Amino acids also control a second aspect of translation by modulating the activity of p70-S6 kinase (p70-S6K). In the presence of amino acids (Iiboshi, Papst et al. 1999) or growth factors (Chung, Grammer et al. 1994), p70-S6K activation results in increased phosphorylation of the S6 ribosomal subunit (Wettenhall, Chesterman et al. 1983; Blenis, Kuo et al. 1987). It was initially

believed that p70-S6K regulated the translation of a specific subset of mRNAs encoding ribosomal genes (Loreni, Thomas et al. 2000), but recent genetic studies have cast doubt upon this reported function (Barth-Baus, Stratton et al. 2002; Pende, Um et al. 2004). p70-S6K is phosphorylated at residue T389 in a rapamycin-sensitive manner (Chung, Grammer et al. 1994), a crucial event in the activation of p70-S6K. p70-S6K may also play a role in feedback inhibition of the mTOR (Chiang and Abraham 2005) and growth factor signaling pathways (Tremblay, Krebs et al. 2005) by direct phosphorylation of substrates. By controlling the activity of competitive inhibitors (4EBPs) and enzymatic activity (mGCN2 and p70-S6K), amino acids are not only the essential building blocks of proteins, but also the ligands regulating intracellular signaling pathways controlling this biosynthetic process.

2. Mammalian Target of Rapamycin

The mTOR pathway is a nutrient-responsive signal transduction cascade (Fig. 1) involved in regulation of cellular growth (Martin and Hall 2005). An extremely large protein (2549 amino acids), mTOR is classified as a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family. Members of this family possess a catalytic domain most closely related to those of lipid kinases but do not possess intrinsic kinase activity towards phospholipids. mTOR functions as a serine/threonine protein kinase and *in vitro* assays suggest mTOR may be a *bona fide* protein kinase (Ali and Sabatini 2005). The N-terminal domain of mTOR consists of 20 HEAT (Huntington-Elongation Factor 1A-protein

phosphatase 2A A subunit-TOR) repeats (Andrade and Bork 1995). Each HEAT repeat forms a pair of antiparallel α-helices that stack in a parallel fashion to form an ordered array of hydrophobic surfaces (Groves, Hanlon et al. 1999). The large number of HEAT repeats, ideal for protein-protein interactions, and the large size of mTOR (289 kDa) suggest this protein functions as part of a large signaling complex.

mTOR kinase activity correlates with positive cell growth and is activated in the presence of nutrients (i.e., amino acid and growth factors). mTOR activity appears to be governed by a series of phosphorylation events and association with specific binding partners (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002). Deprivation of nutrients leads to reduction of mTOR activity and inactivation of downstream effectors (Hay and Sonenberg 2004). mTOR is also be inhibited by the bacterial compound rapamycin (Sabers, Martin et al. 1995). Treatment of cells with rapamycin inhibits protein synthesis and blocks the cell cycle at the same checkpoint induced by amino acid withdrawal (Brown, Albers et al. 1994). Inhibition of mTOR by rapamycin requires the immunophillin FKBP12 (Sabers, Martin et al. 1995), and the FKBP12/rapamycin complex binding site has been mapped to a region termed the FKBP12/rapamycin binding (FRB) domain, located close to the catalytic domain (Helliwell, et al., 1994). Recently, the mTOR-interacting protein raptor (regulatory associated protein of mTOR) has been shown to be essential for mTOR activity and studies suggest that rapamycin induces dissociation of the mTOR/raptor complex (Oshiro, Yoshino et al. 2004). This dissociation uncouples mTOR activity from substrates, indicating that the

regulation of signaling pathways by mTOR is dependent upon the proper co-localization of mTOR kinase activity with target proteins.

3. Activation of mTOR by Nutrients

Initially believed to act as an intracellular amino acid sensor, it now appears that mTOR functions as a signal transduction "hub" within a complex signaling pathway regulated by both amino acids and growth factors (Oldham, Stocker et al. 2002) (Fig. 1). The activation of mTOR by amino acids is independent of PI3K activity, but lipid second messengers play an important, but indirect, role in activation of mTOR. Recent evidence has implicated a nutrient-regulated lipid kinase, hVps34, in the regulation of mTOR-dependent p70-S6K activity (Byfield, Murray et al. 2005) and mTOR itself may be activated by phosphatidic acid generated by phospholipase D1 (Fang, Park et al. 2003). It is still unknown how amino acids activate mTOR and where amino acid-sensing pathways detect ligand. Studies in adipocytes showed mTOR responds preferentially to the branched-chain amino acids leucine and valine, but searches for extracellular leucine receptors have come up empty (Lynch, Fox et al. 2000). The identification of the amino acid-sensing component responsible for mTOR activation will be an important step forward in the understanding of nutrient signaling.

Even though the amino acid sensor has eluded researchers, identifying processes regulated by amino acids and rapamycin has provided crucial insights into the regulation of mTOR activity. mTOR exists *in vivo* as part of a large

macromolecular complex and association of mTOR with several binding partners is dependent upon the presence of amino acids (Oshiro, Yoshino et al. 2004). Two different mTOR-binding proteins, raptor and mLST8 (also known as GβL), were identified (Kim, Sarbassov et al. 2002). Depletion of raptor by RNA interference (RNAi) in 293T cells reduced cell size and decreased phosphorylation of p70-S6K (Hara, Maruki et al. 2002). 4EBP-1 and p70-S6K were co-immunoprecipitated with raptor, indicating that these effectors interact with mTOR through binding to raptor (Nojima, Tokunaga et al. 2003). These observations suggest that raptor functions to position and localize substrates for mTOR to phosphorylate during growth.

Upstream of mTOR lies the TSC (Tuber Sclerosis Complex) comprised of the two proteins hamartin and tuberin (also known as TSC1 and TSC2, respectively). The TSC complex regulates cell growth by inhibiting the activity of mTOR in a nutrient-dependent manner (Gao, Zhang et al. 2002). TSC regulates growth through modulating the activity of the small G protein Rheb (Ras homologue enriched in brain) via a putative GTPase activating protein (GAP) domain in TSC2 (Tee, Anjum et al. 2003; Zhang, Cicchetti et al. 2003). TSC2-mediated down-regulation of Rheb results in decreased phosphorylation of T389 on p70-S6K, hypophosphorylated 4EBPs and reduced protein translation. In the presence of nutrients, TSC2 is phosphorylated on several sites and GAP activity is suppressed. Inactivation of TSC2 GAP activity permits Rheb-mediated activation of mTOR, resulting in p70-S6K phosphorylation (Stocker, Radimerski et al. 2003). Rheb binds to mTOR in a GTP-dependent manner (Long, Lin et al.

2005), but exactly how the TSC complex regulates mTOR activity via Rheb is still unknown. What is clear is that proper TSC function is required for normal cell growth; mutations affecting TSC activity result in the formation of benign tumors involving multiple organ systems (Astrinidis and Henske 2005). By regulating mTOR activity, the TSC heterodimer plays an important role in the regulation of cell growth and tumorigenesis.

Peptide growth factors such as insulin and epidermal growth factor stimulate phosphorylation of p70-S6K though both mTOR and PI3K-dpendent processes. Treatment of cells with insulin increases phosphorylation of T389 above the levels induced by amino acids (Lizcano, Alrubaie et al. 2003) and several sites in a putative auto-inhibitory domain of p70-S6K are phosphorylated in the presence of serum (Dennis, Pullen et al. 1998). Activation of p70-S6K by insulin occurs through the PI3K-dependent pathway linking the insulin receptor (IR) to intracellular signaling components. Production of the lipid mediator phosphatidylinositol 3,4,5-trisphosphate (PIP_3) results in recruitment of protein kinase B (PKB, also known as Akt) and phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane via binding of PIP_3 to pleckstrin homology (PH) domains on both enzymes (Franke, Yang et al. 1995; Alessi, James et al. 1997). PDK1 phosphorylates PKB on in the activation T-loop at Ser308 (Wick, Wick et al. 2002), stimulating PKB to phosphorylate TSC2 on Ser-939 and Thr-1462, relieving TSC-mediated repression of mTOR signaling (Tee, Anjum et al. 2003; Inoki, Ouyang et al. 2005). Activation of mTOR increases T389 phosphorylation, producing a docking site on p70-S6K for PDK1 (Biondi,

Kieloch et al. 2001). Once bound to phospho-T389, PDK1 phosphorylates p70-S6K in the activation T-loop at T229 (Pullen, Dennis et al. 1998; Romanelli, Dreisbach et al. 2002). Therefore, regulation of protein synthesis by growth factors not only activates downstream effectors via a PI3K-dependent mechanism, but also relieves TSC-mediated repression of mTOR. In this manner, amino acids and growth factors work in concert by cross-talk between the mTOR- and PI3K-mediated signaling pathways to stimulate protein translation.

4. Conservation of Nutrient Signaling

The TOR signaling pathway is conserved from yeast to humans and the core components of the pathway are found in many eukaryotic systems (Long, Spycher et al. 2002; Jia, Chen et al. 2004). Homologues of TOR have been identified in humans (Sabatini, Erdjument-Bromage et al. 1994), *Drosophila* (Oldham, Montagne et al. 2000), plants (Menand, Desnos et al. 2002), nematodes (Vellai, Takacs-Vellai et al. 2003) and yeast (Barbet, Schneider et al. 1996). Genetic and pharmacological studies have showed the protein products of these TOR genes are essential for cell growth and viability. In all organisms, loss of TOR activity results in decreased cell growth and protein synthesis. Genetic analysis of TOR function in *Drosophila* reveals the fly homolog of TOR (dTOR) is essential for developmental growth (Oldham, Montagne et al. 2000). Studies in *Caenorhabditis elegans* (Vellai, Takacs-Vellai et al. 2003) and *Drosophila* (Kapahi, Zid et al. 2004) suggest that TOR activity may also influence chronological lifespan. *Sacchromyces cerevisiae* also possesses a TOR-controlled

nutrient signaling pathway. Yeast TOR regulates the phosphorylation state of downstream effector proteins Npr1 and the transcription factor Msn2α (Beck and Hall 1999). The yeast TOR pathway differs from higher eukaryotes in that yeast does not possess a homolog of p70-S6K or 4EBP-1. For the purpose of this study, we chose to study nutrient signaling pathways regulated by dTOR and will thus focus upon these pathways in detail.

To date, the growth pathways characterized in *Drosophila* appear closely related to the pathways characterized in mammalian cells (Neufeld 2004). The availability of genetic manipulation in *Drosophila*, either by the selective breeding of flies or depletion of genes by RNA interference has provided an extra level of insight into the function of these pathways. The fly amino acid-responsive pathway includes orthologs of mTOR (Oldham, Montagne et al. 2000; Zhang, Stallock et al. 2000), 4E-BP1 (Miron, Verdu et al. 2001), the TSC complex (Dobens, Hsu et al. 1997), α4/Tap42 (Cygnar, Gao et al. 2005) and p70-S6K (Stewart, Berry et al. 1996; Watson, Chou et al. 1996), as well as a PI3K-dependent signaling cascades involving PTEN, PDK1 and PKB/Akt (Scanga, Ruel et al. 2000; Radimerski, Montagne et al. 2002). Genetic studies have confirmed a role for the components of the dTOR pathway in the regulation of cell size (Gao, Zhang et al. 2002). Importantly, the *Drosophila* homologue of p70-S6K (dS6K) is required for normal cell growth (Montagne, Stewart et al. 1999). Embryonic fruit flies lacking the dS6K gene exhibited an extreme delay in development, lower body weight and possessed cells of much smaller size than wild-type flies.

5. Regulation of p70-S6K by Phosphorylation

Considering the complexity of the TOR-regulated pathways, it is not surprising that regulation of downstream effectors also requires multiple signaling inputs. It is now well accepted that p70-S6K lies at what can be considered a "bottleneck" of several signal transduction pathways governed positively by amino acids (TOR) and growth factors (insulin and epidermal growth factor), but also sensitive to negative input from hormone and cellular stress pathways (Inoki, Ouyang et al. 2005). The ability of p70-S6K to respond to numerous signals requires a strict control of activation of the kinase, as unabated activity would result in constitutive ribosomal biogenesis, a heavy taxation on cellular energy and metabolic supplies.

Early structural and functional studies on the activation of mammalian p70-S6K revealed several intriguing characteristics of this mitogen-activated protein kinase. It was discovered that S6 kinase activity could be maintained in cell extracts in the presence of protein phosphatase inhibitors (Ballou, Jeno et al. 1988) and treatment with purified Protein Phosphatase 2A (PP2A) catalytic subunit efficiently abolished this kinase activity (Jeno, Ballou et al. 1988). These results strongly supported the hypothesis that p70-S6K was regulated by a cycle of phosphorylation and dephosphorylation. Indeed, eight phosphorylation sites on p70-S6K have been mapped and phosphorylation at these sites vary in response to amino acids or mitogens (Proud 2002), but each with a specific role in what appears to be a multistep process of activation.

Analysis of mammalian p70-S6K primary amino acid sequence suggested the protein can be divided into four different modules (Fig. 2A), each necessary for appropriate kinase activity (Pullen and Thomas 1997). Module I consists of the N-terminal portion of the protein immediately downstream of the catalytic domain. This module has been demonstrated to confer rapamycin sensitivity to p70-S6K (Cheatham, Monfar et al. 1995). The catalytic domain comprises Module II and contains the activation T-loop phosphorylation site at T229. Module III contains two phosphorylation sites, T389 and S404. Module IV, the C-terminus of p70-S6K, acts as an autoinhibitory pseudosubstrate (Price, Mukhopadhyay et al. 1991; Cheatham, Monfar et al. 1995). Interestingly, this region contains considerable sequence homology with the substrate region of the S6 ribosomal subunit. This module contains four serum-sensitive phosphorylation sites, S411, S418, T421 and S424. Phosphorylation at these sites results in increased levels of kinase activity (Cheatham, Monfar et al. 1995).

Mutational studies on exogenously expressed p70-S6K have presented an interesting view of how this kinase is controlled. Under conditions unfavorable for growth, p70-S6K is kept in an inactive state by the binding of autoinhibitory Module IV to the N-terminal Module I (Fig. 2B). This conformation represses kinase activity by preventing phosphorylation of T229 and T389. Phosphorylation of the four clustered sites in Module IV disrupts interaction with Module I, exposing the two phosphorylation sites within Module III (T389 and S404). Phosphorylation at T389 is catalyzed in a mTOR-dependent manner and provides a docking site for PDK1. PDK1 phosphorylates T229 in the activation

T-loop, resulting in fully active p70-S6K (Balendran, Currie et al. 1999). This model is supported by previous observations from mutational studies that showed substituting phosphomimetic residues into these sites increases p70-S6K activity (Cheatham, Monfar et al. 1995). This scheme demonstrates how cross-talk between the mTOR and growth factor pathways results in regulated ribosomal biogenesis. Lack of input from either pathway would result in lowered or incomplete activation of the kinase.

Identification of the *Drosophila* homolog of p70-S6K (dS6K) demonstrated conservation of the overall architecture of the kinase in fruit flies (Watson, Chou et al. 1996), as dS6K can be dissected into four modules corresponding closely to those found in human p70-S6K. Alignment of dS6K primary amino acid sequence with mammalian p70-S6K reveals that *Drosophila* S6K shares only 46% overall identity, with most of the disparity found in the regions comprising Modules I and IV. However, alignment of Modules II and III show 71% identity, demonstrating high functional conservation of the catalytic and linker domains. *Drosophila* S6K also possesses the activation T-loop phosphorylation site at T238 in Module II, as well as the T389 phosphorylation site (T398 in dS6K) in Module III. Interestingly, dS6K lacks the serum-responsive S404 and T421 phosphorylation sites (proline and alanine substitutions are found at the two corresponding residues, P413 and A473), but has a threonine in place of serine at the position corresponding to S411 (T422) and the S418 and S424 phosphorylation sites are conserved (S429 and S466 in dS6K). The lack of conservation of the two regulatory sites (S404 and T421), coupled with the

disparity of sequence in Modules I and IV, suggests dS6K is likely to be activated in a similar manner to the human protein, but some fundamental differences in regulation may exist.

B. Protein Phosphatases

1. Role of Protein Phosphatases in Nutrient Signaling

If p70-S6K is activated by phosphorylation, then logic dictates that it is inactivated by dephosphorylation of the critical residues in Modules II-IV. Accordingly, dephosphorylation of these sites coincides with a loss of p70-S6K activity and mutation of these sites to non-phosphorylatable residues severely impairs p70-S6K function (Cheatham, Monfar et al. 1995). Since all phosphorylation sites on p70-S6K are serine or threonine residues, dephosphorylation should be catalyzed by serine/threonine phosphatases. Studies utilizing broad range phosphatase inhibitors indicate that this phosphatase is of the PP2A-like family (Peterson, Desai et al. 1999). Several phosphorylation sites on p70-S6K, especially T389, are sensitive to phosphatase inhibitors and blockade of phosphatases prevents dephosphorylation of these sites during starvation (Grove, Banerjee et al. 1991), cellular stress (Parrott and Templeton 1999), or pharmacological inhibition of mTOR (Chung, Kuo et al. 1992). However, a role for specific phosphatases could not be assigned from the results of these studies, as the members of the PP2A-like family have similar sensitivity to okadaic acid (OA) and calylculin A (CL-A) (Janssens and Goris 2001). In addition, another major serine/threonine protein phosphatase, PP1, is 2-fold less sensitive to CL-A

and 100-fold less sensitive to OA than PP2A-like family members (Ishihara, Martin et al. 1989), but would still be affected by the concentrations utilized in most studies.

Studies examining nutrient signaling in *S. cerevisae* have provided some of the best insights into the role of protein phosphatases in regulating growth pathways (Zabrocki, Van Hoof et al. 2002). In response to abundant nitrogen sources (i.e., glutamine), TOR controls protein synthesis through activation of the eIF4E translation factor (Altmann, Schmitz et al. 1997), but no yeast homolog of 4EBP-1 has been identified. In addition to regulation of translation, TOR also controls the transcription of numerous genes encoding ribosomal protein subunits (Powers, Dilova et al. 2004). TOR regulates transcription in yeast by phosphorylating several transcription factors (including Gln3 and Msn2α), resulting in sequestration of these factors in the cytoplasm (Beck and Hall 1999). In yeast, TOR kinase activity is not only required for eIF4E-mediated translation, but also as a mechanism to antagonize transcription of genes required for survival in low nitrogen conditions.

S. cerevisiae has provided a powerful system for genetic studies on the role of phosphatases in nutrient signaling. Sequencing of the yeast genome has revealed up to thirty-two putative phosphatase genes and at least five genes closely resemble mammalian PP2A-like phosphatases. The yeast PP2A catalytic subunits are redundantly encoded by two genes, named *PPH21* and *PPH22*, and display a high level of sequence identity to the human PP2A catalytic subunit (Sneddon, Cohen et al. 1990). *PPH21* and *PPH22* protein products form

heterotrimeric complexes with the yeast homologues of the PP2A-A subunit (*TPD3*) and two variable regulatory (B) subunits, encoded by the *CDC55* and *RTS1* genes. Deletion of both *PPH21* and *PPH22* results in a severe reduction in growth and loss of most of the phosphatase activity within the cell (Ronne, Carlberg et al. 1991; Lin and Arndt 1995; Evans and Stark 1997). Additional deletion of a third PP2A-like gene, *PPH3,* results in cell death (Ronne, Carlberg et al. 1991). Studies on *RTS1* (Shu, Yang et al. 1997), *CDC55* (Wang and Burke 1997), and *TPD3* (van Zyl, Huang et al. 1992) showed PP2A regulates the cell cycle, morphology and cytokenesis, as well as cell growth. These studies also indicate that yeast PP2A functions as a trimer comprised of a scaffolding subunit (Tpd3), regulatory subunits (Rts1/Cdc55) and a catalytic subunit (Pph21/22). *In vivo* substrates for Pph21/22 have yet to be identified, but over-expression of Pph22 mimics hyperactive PKA (protein kinase A) signaling and causes an exaggerated swollen phenotype (Ronne, Carlberg et al. 1991), suggesting a role for yeast PP2A in regulation of growth pathways.

2. Tap42 as an Inhibitor of Phosphatase Activity

Yeast PP2A can also form a heterodimeric complex with the *TAP42* gene product (Tap42). Tap42 was initially described as a high copy suppressor of a temperature-sensitive *SIT4* allele, the yeast homolog of protein phosphatase 6 (PP6). Tap42 binds both Pph21/22 independently of the Tpd3 and Rts1/Cdc55 subunits (Di Como and Arndt 1996). Cells possessing a temperature-sensitive allele of *TAP42* (*tap42-11*) displayed a reduction in growth and conferred partial

rapamycin resistance (Di Como and Arndt 1996). Association of Tap42 with Pph21/22 is sensitive to rapamycin (Jiang and Broach 1999), although these results remain somewhat controversial. Association of Tap42 with Pph21/22 decreased in the presence of drug, suggesting Tap42 function within the yeast cells is to inhibit PP2A-like phosphatase activity and the formation of this complex is dependent upon upstream TOR kinase activity. These results suggest that Tap42 may be a component of the mechanism by which TOR represses phosphatase activity. However, it was estimated that less than 2% of Pph21 is in complex with Tap42 in log phase cells, therefore the regulation of Pph21 by Tap42 is not a broad mechanism of phosphatase regulation throughout the cell. Tap42 can also associate with the catalytic subunit of the PP2A-like phosphatase Sit4 (Di Como and Arndt 1996), suggesting that Tap42 may have multiple functions within the cell. Accordingly, roles for Tap42 in regulation of translation (Cherkasova and Hinnebusch 2003; Rohde, Campbell et al. 2004), transcription (Beck and Hall 1999; Shamji, Kuruvilla et al. 2000; Duvel, Santhanam et al. 2003), and actin cytoskeleton integrity (Torres, Di Como et al. 2002) have been recently reported.

The high level of TOR pathway conservation was again confirmed when the human homologue of Tap42 was identified as α4 (also known as *IGBP-1*), a 52-kDa phosphoprotein initially reported as a component of the B cell receptor (BCR) complex (Kuwahara, Matsuo et al. 1994). Comparisons of Tap42 and *a*4 primary amino acid sequences show these proteins share 28% identity. α4 complexes with human PP2A catalytic subunit independently of the A or B

subunits (Murata, Wu et al. 1997), as well as binding to PP4 and PP6 catalytic subunits (Nanahoshi, Tsujishita et al. 1999; Kloeker, Reed et al. 2003), two PP2A-like phosphatases with high sequence identity to PP2Ac. The rapamycin sensitivity of *a*4/phosphatase association is still unresolved (Inui, Sanjo et al. 1998; Kloeker, Reed et al. 2003), but α4 is presumed to be the human homologue of Tap42. Recent studies have demonstrated *a*4 is an essential gene; embryonic stem cells missing both alleles of α4 underwent apoptosis (Kong, Fox et al. 2004). Other studies suggest that *a*4 plays a role in B cell activation and antigen-specific signal transduction (Inui, Maeda et al. 2002). Interestingly, B cells devoid of α4 also displayed rapamycin sensitivity and decreased p70-S6K activation. Other studies suggest α4 is required for early development in T cells (Hua, Inui et al. 2003). However, it is unknown at this point if the apoptosis and defects in immune cell activation observed with loss of *a*4 is a direct effect due to reduction of α4 protein levels. To date, homologues of Tap42 have been described in *Arabidopsis* (Harris, Myrick et al. 1999) and *Drosophila* (Cygnar, Gao et al. 2005). The presence of Tap42 homologues in higher eukaryotes and the similar properties displayed by α4 suggest that repression of phosphatase activity by TOR may operate through an evolutionarily conserved mechanism.

3. Protein Phosphatase 2A

PP2A is a multifunctional protein phosphatase involved in numerous intracellular events (Janssens, Goris et al. 2005). *In vivo*, PP2A exists as a heterotrimeric complex consisting of a scaffolding A subunit, the catalytic C

subunit that confers phosphatase activity, and variable regulatory B subunits. To date, three families of B subunits (B, B56 and B") have been described (Janssens and Goris 2001). These regulatory subunits target PP2A to discrete subcellular locales. Many of the protein-protein interactions with substrate likely occur through the B subunits and evidence exists that binding of a B subunit is required for stability of the A and C dimer (Li, Scuderi et al. 2002; Silverstein, Barrow et al. 2002). Therefore, it is likely that a majority of a cell's functional PP2A activity is in the form of heterotrimeric complexes. Mammals express two isoforms of the A and C subunits and many of the B subunit mRNA transcripts undergo splicing, resulting in a number of different possible combinations of PP2A subunits. However, the *in vivo* functions of most of these phosphatase complexes remain unknown. Ideally, PP2A is best described as a family of heterotrimeric serine/threonine phosphatases with the common components of the A and C core dimer.

PP2A A and C are ubiquitously expressed in mammals, while some B subunits are expressed only in certain tissues (Janssens and Goris 2001). PP2A accounts for a majority of intracellular phosphatase activity and numerous studies have shown PP2A activity is required for cellular survival (Klumpp and Krieglstein 2002). Deletion of the C subunit gene resulted in embryonic lethality in mice (Gotz, Probst et al. 1998) and prolonged treatment of cells with PP2A inhibitors results in cell death (Kiguchi, Glesne et al. 1994; Inomata, Saijo et al. 1995) and cell cycle arrest (Zheng, Woo et al. 1991; Ishida, Furukawa et al. 1992). It has been estimated that as much as 99% of all phosphorylation events

within a cell occur on serine or threonine residues within proteins. Since the number of PP2A genes is markedly smaller than the number of serine/threonine kinase genes (Hunter 1995), mammalian PP2A likely acts as a generic phosphatase activity, where specificity is provided through complex interactions with numerous substrates.

Analysis of the *Drosophila melanogaster* genome has identified what appears to be a highly conserved set of PP2A subunit genes. The scaffolding and catalytic subunits are expressed from only one allele each (Chen, Chen et al. 1992). Homologues of the three B subunit families are found, with the B56 family possessing two related genes (B56-1 and B56-2). It is unknown if the two B56 genes are redundant, however knockdown studies suggest independent functions for each gene (Berry and Gehring 2000; Hannus, Feiguin et al. 2002; Sathyanarayanan, Zheng et al. 2004). To date, a total of seven PP2A subunits have been identified in the *D. melanogaster* genome, much fewer than found in mammals (Chen, Chen et al. 1992). However, this simplified version of PP2A signaling affords researchers the opportunity to narrow down functions of specialized PP2A heterotrimers.

Genetic studies of PP2A in both whole flies and *Drosophila* cell cultures have identified a number of interesting and essential functions for PP2A. In agreement with mammalian studies (Gotz, Probst et al. 1998), loss of C subunit results in embryonic lethality in flies and prevents attachment of microtubules to centrosomes (Snaith, Armstrong et al. 1996). Studies on *Drosophila* B subunits have revealed specific roles for regulatory subunits in flies. *Twins*, the fly Bα

homolog, regulates β-catenin (*Armadillo* in flies) protein levels in response to the Wnt signaling pathway (Bajpai, Makhijani et al. 2004). There is also evidence that a *Twins*-associated PP2A heterotrimer regulates transcription by regulating the phosphorylation state of histone H3 (Nowak, Pai et al. 2003). Loss of a single B56 gene does not appear to negatively impact cellular processes, but knockdown of both B56 genes in *Drosophila* S2 cells results in apoptosis (Li, Scuderi et al. 2002; Silverstein, Barrow et al. 2002). However, B56-1 is required for regulation of the transcription factor *Sex combs reduced* (Berry and Gehring 2000), a gene required for proper segmentation in embryonic flies (Hughes and Kaufman 2002). The B56-2 homologue, known as *Widerborst*, also regulates fly development (Hannus, Feiguin et al. 2002) and circadian rhythm (Sathyanarayanan, Zheng et al. 2004). It is worthy to note that several of these studies identified PP2A subunits through interaction screening and only recovered one member of the B56 family each. These results support the conclusion that the *Drosophila* B56 family of regulatory subunits is comprised of two genes, each with a specific function. The fourth B subunit, dmPR72, has not been studied in detail and *in vivo* functions of this PP2A subunit remain unknown. However, loss of dmPR72 does not result in apoptosis or affect cell growth in S2 cells (Li, Scuderi et al. 2002; Silverstein, Barrow et al. 2002).

Other members of the PP2A-like family are present in the *D. melanogaster* genome. Homologues of PP4/Pph3 (dPP4) and PP6/Sit4 (dPP6) catalytic subunits have been cloned (Cohen, Brewis et al. 1990; Mann, Dombradi et al. 1993) and a small number of knockdown studies have been carried out on

the dPP4 gene. Loss of dPP4c is semi-lethal to flies and resulted in the phenotype described as *centrosomes minus microtubules* (*cmm*). Early fly embryos lacking dPP4c displayed centrosomes unable to connect to the mitotic spindle, resulting in a block in mitosis (Helps, Brewis et al. 1998). RNAi studies in S2 cells reported that, unlike PP2Ac, loss of dPP4 catalytic subunit did not result in apoptosis, but reduced cell growth by 20% (Silverstein, Barrow et al. 2002). These results, in combination with the identification of a PP4-interacting regulatory (R) subunit in the fly genome (Hastie, Carnegie et al. 2000) support the hypothesis that dPP4c is indeed a component of functional phosphatase complexes *in vivo*.

4. Sequestration Model of dTap Activity

It has been proposed that Tap42 acts as an inhibitor of PP2A function in yeast (Zabrocki, Van Hoof et al. 2002). This view is supported by the observations that Tap42 associates with PP2A independently of regulatory subunits and interaction of Tap42 with PP2Ac is sensitive to the presence of rapamycin (Di Como and Arndt 1996). These proposals can be summarized as a "sequestration" model of Tap42 function (Fig. 3). During cell growth, Tap42 is phosphorylated in a TOR-dependent manner (Jiang and Broach 1999), driving association with phosphatase catalytic subunit. This binding of Tap42 with PP2Ac sequesters enzymatic activity away from the A and B subunits, preventing PP2A from dephosphorylating substrate. During periods of starvation, Tap42 is dephosphorylated, presumably by another PP2A trimer, reducing the stability of the complex. This reduction in affinity of Tap42 for PP2Ac allows catalytic

subunit to re-form trimeric PP2A with A and B regulatory subunits. This PP2A trimer then proceeds to dephosphorylate appropriate substrate. Therefore, loss of Tap42 would result in increased phosphatase activity and hypophosphorylation of substrate.

The ease of RNA interference in cultured *Drosophila* cells has allowed for dissection of signal transduction pathways by selective ablation of genes (Clemens, Worby et al. 2000). Since other PP2A-like phosphatases share roughly the same sensitivity to inhibitors as PP2A (reviewed in Cohen, 1997), assigning functions to specific phosphatase family members has been difficult. Indeed, perhaps some of the functions assigned to PP2A in previous inhibitor studies may actually be carried out by PP4 or PP6. In mammals, the high number of B subunit splice variants increases the likelihood of redundancy, potentially complicating knockdowns and functional studies. Fortunately, the reduced number of PP2A genes in *D. melanogaster* implies that knockout of a single gene will provide insight into the functions of an entire PP2A family. Addition of okadaic acid or calylculin A to cells will inhibit all PP2A heterotrimers, regardless of the bound B subunit. In contrast, targeted knockdown of PP2A regulatory subunits will eliminate the broad array of non-specific phosphatase inhibition. This provides the exciting possibility of narrowing down the specific regulatory subunit family responsible for dephosphorylation of T398 of dS6K.

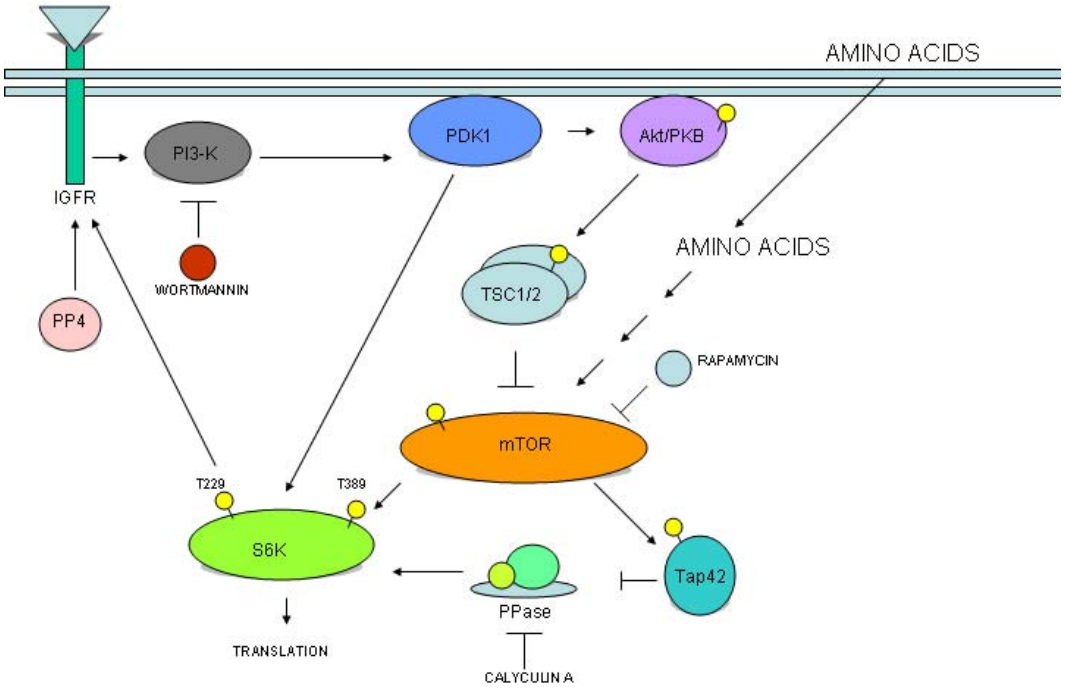

Figure 1. Protein synthesis is regulated by several signaling inputs. Growth factors stimulate protein translation by cell surface receptors and PI3K-dependent mechanisms, whereas amino acids activate mTOR through an unknown mechanism involving the TSC complex. The activity of mTOR is also regulated by intracellular energy levels.

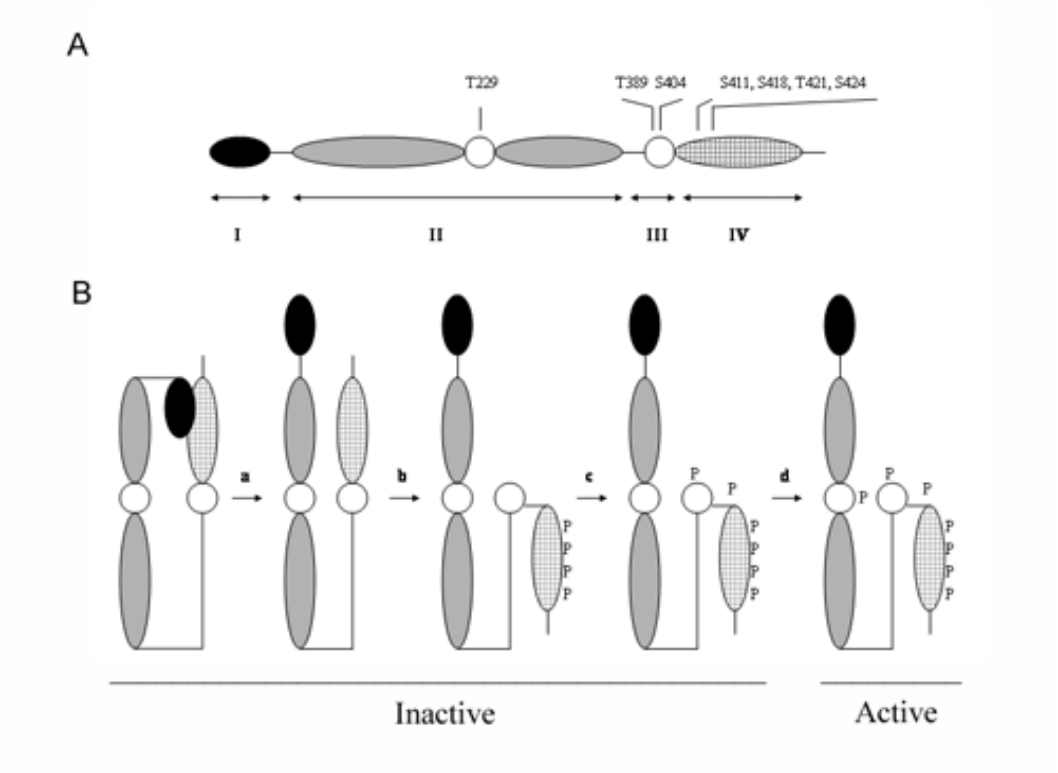

Figure 2. Modular regulation of p70-S6K by phosphorylation. (A) Schematic diagram representing the four modules of mammalian p70-S6K. (B) Proposed mechanism of S6K activation. Interactions between Modules I and IV prevent phosphorylation of the T-loop in the p70-S6K catalytic domain. This repressive conformation is relieved by phosphorylation of the four clustered residues in Module IV, exposing the T389 and S404 residues in Module III. Phosphorylation of T389 by an mTOR-dependent mechanism provides a docking side for PDK1. PDK1 phosphorylates p70-S6K in the activation T-loop at T229, resulting in full activity of the kinase. Adapted from Pullen and Thomas, 1997.

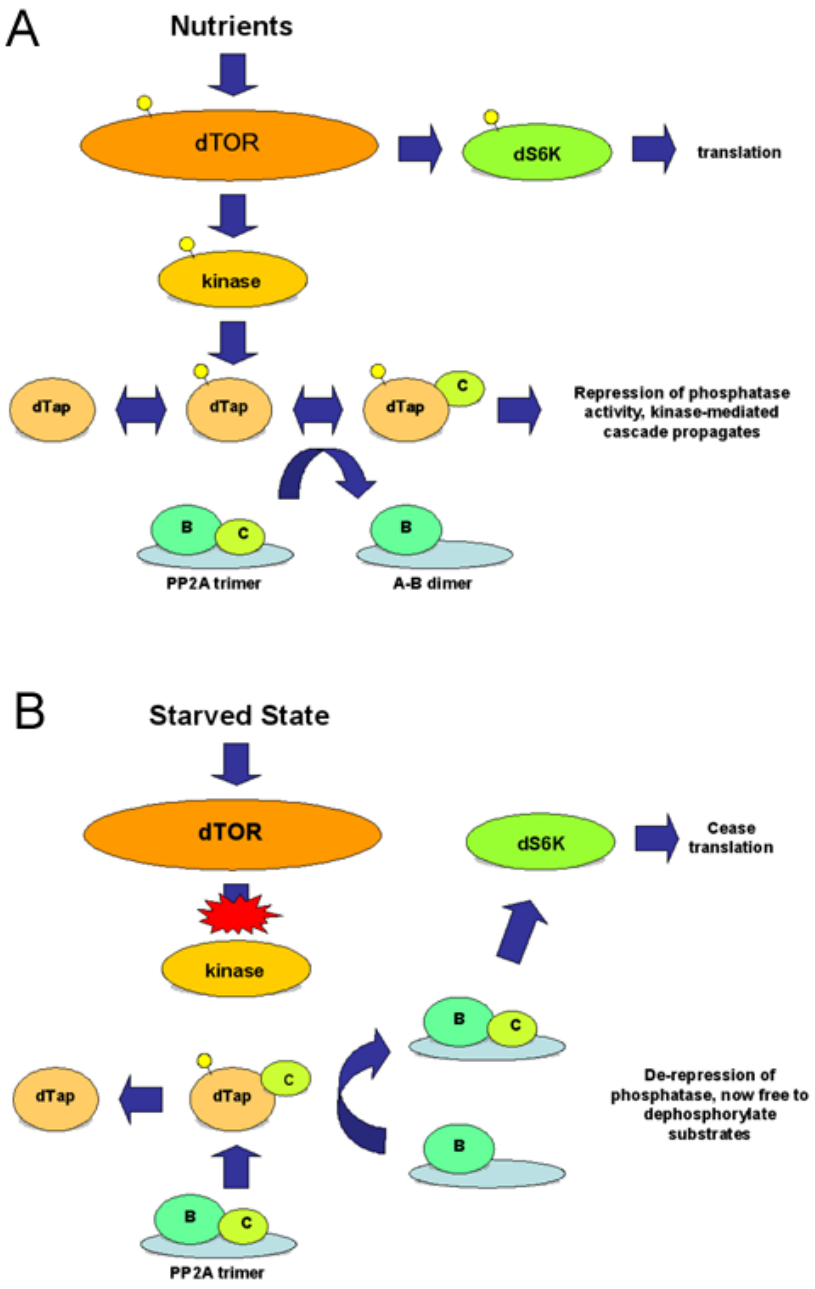

Figure 3. Sequestration model of dTap-mediated repression of PP2A activity. dTap is proposed to act as an inhibitor of PP2A by sequestering the enzyme away from regulatory subunits of PP2A that target PP2A to phosphoproteins, including S6 kinase (S6K) (top panel). In the presence of adequate nutrients, the TOR-dependent phosphorylation of dTap drives association with PP2A catalytic subunit. Under conditions of low nutrient availability, inhibition of TOR activity results in dephosphorylation of dTap, allowing PP2A catalytic subunit to re-form trimeric PP2A (bottom panel). This PP2A trimer then catalyzes dephosphorylation of dS6K at residue T398.

Chapter 2

Regulation of Drosophila S6 Kinase by Nutrients

A. Introduction

In *Drosophila*, amino acids activate dS6K through a rapamycin-sensitive pathway regulated by dTOR (Stewart, Berry et al. 1996). Phosphorylation of T398 is dependent upon the presence of free amino acids and can be stimulated by insulin and serum components through a PI3K-dependent pathway (Radimerski, Montagne et al. 2002; Lizcano, Alrubaie et al. 2003). The effect of amino acids on dS6K activation is blocked by rapamycin, resulting in dephosphorylation of dS6K. What is not completely clear is the question of whether signaling by insulin to dS6K requires the activity of amino acid-responsive pathways. Insulin can increase phosphorylation of T398 in the presence of amino acids but its effects in the absence of amino acids are not entirely clear. Early studies on *Drosophila* nutrient signaling suggested that activation of dS6K by insulin in Kc.167 cells requires amino acids (Radimerski, Montagne et al. 2002), but Lizcano and collegues demonstrated in both Kc.167 and Schneider S2 cells that insulin stimulates phosphorylation of dS6K at T398 in cells starved of amino acids (Lizcano, Alrubaie et al. 2003). The crucial element of this puzzle yet to be resolved is whether active dTOR is required for insulin-stimulated activation of dS6K.

This chapter had three goals, 1) determine the requirements for phosphorylation of dS6K by amino acids and insulin by using the phosphorylation state of T398 as a marker, 2) determine time courses of dephosphorylation and

activation of dS6K at T398 for use in future experiments, and 3) verify that dephosphorylation of T398 in *Drosophila* S6K is catalyzed by a calylculin A-sensitive phosphatase.

B. Experimental Procedures

1. Materials

Rapamycin, wortmannin, amino acids and insulin were purchased from Sigma. Calyculin A was purchased from Cell Signaling Technology (Danvers, MA). Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA). ECL and ECL Plus were purchased from Amerhsam (Arlington Heights, IL). Autoradiographic film was purchased from Phenix Research Products (Hayward, CA).

2. Antibodies

Anti-phospho-p70 S6K (T389) (1A5) and anti-β-actin were purchased from Cell Signaling Technology (Danvers, MA). A polyclonal anti-*Drosophila* S6K antibody (Ab92) was a generous gift from Mary Stewart (North Dakota State University, Fargo, ND). Anti-PP2Ac (1D6) was purchased from Upstate Biotechnology (Charlottesville, VA). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham (Arlington Heights, IL). Anti-mouse Alexfluor467 secondary antibody was purchased from Molecular Probes (Eugene, OR).

3. Cell Culture

Schneider S2 cells (a gift of T. MeGraw, UTSW, Dallas, TX) were maintained in Schneider's *Drosophila* Medium (SDM) supplemented with 10% FBS. SDM was prepared as described previously (Echalier, 1997), with peptone omitted from the recipe. For amino acid starvation, cells were resuspended in an SDM solution lacking amino acids, peptone and yeastolate (SDM Minus). Both yeastolate and peptone contain amino acids; therefore these materials were omitted from starvation medium. The media used in restimulation assays were SDM Minus supplemented with either 1x amino acids (SDM+AA) or 1uM insulin (SDM+Ins), or a mixture of both (SDM+AA+Ins). The concentration of each amino acid designated as 1x is as follows (in mg/L): beta-Ala, 500; Gly, 250; L-Arg, 400; L-Asp, 400; L-Cys, 60; L-Cystine, 100; L-Glu, 800; L-Gln, 1800; L-His, 400; L-Ile, 150; L-Leu, 150; L-Lys, 1650; L-Met, 800; L-Phe, 150; L-Pro, 1700; L-Ser, 250; L-Thr, 350; L-Trp, 100; L-Tyr, 500; L-Val, 300. A mixture of these amino acids, all at the concentrations indicated above, is defined as a "1x amino acids".

4. Dephosphorylation assays

Cells were pooled and harvested by centrifugation for 5 minutes at 2400 x g and medium removed. Cells were then resuspended in SDM Minus, SDM+AA or SDM+10% FBS at a concentration of $5x10^6$ cells/ml and aliquots collected at the time points designated.

5. Restimulation Assays

Cells were harvested by centrifugation for 5 minutes at 2400 x g and growth medium removed. Cells were starved in SDM Minus at a concentration of $5x10^6$ cells/ml for the times designated, then an equal volume of 2x SDM Minus, 2x SDM+AA, 2x SDM+Ins, or 2x SDM+AA+Ins was added and aliquots collected at the time points indicated.

6. Quantitative Restimulation Assays

For quantitative assay, cells were starved of amino acids for 30 minutes in SDM Minus at a concentration of $5x10^6$ cells/mL. Prior to restimulation, zero time points were taken and cells were then pelleted by centrifugation for 30 seconds at 18,000 x g and starvation medium removed. The cell pellet was then resuspended at a concentration of $5x10^6$ cells/mL in SDM Minus supplemented with 1x amino acids, 100 nM insulin, or a mixture of 1x amino acids and 100 nM insulin. Samples were collected at the time points indicated.

7. Cell Lysis and Immunoblotting

Treatments were terminated by pelleting cells by centrifugation for 30 seconds at 18,000 x g. Medium was removed and cells were lysed in 5% SDS sample buffer (0.25M Tris-HCl (ph 6.7), 10% glycerol, 5% sodium dodecyl sulfate, 0.1% bromophenol blue, 2% β-mercaptoethanol) by repeated pipetting. Lysates were briefly vortexed and heated for 3 minutes at 95^oC. Samples were allowed to cool to room temperature followed by 30 seconds of vigorous

vortexing to shear genomic DNA. Lysates were then centrifuged for 10 minutes at 14,000 rpm at room temperature to pellet insoluble debris.

Whole cell lysates were probed for total S6K, phospho-T398-S6K, PP2Ac, and β-actin by immunoblotting. Lysate proteins resolved by electrophoresis on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in blocking solution (1x TBS, pH 8.0; 5% nonfat dry milk) for 1 hour at room temperature, followed by two 5-minute washes in TBS-T (TBS + 0.2% Tween-20). Blots were incubated overnight at 4^{o}C with antibodies diluted in blotting solution (1x TBS, 5% bovine serum albumin, 0.1% Tween-20) with antibodies at the following dilutions: anti-phospho-T389, 1:2000; anti-dS6K, 1:3000; β-actin, 1:2000, anti-PP2Ac, 1:2000. The membranes were washed three times with 1x TBS-T. Bound antibodies were detected by incubating with HRP-conjugated anti-rabbit (1:5000) or HRP-conjugated anti-mouse (1:5000) secondary antibodies in blotting solution for 1 hour at room temperature. Immunoreactivity was visualized by incubating the membrane with ECL reagent (GE Healthcare, Piscataway, NJ) and exposure to autoradiography film.

For quantitative Western blots, membranes were probed simultaneously with anti-phospho-S6K (1:2000) and anti-dS6K (1:3000) antibodies, followed by incubation with a mixture of HRP-conjugated anti-rabbit (1:5000) and anti-mouse Alexfluor467 secondary antibodies (1:1000). Before detection, blots were incubated with ECL-Plus for 5 minutes at room temperature. Proteins were visualized by scanning membranes on a Molecular Dynamics Storm 860 fluorescence scanner. Alexflour467 signal was detected by scanning at 540nm

and HRP-rabbit secondary signal was visualized by detection of a fluorescent intermediate in the ECL Plus light producing reaction pathway by scanning at a wavelength of 467nm. Quantitation of band intensities was performed using Image Master 1D.4 Elite Software. The ratio of phospho-S6K intensity to the non-phosphospecific Ab (total S6K) intensity was calculated for each lane and fold change was determined by normalizing phospho-S6K:S6K intensity ratio for each experimental lane to the phospho-S6K:S6K ratio of an untreated control lane. Data was then represented in a graphical format using Sigmaplot software (Systat Software, Inc.).

C. Results

1. Phosphorylation of dS6K at T398 is Stimulated by Nutrients

dS6K has been previously shown to undergo phosphorylation at residue T398 in response to the addition of amino acids, insulin and serum. It has also been noted that dS6K is also activated by the SDM medium components yeastolate and tryptone, both believed to contain free amino acids (Echalier, 1997). In the absence of amino acids and growth factors, T398 is dephosphorylated by a calylculin A-sensitive phosphatase (Peterson, Desai et al. 1999). In order to determine optimal starvation and restimulation conditions, S2 cells were starved of amino acids and serum and response to SDM components was examined using the phosphorylation state of T398 as a marker (Fig. 4). Lysates from control cells maintained in SDM+10% (lane 1) display a high level of anti-phospho-S6K immunoreactivity, with the most prominent band migrating

near 65-kDa and four lower immunoreactive bands in the 50- to 55-kDa range. These lower migrating bands could possibly be hypophosphorylated forms of dS6K that are phosphorylated at T398 upon addition of nutrients, but the small size suggests that these may be non-specific bands detected with the phospho-T398 antibody. In cells deprived of amino acids, a marked decrease in anti-phospho-S6K immunoreactivity is observed (lane 2), whereas T398 is phosphorylated to varying degrees in the presence of amino acids, yeastolate and peptone (lanes 2-5), with peptone being the most potent activator of the three. Addition of a mixture of both yeastolate and peptone to starved cells resulted in a greater increase in T398 immunoreactivity (lane 6) than either yeastolate or peptone alone. β-actin was blotted as a loading control. These results confirm the role of amino acids in stimulating phosphorylation of dS6K at T398, since cells deprived on amino acids and serum showed extremely low levels of phospho-S6K immunoreactivity. The ability of yeastolate and peptone to induce T398 phosphorylation indicates that these components should be excluded from starvation medium in order to maximize dephosphorylation of dS6K when necessary.

2. Dephosphorylation of T398 During Inhibition of dTOR Activity

In order to establish a time course of T398 dephosphorylation, cells were starved of amino acids and the phosphorylation state of T398 monitored by Western blotting (Fig. 5A). In cells starved of amino acids, T398 was dephosphorylated within 20 minutes (Fig, 2A, bottom panel), compared to robust

phosphorylation of T398 maintained in cells cultured in SDM + 10% FBS. A time course study of rapamycin-mediated dS6K dephosphorylation was undertaken to compare the rate of dephosphorylation in the presence of a direct protein inhibitor (rapamycin) versus an environmental stress (absence of amino acids). Treatment of cells with 20 nM rapamycin resulted in an rapid dephosphorylation of T398, greatly reducing the amount of phospho-S6K signal within 5 minutes, whereas cells treated with an equal volume of vehicle (DMSO) maintained dS6K phosphorylation (Fig. 5B). These results confirm that maintenance of T398 phosphorylation is dependent upon dTOR kinase activity, as inhibition of dTOR by both starvation and rapamycin resulted in inactivation of dS6K.

3. Phosphorylation of T398 by Insulin does not Require Amino Acids

Previous studies have reported conflicting data regarding the requirement by insulin for the presence of amino acids in order to phosphorylate T398 (Radimerski, Montagne et al. 2002; Lizcano, Alrubaie et al. 2003). Radimerski and co-workers reported activation of dS6K requires amino acids, where the Lizcano and collegues showed in two different *Drosophila* cell lines that the actions of insulin upon dS6K do not require dTOR to be activated by amino acids first. In order to test the ability of insulin to stimulate T398 phosphorylation, S2 cells were starved of amino acids and restimulated with amino acids, insulin or a combination of both (Fig. 6). Both amino acids and insulin were able to induce phosphorylation of T398 individually and the effect of insulin was significantly

larger than that of amino acids alone (compare Fig. 6B and C). Phosphorylation of T398 in response to insulin was faster than the response to amino acids, with phosphorylation by insulin approaching a maximum within 10 minutes (Fig. 6C), whereas amino acid-induced phosphorylation of dS6K does not approach a maximum until 20 minutes (Fig. 6B). Addition of a combination of both amino acids and insulin resulted in a pattern of T398 similar to that of insulin alone (Fig. 6D). These results, in agreement with the findings of Lizcano and collegues (Lizcano, Alrubaie et al. 2003), indicate that in S2 cells insulin does not require the presence of amino acids to induce phosphorylation of T398.

4. Quantitation of T398 Response to Amino Acids and Insulin

Because amino acids and insulin both stimulate T398 phosphorylation to varying degrees, it would be useful to determine the fold change in dS6K phosphorylation induced by amino acids or insulin in amino acid-starved cells. A quantitative Western blotting method was employed in order to calculate the fold change in response to these two stimuli, both individually and in combination. To do so, S2 cells were starved of amino acids for and then restimulated with 1x amino acids, 1uM insulin, or a mixture of both 1x amino acids and 1uM insulin (Fig 7). Addition of 1x amino acids of to starved cells resulted in a maximal 3-fold increase in T398 phosphorylation when compared to starved control cells. Treatment with 1uM insulin resulted in a 9-fold increase of T398 phosphorylation, while interestingly, addition of a combination of 1x amino acids and 1uM insulin to starved cells did not stimulate T398 phosphorylation to the

levels seen by insulin alone, resulting in a roughly 4-fold increase in dS6K phosphorylation. These observations are in agreement with previous studies that reported insulin to have a 5-fold greater capability to induce phosphorylation at T398 (Lizcano, Alrubaie et al. 2003).

5. Dephosphorylation of T398 by a Calyculin A-sensitive Phosphatase

Studies in skeletal myoblasts and Jurkat T cells demonstrated that p70-S6K dephosphorylation during starvation can be blocked by inhibition of protein phosphatases (Peterson, Desai et al. 1999). Considering the similarity of *Drosophila* TOR signaling components to the mTOR pathway, it is likely that dS6K is also regulated by a phosphatase in a dTOR activity-dependent manner. To test this hypothesis, cells were pre-treated with 50 nM calylculin A and then starved for amino acids (Fig. 8). Dephosphorylation of T398 was blocked by the presence of the phosphatase inhibitor, maintaining high levels of phospho-T398 in the absence of amino acids, even after 60 minutes of starvation (Fig. 8C, lane 24). These results are consistent with the hypothesis that dS6K is dephosphorylated at T398 by a calylculin A-sensitive phosphatase under conditions that inhibit dTOR activity.

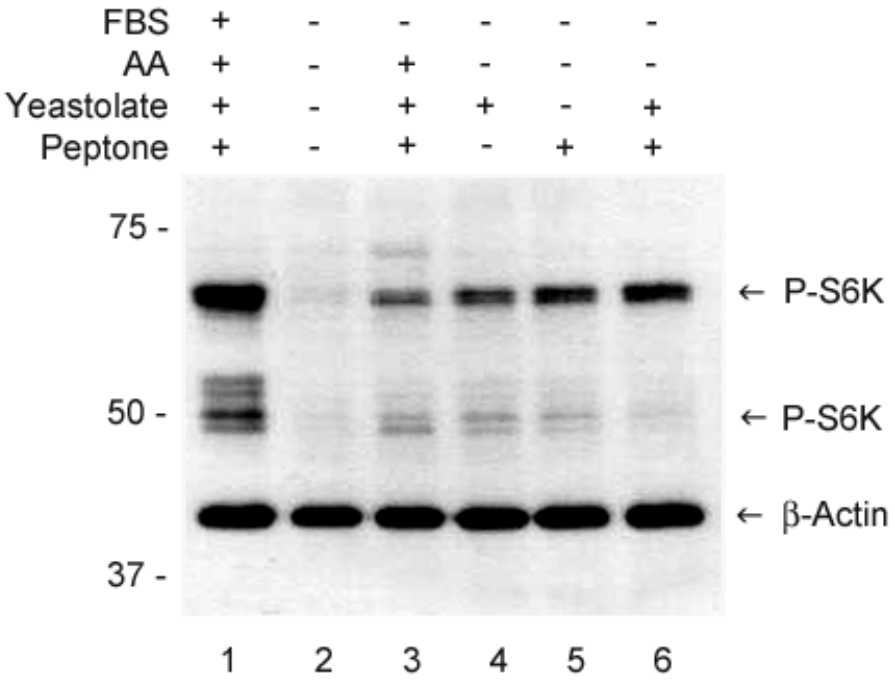

Figure 4. Response of dS6K to SDM medium components. *Drosophila* S2 cells were starved of amino acids and serum for 30 minutes at room temperature. Starved cells were then stimulated by addition of an equal volume of 2x SDM Minus, supplemented with 2x amino acids, 2x yeastolate, 2x peptone or a mixture of 2x yeastolate and 2x peptone and incubated for 30 minutes. Control cells were maintained in either SDM + 10% FBS or SDM Minus for 30 minutes. Cells were collected by centrifugation and lysed by repeated pipetting in 5% SDS sample buffer. Lysates were separated by SDS gel electrophoresis and probed by immunoblotting with anti-phospho-S6K and anti-β-actin antibodies.

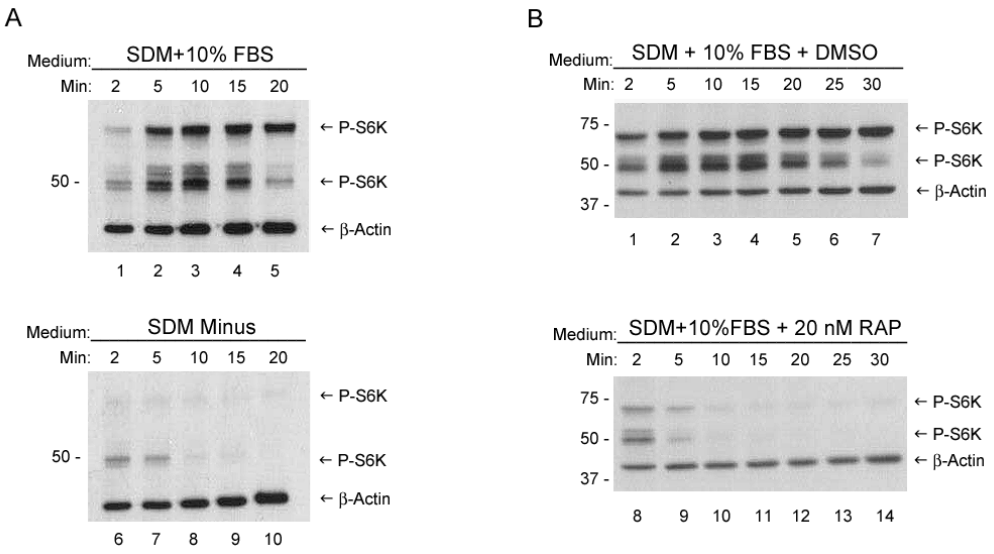

Figure 5. dS6K is dephosphorylated at T398 under conditions that inhibit dTOR. (A) Dephosphorylation of T398 during amino acid starvation. S2 cells were harvested and resuspended in amino acid- and serum-free medium (SDM Minus) while control cells were resuspended in SDM+10% FBS. Aliquots were collected at times designated and lysates prepared as described previously. Lysates were separated by electrophoresis on 10% SDS-PAGE and proteins transferred to nitrocellulose membrane. Membranes were probed simultaneously with anti-phospho-S6K and anti-β-actin antibodies and bands visualized using chemiluminesence. (B) T398 is a rapamycin-sensitive phosphorylation site. Cells were pooled and rapamycin was added to a final concentration of 20 nM. An equal volume of vehicle (DMSO) was added to control cells. Lysate preparation and immunoblotting were carried out as previously described.

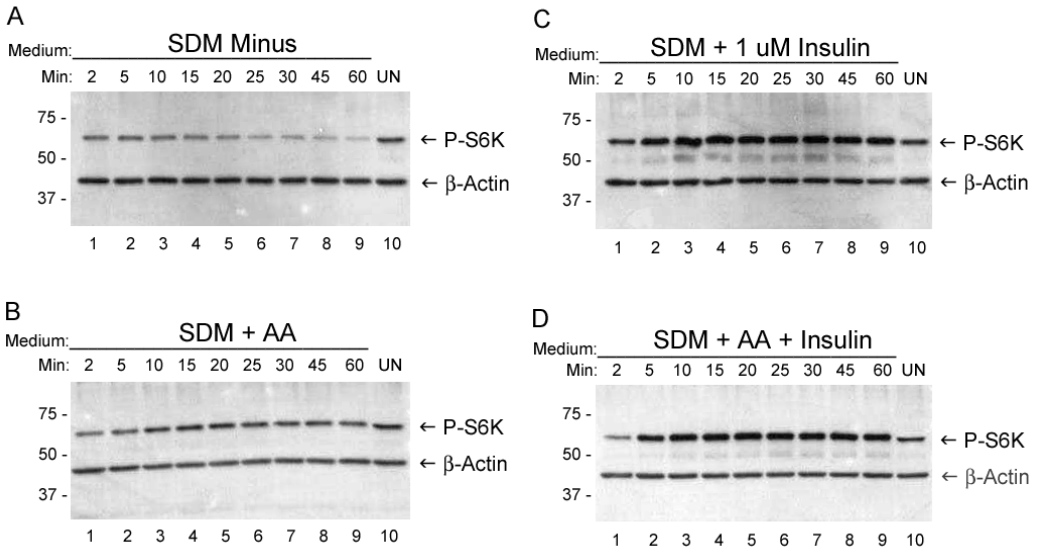

Figure 6. Activation of dS6K by insulin in S2 cells does not require amino acids. Cells were starved for 30 minutes and restimulated with 1x amino acids, 1uM insulin, or a combination of both. Untreated cells (UN) were incubated in SDM +10% for the duration of the time course (60 minutes). Aliquots were collected at the times indicated and lysates prepared. Lysates were separated by electrophoresis on 10% SDS-PAGE and proteins transferred to nitrocellulose membrane. Membranes were probed simultaneously with anti-phospho-S6K and anti-β-actin antibodies and the proteins visualized using chemiluminesence.

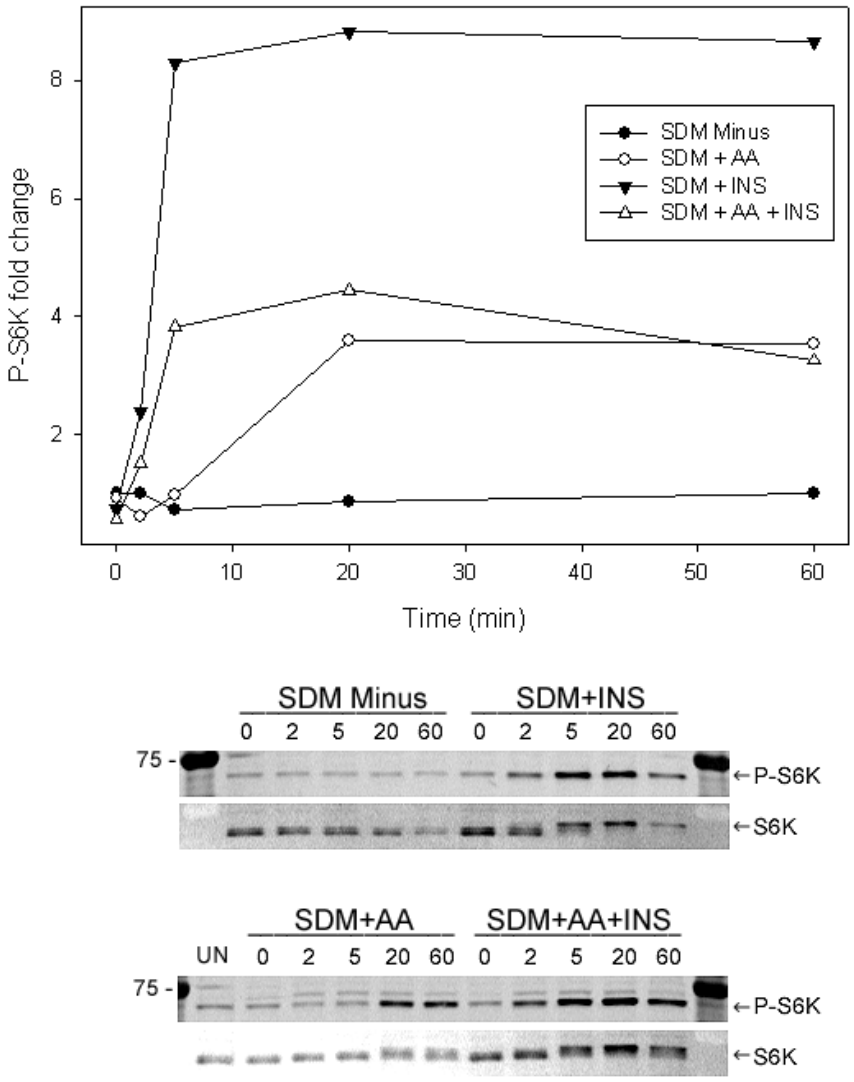

Figure 7. Quantitative assessment of nutrients on T398 phosphorylation. dS6K is activated by both amino acids and insulin. Cells were starved for 30 minutes and then treated with SDM Minus supplemented with 1x amino acids, 1uM insulin, or a combination of 1x amino acids and 1uM insulin. Control cells were treated with SDM Minus. Aliquots were collected at the times indicated and lysates prepared. Lysates were resolved by electrophoresis on 10% SDS-PAGE and proteins transferred to nitrocellulose membranes. Membranes were probed simultaneously with anti-phospho-S6K and anti-dS6K. Secondary antibodies were HRP-anti-rabbit and Alexfluor467 anti-mouse. Membranes were incubated in ECL Plus for 5 minutes and bands were visualized using the Storm scanner.

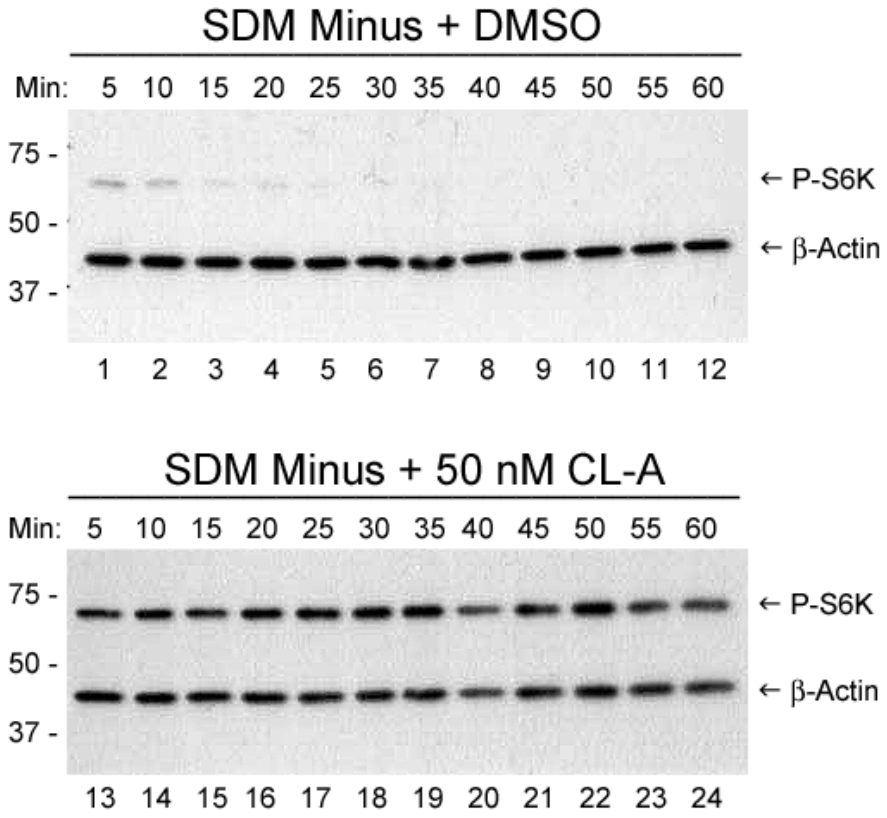

Figure 8. Dephosphorylation of T398 by a calyculin A-sensitive phosphatase. S2 cells were pre-treated with either 50 nM calylculin A (CL-A) or an equal volume of vehicle (DMSO). Cells were then transferred to SDM Minus supplemented with 50nM CL-A or DMSO. Cells were collected at the times indicated and lysates separated by electrophoresis on 10% SDS-PAGE and proteins transferred to nitrocellulose membrane. Membranes were probed simultaneously with anti-phospho-S6K and anti-β-actin antibodies and the protein bands visualized using chemiluminesence.

Chapter 3

Testing of the dTap Sequestration Model

A. Introduction

The discovery of RNA interference has provided researchers with a powerful tool for the dissection of signal transduction pathways by selective ablation of components (Worby, Simonson-Leff et al. 2001). Indeed, knockdown of components of the dTOR pathway in *Drosophila* cells in culture provided crucial insights into cellular responses to nutrients (Montagne, Stewart et al. 1999; Oldham, Montagne et al. 2000; Scanga, Ruel et al. 2000). Previous studies have shown that disruption of a specific phosphatase activity can be achieved by knockdown of catalytic or regulatory subunits (Li, Scuderi et al. 2002; Silverstein, Barrow et al. 2002). This selective ablation allows an examination of individual phosphatase subunits that is not possible when general serine/threonine phosphatase inhibitors are used.

The sequestration model of dTap regulation of PPases implies three concepts: 1) dS6K is dephosphorylated at T398 by trimeric PP2A during amino acid starvation, 2) ablation of the appropriate regulatory subunit will prevent targeting of PP2A to dS6K and inhibit dephosphorylation of T398, and 3) loss of dTap will result in increased phosphatase activity and cause hypophosphorylation of dS6K under conditions conducive to growth. This model assumes that dTap acts as an inhibitor of PP2A by sequestering catalytic activity away from PP2A regulatory subunits.

A major goal was to test the sequestration model of dTap function and study the role of dTap in the TOR signaling pathway in higher eukaryotes. In view of the reduced number of PP2A subunits in *Drosophila*, we decided to utilize the S2 cell culture system. In order to carry out these studies, it was necessary to generate reagents and characterize this system. The goals of this chapter were to 1) identify and clone the *Drosophila* homolog of Tap42/α4, 2) optimize conditions for efficient knockdown of dTap by RNA interference, 3) test the sequestration model of dTap activity, 4) identify phosphatase subunits involved in regulation of dS6K during amino acid starvation, and 5) characterize the effects of dTap knockdown on cell growth and viability.

B. Experimental Procedures

1. Materials

Pfu Turbo was purchased from Stratagene (La Jolla, CA). Large-scale T7 transcription kits were purchased from EMD Biosciences (San Diego, CA). Rapamycin, DMSO, and ampicillin were obtained from Sigma (St. Louis, MO). The pAc5.1 plasmid, fetal bovine serum, TOPO-Zero Blunt II cloning kit and Superscript One-Step RT-PCR kit were purchased from Invitrogen (Carlsbad, CA). Adult and 4-8 hour embryonic *Drosophila* cDNA libraries were a gift of the Greg Tall (UTSW, Dallas, TX). The pZJM vector was a generous gift of Meg Phillips (UTSW, Dallas, TX). Quantitative PCR (qPCR) experiments were carried out using an Applied Biosystems 7500 Real Time PCR System (Foster City, CA). Tripure Isolation Reagent was purchased from Roche Applied Science

(Indianapolis, IN). Effectene transfection reagent and Qiaquick gel extraction kit were purchased from Qiagen (Valencia, CA). Restriction enzymes and T4 ligase were purchased from New England Biolabs (Ipswich, MA). Wizard Miniprep and BCA Protein Assay kit was purchased from Pierce (Rockford, IL). zVAD-fmk was purchased from R&D Systems (Minneapolis, MN).

2. Cell Culture

Drosophila S2 cells were maintained in SDM +10% FBS at 25^{o}C. SDM+10% FBS, SDM+AA, SDM+15% FBS and SDM Minus were prepared as described previously (section 2.2).

3. Antibodies

Anti-phospho-T398-S6K and anti-β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA). Ab92, a rabbit polyclonal antibody that recognizes dS6K, was a gift of Mary Stewart (North Dakota State University, Fargo, ND). F725, a rabbit polyclonal antibody that recognizes Drosophila PP2A A subunit, and M878, a polyclonal rabbit antibody that recognizes *Drosophila* B56-1, have been described previously (Silverstein, Barrow et al. 2002). 1F6 is a monoclonal antibody raised against the C-terminus of mammalian PP2Ac that cross-reacts with dPP2Ac. Anti-V5 antibody was purchased from Invitrogen. HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Biosciences (Pittsburgh, PA).

4. Clones

Drosophila cDNA clones corresponding to PP4 and the PP2A subunits have been described previously (Silverstein, et al., 2002). A plasmid containing the full-length sequence of dPP6 (*SD01279*) was purchased from the *Drosophila* Genomics Resource Center (Indiana University, Bloomington).

5. Primers

All primers were purchased from Integrated DNA Technology (Coralville, IA). The nucleotide sequence of primers utilized in RNAi studies can be found in Table 1. The forward and reverse primers for PCR-based cloning of full-length dTap from cDNA libraries corresponded to the predicted 5' and 3' ends of the gene and were comprised of the sequences 5'-*ATGGCTGAGGGTAATACCGCTGG*-3' (sense strand) and 5'-*GACGGCAACCGTCATAACCGTAGTTAA*-3' (anti-sense strand). Primers for generation of dTap-V5 corresponded to the 5' and 3' ends of dTap, with the addition of EcoRV and XbaI restriction sites into the primers upstream of dTap coding sequence. The nucleotide sequences of the cloning primers are 5'-*GATATCATGGCTGAGGGTAATACCGCTG*-3' (sense strand) and 5'-*TCTAGACCTCGTGACTACGGTTCTGACGGT*-3' (anti-sense strand). The sequence of primers used to amplify a region of the *stubarista* (Sta) gene are 5'-*ATGTCGGGAGGCTTAGATAT*-3' (sense strand) and 5'-*GATCTGGTTGGTGAAGGCAC*-3' (anti-sense strand). The nucleotide sequence of dPP6 was scanned using the Primer 3 analysis tool (http://frodo.wi.mit.edu/cgi-

bin/primer3/primer3_www.cgi) and primers were designed to produce a 500 bp cDNA fragment.

For production of cDNA fragments for use in synthesis of dsRNA, cDNAs were first produced using primers corresponding to the three target regions of dTAP, bp 88-637 (dTap-1), bp 203-741 (dTap-2) and bp –85 to 599 (dTap-UTR). The primer sequences are as follows: dTap-1, 5'-*GTTACCGAACTGCCCTTCAA*-3' (sense strand) and 5'-G*CTTCATTACGCCCAATGTT*-3' (anti-sense strand); dTap-2, 5'-*AGTTGATCGACGAGGTGTCC*-3' (sense strand) and 5'-*GGTGGACGATGCAGATT*-3' (anti-sense strand); dTap-UTR, 5'-*GTCGGCATCGTTGCACATTATT*-3' (sense strand) and 5'-*ATTGAAGGCTGCCTCTACCA*-3' (anti-sense strand). For dTap-1 and dTap-UTR, the cDNAs obtained from PCR using pMT-dTap-V5 plasmid as a template were then used as template for PCR using primers corresponding to dTAP-1 and dTAP-UTR, but with the T7 promoter sequence 5'-*GAATTAATACGACTCACTATAGGGAGA*-3' added 5' to the dTap-specific primer sequence. T7-dTap2 primers provided very low yields of cDNA, subsequently the dTap-2 fragment was cloned into the pZJM vector, a plasmid containing flanking T7 sites for production of dTap-2 dsRNA. The primers used for the cloning of dTap2 into PZJM corresponded to regions bp 203 to 805, and included the addition of *Xho*I and *BstB*I restriction sites into the primer sequence. Primers for cloning of dTap2 into pZJM consisted of the nucleotide sequences 5'-*TTCGAAAGTTGATCGACGAGGTGTCC*-3' (sense strand) and 5'-*CTCGAGGGTGGACGATCAGAAGATT*-3' (anti-sense strand).

For quantitative measurement of dTap knockdown via quantitative PCR (qPCR), four sets of primers were designed using the Primer3 analysis software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). These primers were designed to produce 50 bp cDNA fragments and corresponded to regions of the dTap gene located outside of those targeted by the three dsRNAs utilized (dTap-1, dTap-2 and dTap-UTR). The sequences of these primers are as follows: qTAP-1, 5'-*GGAGAAGGTTGCCAAGATGA*-3' (sense strand) and 5'-*CCTCGTCCTCTTTTTCGTTG*-3' (anti-sense strand); qTAP-2, 5'-*ATCTTCTGCATCGTCCACCT*-3' (sense strand) and 5'-*CTGATGATGGTGGTGGTGTC*-3' (anti-sense strand); qTAP-3, 5'-*TGCAGCCCTTCATCATAACA*-3' (sense strand) and 5'-*TAATGGGCAAACTGGGGTAT*-3' (anti-sense strand); qTAP-4, 5'-*CTACAGGCGGAGCAGGATG*-3' (sense strand) and 5'-*CGCACCACATCCTTGTATTC*-3' (anti-sense strand). Initial validation experiments identified qTAP-1 as the optimal primer set; therefore qTAP-1 primers were utilized for subsequent qPCR analysis of knockdown mRNAs.

6. Cloning of dTap into pAc5.1 Expression Vector

PCR primers were designed to produce full-length dTap cDNA with *Eco*RV and *Xba*I restrictions sites 5' and 3' of dTap sequence, respectively. The anti-sense primer also contained a point mutation in the stop codon allowing transcription to proceed into the V5 epitope-encoding region of the plasmid. Cloning vector and cDNA were incubated with *Eco*RV and *Xba*I, and digested

vector and cDNA were separated from enzymes by electrophoresis on a 1% agarose gel. Insert and vector were cut out of gel and purified away from agarose using the Qiaquick kit. Insert was cloned into the pAc5.1 vector using T4 ligase overnight at 4^{o}C. TOP-10 cells were then transformed with 3 ul of each ligation reaction by heat shock for 1 minute at 42^{o}C, followed by 2 minute incubation on ice. Cells were then plated on LB-AMP agar plates and incubated overnight at 37^{o}C. Clones displaying antibiotic resistance were picked, cultures grown and minipreps carried out using the Wizard kit. T7 sequencing verified that the V5 epitope was in frame with dTap coding sequence. This construct will be defined as "dTap-V5".

7. Transfection of S2 cells with dTap-V5

Transfection of S2 cells with pAc5.1-dTap-V5 was carried out using the Effectene reagent, using the manufacturer's protocol. Briefly, 3x10^{6} cells were plated in 60 mm culture dishes the day before transfection in SDM+10% FBS and allowed to incubate overnight at 25^{o}C. On the day of the experiment, cells were transfected with 1 ug of pAc5.1-dTap-V5 or pAc5.1 empty vector. Parallel control cells were transfected with a version of pMT containing the EGFP gene (pMT-EGFP) as a visual marker for transfection efficiency. After transfection, cells were incubated at 25^{o}C for the duration of experiment. Cells transfected with pAc5.1 or pAc5.1-dTap-V5 were incubated for 48 hours before harvest without changing the medium.

8. Production of dsRNA

Synthesis of dsRNAs was carried out using the large-scale T7 transcription kit from Novagen, using a modified version of the protocol provided by the supplier. Briefly, 3 ug of either T7-EGFP cDNA or *Kpn*I-linearized pZJM-dTap-2 was used as template. DNA was mixed with contents of the transcription kit and allowed to incubate at 37^{o}C for three hours, at which point 15 uL of RNAse-free DNAse was added to each reaction. Tubes were briefly vortexed and incubated for an additional 10 minutes at 37^{o}C. Tubes were then removed from the water bath and 315 uL of 25:24:1 phenol:chloroform:isoamyl alcohol mixture was added to the solution. Tubes were then vortexed vigorously for 15 seconds and centrifuged at 18,000 x g for 30 seconds at room temperature, to help separate the phases. The upper (aqueous) phase was removed and transferred to a 1.5 mL Eppendorf tube. One-tenth volume of 3M NaOAc was added to the aqueous phase and tubes vortexed briefly. In order to precipitate the RNA, 2.5 volumes of cold 100% ethanol was added and the tubes vortexed briefly. The tubes were then incubated for 20 minutes at -80^{o}C, followed by centrifugation at 14,000 rpm for 10 minutes at 4^{o}C. The supernatant was removed by pipetting and the pellet was washed with 1 mL of 75:25 ethanol:$H_2$0. After removal of the ethanol, the RNA pellets were allowed to air dry. The RNA was resuspended in 300 uL of nuclease-free water by repeated pipetting, and then denatured by heating at 65^{o}C for 30 minutes. Denatured samples were cooled to room temperature to allow annealing of the single-standed RNA. The optical density at 260 nm of dsRNA in

each sample was determined and used to calculate the concentration of dsRNA. All dsRNA solutions were stored at -20^{o}C.

9. RNAi treatment of S2 cells

1x10^{6} cells were plated in 1 mL serum-free medium (SDM+AA) in six-well dishes (60 mm wells) and 15 ug of either dsRNA directed against target genes or control EGFP dsRNA were added to each well. Plates were then swirled to mix dsRNA into medium. Cells were incubated at 25^{o}C for three hours, at which point 2 mL of SDM containing 15% FBS was added to each well for a final serum concentration of 10%. Plates were swirled to mix. Cells were then incubated at 25^{o}C for the duration of the experiment. At 24, 48 and 72 hours post-plating, an additional 15 ug of target gene dsRNA or EGFP dsRNA was added to cells. Cells were allowed to incubate in the presence of dsRNA for a total of 96 hours. For double knockdown experiments, cells were treated with 120 ug dsRNA in the presence of 20 uM ZVAD-fmk.

10. Isolation of Total RNA

The Tripure Isolation Reagent was utilized to isolate total RNA from dsRNA-treated cells using a slight modification of the protocol provided by the vendor. Cells were harvested and pelleted by centrifugation for 5 minutes at 5,000 x g. The medium was aspirated and the cell pellet resuspended in 1 mL of Tripure reagent. The cells were vortexed briefly and allowed to incubate at room temperature for 5 minutes. The lysates were transferred to autoclaved 1.5 mL

Eppendorf tubes. 200 uL of chloroform was added and the tubes shaken briskly by hand for 30 seconds. The lysates were incubated for 10 minutes at room temperature and phases separated by centrifugation at 13,000 x g for 15 minutes at 4^{o}C. The upper (aqueous) phase was removed and transferred to a clean 1.5 mL Eppendorf tube. Nucleic acid was precipitated by the addition of 500 uL isopropanol, followed by incubation at room temperature for 10 minutes. The tubes were centrifuged at 12,000 rpm for 10 minutes at 4^{o}C and the chloroform/isopropanol was removed from the RNA pellet. The pellet was washed with 1 mL of 70:30 mixture of ethanol:$H_2$0 and the pellet isolated by centrifugation at 7500 rpm for 5 minutes at 4^{o}C. The pellets were dried and resuspended in 30 uL of nuclease-free water. The resuspended RNA was heated at 55^{o}C for 10 minutes to ensure solubilization. Samples were then allowed to cool to room temperature and purified RNA stored at -80^{o}C.

11. Cell Counts

For rapamycin sensitivity growth curves, cells were gently triturated to suspend adherent cells. 10 μL of cell suspension was removed and counted with a hemocytometer without dilution. Cell numbers were calculated by counting the contents of the four large outer squares (64 smaller squares total). Only cell counts over 100 were considered statistically relevant. The number of cells in the 64 small squares was then divided by 0.1 and multiplied by a factor of 1000. This formula provides an estimate of the cell density in cells/mL.

12. Amino Acid Starvation Assays

Cells were harvested by scraping plates and transferred to 50 mL Falcon tubes. Cells were pelleted by centrifugation for 5 minutes at 2,800 x g at 25^{o}C. The medium was aspirated and cell pellets resuspended in 2 mL of SDM + 10% FBS or SDM Minus. The cells were incubated at 25^{o}C for 60 minutes, after which cells were pelleted by centrifugation at 14,000 rpm for 30 seconds at 25^{o}C. The medium was aspirated and cell pellets resuspended in 100 ul cold lysis buffer A (20 mM Tris-HCl, pH 7.4, 0.2% NP-40, 20% glycerol, 200 mM NaCl, 1 mM EDTA). Clarified cell lysates were prepared as previously described. Protein concentration of clarified lysates was determined using the BCA assay. 5x SDS sample buffer (5% SDS, 0.5% β-mercaptoethanol, 50% glycerol, 312.5 mM Tris-HCl (pH 6.7), 0.5% bromophenol blue) was added to the lysates and tubes heated for 3 minutes at 95^{o}C. For Western blotting, 25 ug of protein was loaded on each lane. Samples were stored at -20^{o}C.

13. Amino Acid Starvation Time Courses

The cells were harvested by gently scraping plates and the contents of three 35 mm wells of a six-well plate were pooled together in a 50 mL Falcon tube. Cells were counted using a hemocytometer and cell density was calculated. A volume corresponding to 1.5 x 10^{7} cells was transferred to a new 50 mL Falcon tube by pipetting. Cells were then pelleted by centrifugation for 5 minutes at 2,800 x g at 25^{o}C. Medium was then aspirated and cells resuspended in 3 mL SDM+AA by repeated pipetting (final density 5x10^{6} cells/mL). Cells were then

allowed to recover for 30 minutes at room temperature. "Time 0" samples were collected by transferring 250 uL ($1.3x10^6$ cells) of cell suspension to a clean 1.5 mL Eppendorf tube and pelleted by centrifugation at 18,000 x g for 30 seconds at RT. Medium was aspirated and the cells were lysed in 100 uL 5% SDS sample buffer. To begin the starvation assay, 2 ml of cell suspension was transferred to a 2 mL Eppendorf tube and cells pelleted by centrifugation at 18,000 x g for 30 seconds at RT. SDM+AA media was aspirated from tube and cells resuspended in 2 mL of SDM Minus (final density $5x10^6$ cells/mL). Cells were then allowed to incubate at RT and samples were collected at time points indicated. To collect sample, cell suspension was briefly swirled and 125 uL (612,500 cells) was transferred to a clean 1.5 mL Eppendorf tube and pelleted by centrifugation at 18,000 x g for 30 seconds at RT. Medium was aspirated and the cells pelleted lysed in 50 uL 5% SDS sample buffer. Samples were then heated for 3 minutes at 95^oC, and then allowed to cool to RT. Samples were vortexed vigorously for 30 seconds to shear genomic DNA and centrifuged for 10 minutes at 14,000 rpm at RT. Samples were stored at -20^oC

14. Rapamycin Resistance Assays

For rapamycin resistance studies, cells were treated with 60 ug dsRNA for 96 hours and cells harvested and counted as described above. A volume corresponding to a total of 1.5×10^7 cells was transferred to a new 50 mL Falcon tube by pipetting. Cells were then pelleted by centrifugation for 5 minutes at 2,800 x g at 25^oC. Media was aspirated and cells resuspended in 3 mL of

SDM+10% FBS (final density $5x10^6$ cells/mL). The cells were then allowed to recover for 30 minutes at room temperature. "Time 0" samples were collected as described above. Rapamycin was added to the remaining cell suspension at a final concentration of 20 nM. The cells were incubated at 25°C and samples were collected at the time points indicated as described above. The samples were stored at -20°C.

15. Preparation of Clarified Lysates

Forty-eight hours post-transfection, cells were harvested and pelleted by centrifugation at 3500 rpm for 5 minutes at 4°C. Medium was aspirated and cell pellets resuspended in 100 uL lysis buffer A and transferred to 1.5 mL Eppendorf tubes. Lysates were incubated on ice for 20 minutes, followed by centrifugation at 18,000 x g for 10 minutes at 4°C. Clarified lysates were transferred to new 1.5 mL Eppendorf tubes and stored on ice. Protein concentration of the lysates was determined using the BCA Protein Assay kit.

16. Western Blotting

Clarified lysates were mixed with equal volume of 2x SDS sample buffer (135 mM Tris-HCl, pH 6.7, 0.2% SDS, 20% glycerol, 0.2% β-mercaptoethanol, 0.2% bromophenol blue) and heated for 3 minutes at 95°C. Tubes were allowed to cool and were then subjected to centrifugation at 14,000 rpm for 10 minutes at room temperature. For Western blotting, 50 ug of protein was resolved by electrophoresis on a 10% SDS polyacrylamide gel. Proteins were transferred to

nitrocellulose membranes and membranes were blocked with 5% non-fat dry milk in TBS for 30 minutes at room temperature. Membranes were incubated overnight at 4^{o}C with anti-V5 antibody diluted 1:5000 in 5% non-fat dry milk. Membranes were washed three times in 1x TBS-T and incubated for 1 hour at room temperature with HRP-conjugated anti-mouse secondary antibody diluted in 5% non-fat dry milk. Membranes were washed again with 1x TBS-T and immunoreactive bands visualized using ECL reagent.

C. Results

1. Identification of the Drosophila TAP homolog

The amino acid sequences of *S. cerevise* Tap42 (AC: AAC49396) and human α4 (AC: NP_001542) were aligned with predicted proteins from the *Drosophila melanogaster* genome using the BLAST search tool at Flybase (http://www.flybase.net). The searches identified a predicted open reading frame from a gene on chromosome 2L designated *CG31852-PA* (AC: NP_723811) with homology with Tap42 (E value: 1e^{-07}) and α4 (E value: 2e^{-19}). BLAST searches with the *ALPHA4* and *TAP42* nucleotide sequences with the *D. melanogaster* genome also identified *CG31852-PA* as the highest scoring match, with E values of 6e^{-14} and 1e^{-04}, respectively. The mRNA nucleotide sequence of the computed gene CG31852-AA is composed of 1,143 base pairs and is predicted to encode a protein of 380 amino acids. Alignment of primary amino acid sequences from Tap42, Alpha4 and *CG31852-PA* using the CLUSTALW program at Pôle Bioinformatique Lyonnais (PBIL) (http://npsa-pbil.ibcp.fr/cgi-

bin/npsa_automat.pl?page=npsa_clustalw.html) showed that *CG31852-PA* shares 28% identity with human α4 and 21% identity with Tap42 (Fig. 9). From these results it was concluded that *CG31852-PA* encodes the *D. melanogaster* homolog of human α4 and yeast Tap42, and was designated *dTAP* (for *Drosophila* two A and related phosphatase-associated protein). *CG31852-PA* will be referred to as dTap from this point on.

2. Cloning of a dTAP cDNA

Forward and reverse primers were designed corresponding to the 5' and 3' ends of the predicted nucleotide sequence of dTap. A cDNA fragment was obtained using these primers when a 4-8 embryonic fruit fly genomic library was utilized as template for PCR. This cDNA fragment migrated on a 1% agarose gel near the predicted size of 1143 base pairs. The same primer set was utilized to clone the dTap mRNA sequence by reverse transcriptase PCR (RT-PCR) from total RNA isolated from S2 cells. Vectors containing the insert were sequenced using T7 forward and reverse primers. Sequencing analysis of the cDNA obtained by RT-PCR confirmed that the cDNA matched the mRNA nucleotide sequence of dTAP provided in Genbank (http://www.ncbi.nih.gov).

3. Exogenous expression of dTAP-V5 in S2 cells

Full-length *dTAP* cDNA was cloned into the pAc5.1 *Drosophila* expression vector. Insertion of the gene into this vector results in the addition of a C-terminal V5 epitope tag to dTap (dTap-V5). Clarified lysates from Schneider

S2 cells transfected with either pAc5.1-dTap-V5 or empty pAc5.1 were probed with anti-V5 antibody to monitor protein expression (Fig. 10). Control lysates from cells transfected with empty vector did not display anti-V5 immunoreactivity, whereas lysates from dTap-V5 transfected cells showed at least 3-4 immunoreactive bands ranging from 39- 45-kDa (Fig. 10).

4. Knockdown of dTap by RNAi

The nucleotide sequence of dTap was scanned using the Primer3 tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and three regions were chosen as potential dsRNA targets. The primers were designed to produce roughly 500 bp cDNA fragments that corresponded to nucleotides 88-637, 203-741 and the 5' UTR of the dTap mRNA. Double stranded RNAs were subsequently synthesized using PCR products prepared with these primers. S2 cells cultured in SDM+10% FBS were treated for 96 hours with a total of 60 ug of dsRNA. Three dsRNAs corresponding to three different regions of dTap (dTap-1, dTap-2 and dTap-UTR) were tested for knockdown efficiency. Control cells were treated with dsRNA corresponding to a region of the EGFP gene. Cells treated with EGFP or the three dTAP dsRNAs for 4 days did not display gross morphological defects. However, in the cells treated with dTap dsRNA, a larger number of cells had surface blebs than cells treated with the EGFP control dsRNA. This blebbing is a marker for apoptosis in S2 cells. After 96 hours, RNA was harvested from cells and analyzed by quantitative PCR (qPCR) using the qTAP-1 primer set (Fig. 11). Both dsRNAs tested reduced the levels of dTap

mRNA to varying degrees. Double stranded RNA synthesized using T7-dTap-2 cDNA as template was the most efficient of the three, reducing levels of dTap mRNA by almost 90%. Levels of dTap mRNA in cells treated with EGFP dsRNA were not affected compared to the non-treated cells. These results demonstrated that dTap mRNA was knocked down efficiently in S2 cells using the dTap-2 dsRNA.

5. Ablation of Phosphatase Subunits via RNAi

To identify the phosphatase responsible for dephosphorylation of T398 of dS6K, RNAi was employed to selectively ablate PP2A subunits, PP4, PP6, and dTap in *Drosophila* S2 cells. Cells were treated with dsRNA directed against the target genes and knockdown efficiency was verified by Western blotting or RT-PCR (Fig. 12). Three *Drosophila* proteins, dPP2Ac, dBα, and dB56-1, can be detected by antibodies generated against mammalian homologs. Western blotting of lysates from cells treated with 15 ug dsRNA daily for four days reduced the amount of PP2Ac and B56-1 protein to barely detectable levels (Fig. 12A and B). Knockdown of the PP2A catalytic subunit also resulted in a reduction of PP2A A subunit (Fig. 12A), in agreement with previously reported results (Silverstein, Barrow et al. 2002). Treatment of cells with dsRNA directed against dB56-1 reduced the protein levels of a band detected by Western blotting migrating near 75-kDa, the predicted molecular weight of dB56-1. The identification of this protein as dB56-1 is supported by the loss of this same band in cells treated with dsRNA against PP2Ac. Two previous RNAi studies in S2 cells have demonstrated

loss of PP2A B subunit proteins when A or C subunit protein levels are reduced (Silverstein, et al. 2002, Li, et al. 2002).

Total RNA was isolated from RNAi cells treated with dsRNA against individual genes and the knockdown efficiencies of dTap, dPP4, dPP6 and three PP2A subunits (Bα/B56-2/PR72) were assayed by RT-PCR (Fig. 12C-H). Daily treatment of cells with 15 ug dsRNA for four days reduced mRNAs levels of all genes tested.

6. Effects of RNAi on dS6K Phosphorylation

RNAi was employed in S2 cells to specifically ablate dTap, the four PP2A regulatory subunits (Bα/B56-1/B56-2/PR72) and the homologs of the three Tap42-interacting phosphatases (PP2A/PP4/PP6). After RNAi treatment, cells were starved of amino acids and T398 phosphorylation monitored by Western blotting. Loss of PP2Ac resulted in a 1.6-1.9 fold increase in phospho-T398 in cells maintained in complete medium when compared to untreated control cells (Fig. 13). T398 phosphorylation remained elevated compared to control when PP2Ac RNAi cells were deprived of amino acids. Treatment of cells with the control EGFP dsRNA had no affect on basal phosphorylation or the dephosphorylation of T398 during starvation. Quantitative western blotting of PP2Ac knockdown lysates showed that the hyperphosphorylation of T398 is significantly higher ($p \leq 0.05$) than EGFP-treated control cells at each time point (Table 2). These results support the conclusion that PP2Ac is responsible for dephosphorylation of T398 during amino acid deprivation. However, knockdown

of PP2Ac did not completely prevent dephosphorylation of dS6K, as T398 was still partially dephosphorylated during amino acid deprivation in PP2Ac RNAi cells. In contrast to amino acid starvation, knockdown of PP2Ac only partially inhibited dephosphorylation of T398 during treatment with 20 nM rapamycin (Fig. 13).

Treatment of cells with dTap-2 dsRNA did not affect basal levels of T398 phosphorylation or prevent dephosphorylation of T398 during amino acid starvation (Fig. 14). Analysis of lysates from dTap-treated S2 cells did not display any statistical difference between knockdown and EGFP control cells (Table 2, first column). These results are in conflict with the sequestration model which predicts hypophosphorylation of T398 when dTap is lost. That data indicates that dTap does not play a significant role regulating either the basal levels of dS6K phosphorylation in complete medium, or dephosphorylation of dS6K during amino acid starvation.

Even though it has been proposed that Tap42/a4/dTap acts as an inhibitor of phosphatase activity, a dTap-PP2Ac dimer could act as a functional dS6K phosphatase. α4-PP2Ac was reported to bind mammalian p70-S6K in an *in vitro* binding assay (Yamashita, Inui et al. 2005). In order to test this hypothesis, both genes were knocked out simultaneously in S2 cells in the presence of the caspase inhibitor zVAD-fmk (Fig. 15). Cells treated with PP2Ac dsRNA in the presence of zVAD-fmk do not undergo apoptosis, but cell proliferation is severely blunted. Analysis of lysates from dTap/PP2Ac double knockout cells displayed an increase in basal T398 phosphorylation comparable to that of PP2Ac knockout alone (Fig.

15). In dTap/PP2Ac dsRNA-treated cells T398 appeared to be dephosphorylated to a greater extent upon amino acid withdrawal than cells lacking PP2Ac alone. These results suggest that dTap and PP2Ac do not act in concert to catalyze T398 dephosphorylation during starvation.

Interestingly, knockdown of the PP2A dB56-2 regulatory subunit and the PP4 catalytic subunit resulted in hypophosphorylated T398 during starvation (Figs. 16 and 17). Loss of dB56-2 did not affect basal levels of T398 phosphorylation, but resulted in hypophosphorylated T398 within 2 minutes of amino acid withdrawal. Phosphorylation of T398 in cells treated with dB56-2 dsRNA remained at a significantly lower level throughout starvation than EGFP-treated control cells (Fig. 17). In the case of dPP4c, basal levels of T398 phosphorylation were lower than control cells, but did not reach statistical significance. T398 phosphorylation was significantly lower than EGFP-treated control cells within 2 minutes after amino acid withdrawal and continued up to 20 minutes after withdrawal (Table 2). However, after 60 minutes of starvation, T398 phosphorylation in dPP4 knockdown cells was not significantly different from the control lysates. Interestingly, knockout of PP4 did not result in hypophosphorylation of T398 when cells were treated with rapamycin in the presence of amino acids and serum. These results suggest that both dPP4c and a dB56-2-associated PP2A heterotrimer are required for maintenance of dTOR activity (discussed below). Most importantly, loss of dPP4 or dB56-2 did not result in an increase of T398 phosphorylation during starvation, indicating that these genes are not required for the dephosphorylation of dS6K at T398.

Knockdown of dPP6 did not affect dephosphorylation of T398 during amino acid starvation or treatment of cells with rapamycin (Fig. 18). The fact that neither loss of dPP4 nor dPP6 results in hyperphosphorylation of T398 indicates that only PP2Ac is required for dephosphorylation of T398 during inhibition of dTOR.

Ablation of the three remaining PP2A regulatory subunits (dB56-1, dBα and dmPR72) did not appear to affect basal dS6K phosphorylation or dephosphorylation of T398 during amino acid starvation (Figs. 19-21). There is a slight trend towards hypophosphorylation of T389 when dBα is knocked down (Fig. 20), however, none of the time points are to be statistically different from EGFP-treated control lysates (Table 2, sixth column from left). These results, when combined with the dB56-2 knockout data (above), suggest that none of the four identified *Drosophila* PP2A regulatory subunits target PP2A to dephosphorylate T398. These results argue against the hypothesis that a known trimeric form of PP2A is responsible for regulating dS6K phosphorylation (discussed below).

Previous knockout studies in S2 cells reported that knockdown of either dB56-1 or dB56-2 individually did not have a major affect on cell growth. However, simultaneous loss of both subunits resulted in apoptosis (Li, Scuderi et al. 2002; Silverstein, Barrow et al. 2002), raising the possibility that dB56-1 and dB56-2 may be functionally redundant, and loss of only one family member would not be sufficient to affect T398 phosphorylation. This suggests that both dB56-1 and dB56-2-associated PP2A trimers may target phosphatase to dS6K. In order to test this hypothesis, dB56-1 and dB56-2 were knocked down

simultaneously in S2 cells. In light of previous reports that loss of both genes results in apoptosis, double knockouts were performed in the presence of caspase-3 inhibitor zVAD-fmk. Knockdown of both dB56 genes in the presence of ZVAD-fnk did not result in apoptosis, however cell proliferation was severely reduced. Cell counts revealed that dB56 double knockout cells grew to only half the density ($1x10^6$ cells/mL) of EGFP dsRNA-treated cells ($2x10^6$ cells/mL). Knockdown of both dB56 genes did not affect basal dS6K phosphorylation and did not prevent dephosphorylation of T398 during amino acid withdrawal. Quantitation of phospho-T398 intensity in lysates from dB56-1/2 knockdown cells showed decreased levels of T398 phosphorylation when compared to control (Fig. 22), but the values of P-T389 intensity from B56-1/2 double knockout lysates are not statistically different than control lysates (Table 2, second column from right). These results indicate that dB56-1 and dB56-2 do not act in a partially redundant manner to regulate dephosphorylation of dS6K during amino acid deprivation.

The most intriguing possibility from the results described above is that although PP2Ac is responsible for dephosphorylating T398 during amino acid starvation, none of the known PP2A regulatory subunits or dTap are required for this activity. However, it must be noted that in the experiments described above, S2 cells were deprived of serum for 30 minutes before amino acid withdrawal and for an additional 60 minutes during starvation. During the course of RNAi treatments, it was observed that S2 cells deprived of serum underwent morphological changes, most striking was a flattening of the cell and increased

adherence to tissue culture plates. Although the cause of this morphological change is unknown, a likely candidate pathway for down regulation would be the Ras/ERK pathway. Serum deprivation could induce a form of cellular stress, as S2 cells deprived of serum were observed to undergo morphological changes such as a flattening of the cell and increased adherence to tissue culture plates. The phosphorylation of p70-S6K has been shown to be sensitive to stress in mammalian cells (Patel, McLeod et al. 2002; Corradetti, Inoki et al. 2005) and in particular to the formation of actin stress fibers (Berven, Willard et al. 2004).

In order to eliminate the effects of possible stress due to serum deprivation, the macroglide compound rapamycin was utilized to inhibit dTOR in S2 cells treated with dTap-2 dsRNA. This inhibition of dTOR mimics amino acid withdrawal, but allows monitoring of T398 dephosphorylation in the presence of both serum and amino acids. However, knockdown of dTap in S2 cells did not prevent the dephosphorylation of T398 by 20 nM rapamycin (Fig. 15). Quantitative analysis of lysates showed that T398 is dephosphorylated to the same extent as EGFP dsRNA-treated cells. Basal levels of T398 phosphorylation may be slightly higher than control cells, in agreement with previously reported results (Cygnar, Gao et al. 2005). These results are not in agreement with the hypothesis that dTap acts as a regulatory subunit of a heterodimeric phosphatase, as loss of dTap did not appear to affect T398 dephosphorylation activity during dTOR inhibition. Most importantly, the increased basal level of T398 in dTap knockdown cells argues against the sequestration model of dTap activity.

7. Effects of RNAi on Cell Growth

Knockdown of PP2A results in apoptosis in *Drosophila* S2 cells (Silverstein, Barrow et al. 2002). Loss of PP2A catalytic subunit induces a striking apoptotic phenotype, including cell blebbing and massive cell death, whereas loss of the A subunit causes a less severe apoptotic phenotype. RNAi-mediated knockdown of both dB56 genes also causes apoptosis. In agreement with these results, treatment of cells with dsRNA against PP2Ac displayed a severe reduction in growth and a large number of blebbing cells (Fig. 23). Cell death induced by loss of PP2Ac is dose-dependent, with four daily treatments of 15 ug dsRNA causing a much more severe defect than a single treatment. Cells treated with 60 ug PP2Ac dsRNA showed greatly reduced cell growth and a large number of apoptotic bodies were observed. A small number of cells treated with 15 ug PP2Ac dsRNA appeared swollen and segmented into several compartments, resembling a honeycomb-like shape. Addition of control EGFP dsRNA did not affect cell growth or activate apoptosis. A single treatment of S2 cells with 15 ug dsRNA against dTap did not adversely affect growth; however, addition of 60 ug dsRNA against dTap reduced growth and increased the number of apoptotic bodies observed. Interestingly, loss of α4, the mammalian homolog of dTap, in embryonic stem cells also results in apoptosis (Kong, Fox et al. 2004) and our results agree with previously reported data that loss of dTap causes death in *Drosophila* cells (Cygnar, Gao et al. 2005).

8. Knockdown of dTap Confers Rapamycin Resistance

Previous studies reported that loss of Tap42 in *S. cerevisiae* conferred rapamycin resistance (Di Como and Arndt 1996). In order to test if the loss of dTap conferred rapamycin resistance to S2 cells, dTap was knocked down using RNAi. S2 cells were treated with dTap dsRNA for 72 hours and incubated with 20 nM rapamycin for an additional 24 hours. (Fig. 24). Knockdown of dTap does not inhibit cell proliferation and may cause a slight increase in cell numbers. Cells treated with dTap dsRNA grew to a higher density in the presence of rapamycin when compared to EGFP control cells. These results indicate that knockdown of dTap may confer resistance to the growth inhibitory effects of rapamycin. This result is in agreement with studies showing that loss of Tap42 confers rapamycin resistance in yeast (Di Como and Arndt 1996).

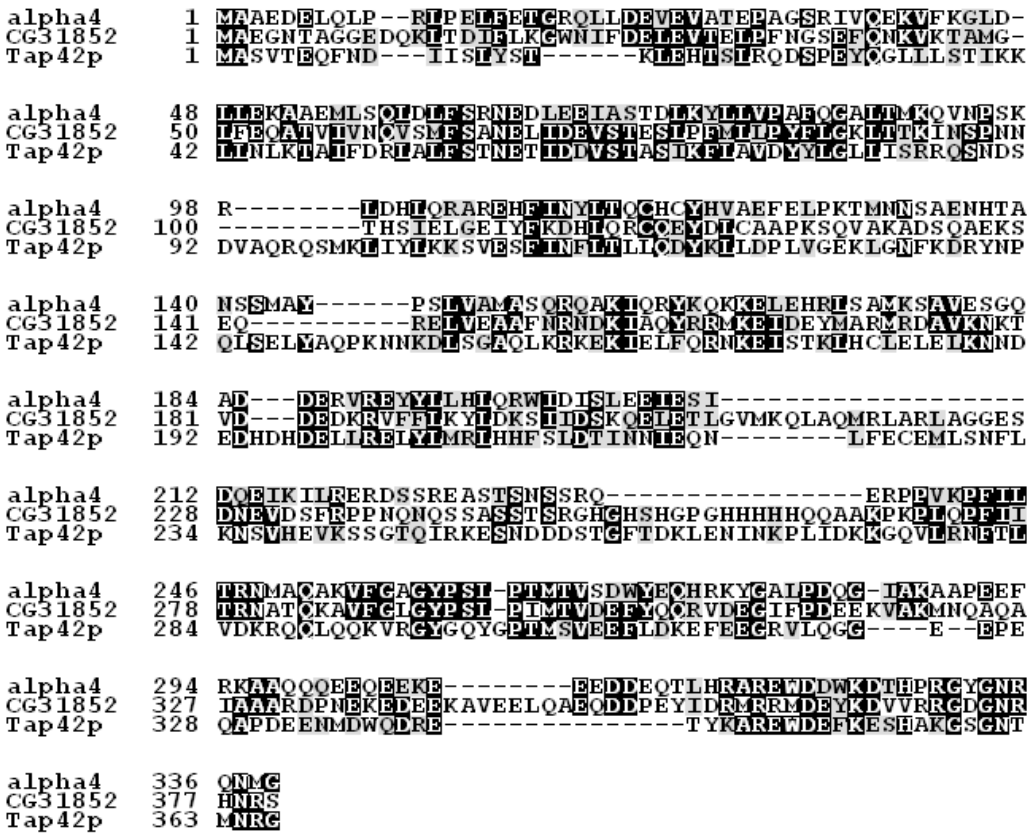

Figure 9. Alignment of *Drosophila melanogaster CG31852-PA* with *S. cerevisae* Tap42 and human α4. Amino acids sequences were aligned using the CLUSTALW program. Identical residues are shaded with black boxes. Individual alignments of *CG31852-PA* with α4 and Tap42 revealed identities of 28% and 21%, respectively.

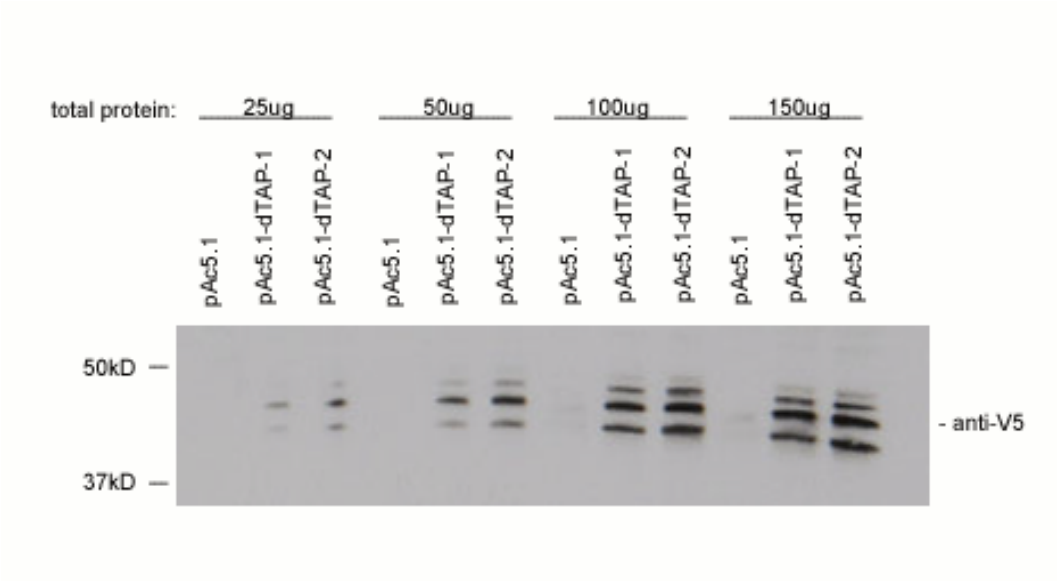

Figure 10. Expression of recombinant dTap-V5 in *Drosophila* S2 cells. Cells were transfected with 1 ug of pAc5.1-dTap-V5 or empty pAc5.1 plasmid using the Effectene transfection reagent. Cells were harvested 48 hours post-transfection and clarified lysates prepared. The indicated amounts of lysate proteins were separated by SDS-PAGE and proteins transferred to nitrocellulose. Membranes were probed with anti-V5 antibody to monitor expression of exogenous dTap.

TABLE 1. Genes and RNAi primers

Gene	Accession number	dsRNA primers
dPP2Ac	X55199	F: TCTTGATCAATGGATTGAGC R: CTGTCATCGGGATCGGACCA
dBα	D13004	F: ACATCATATCCTGCGTGGAA R: ACAGATTGATCCGCAGGTCG
dB56-1	AJ2777140	F: GAGGAGAGCACATCCAAAGA R: GGAGAAGTCGAACAGTGTGC
B56-2	LD40774	F: TCATCAGCAGCAGTCGAATA R: CAACATCTTTTCGCTTAAAC
PR72	CG4733	F: TAGAAGTGCTCGTAGCTAAA R: CCCAGGAGCAGAATGTGCTG
PP4	Y14213	F: CAGAAACCCGGTTGGCAAAA R: CGATGCTCGCGAAAAGGCTG
dTap	CG31852	F: AGTTGATCGACGAGGTGTCC R: GGTGGACGATGCAGATT
Stubarista	AAA28741	F: ATGTCGGGAGGCTTAGATAT R: GATCTGGTTGGTGAAGGCAC

F = Forward (5’ sense) primer, R = Reverse (5’ anti-sense) primer

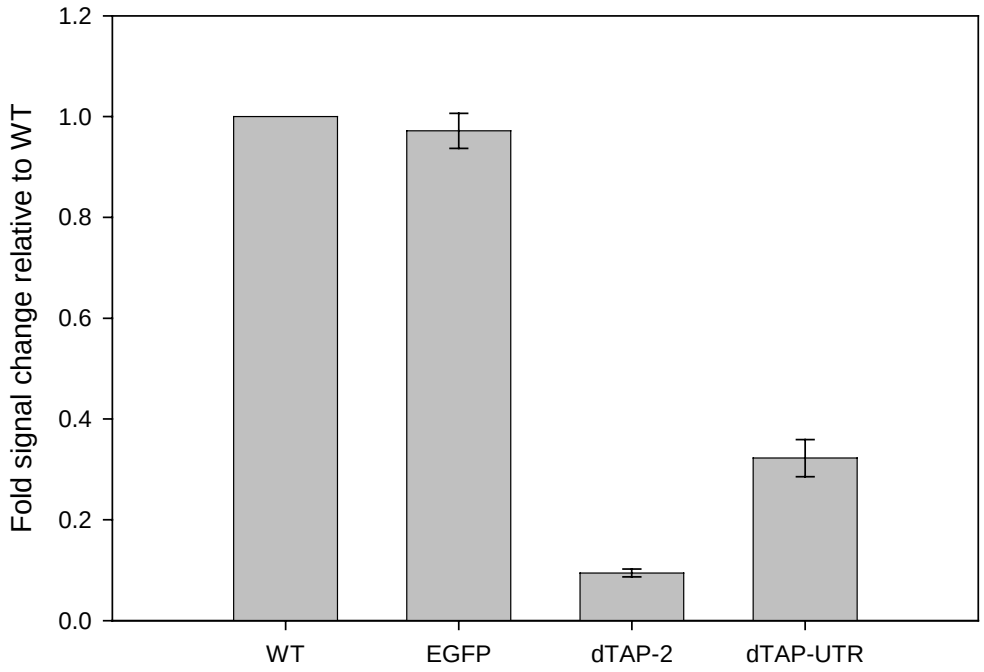

Figure 11. Knockdown of dTap mRNA by RNAi. S2 cells we treated in triplicate with 15 ug of EGFP, dTap-2, or dTap-UTR dsRNA daily for 4 days. Untreated control cells were plated at the same time as experimental samples. Total RNA was isolated and subjected to qPCR using the qTAP-1 primer set. A primer set against actin was utilized as qPCR control for determination of ΔCt. Fold change was calculated using the ΔΔCt method. Results are represented as percent signal relative to RNA isolated from untreated cells.

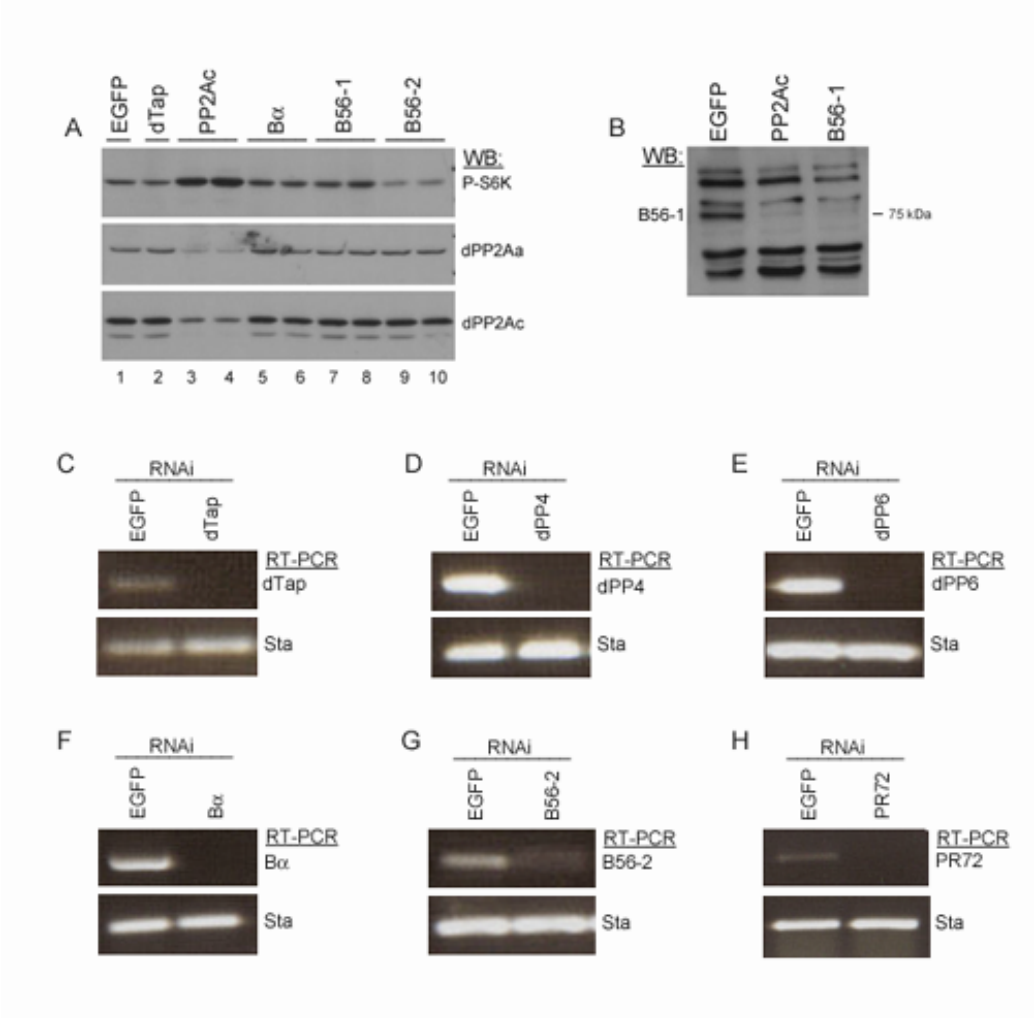

Figure 12. RNAi knockdown of dTap, PP2A, PP4, and PP6 subunits in S2 cells. Cells were treated with 60 ug dsRNA directed against PP2A subunits, dPP4, dPP6 and dTap for 96 hours. Clarified lysates were prepared and subjected to Western blotting with antibodies against PP2Ac, PP2A A subunit, P-S6K or dB56-1 (A and B). Total RNA was isolated and subjected to RT-PCR using gene-specific primers (C-H). *Stubarista*, an unrelated gene, was used as a loading control for RT-PCR experiments.

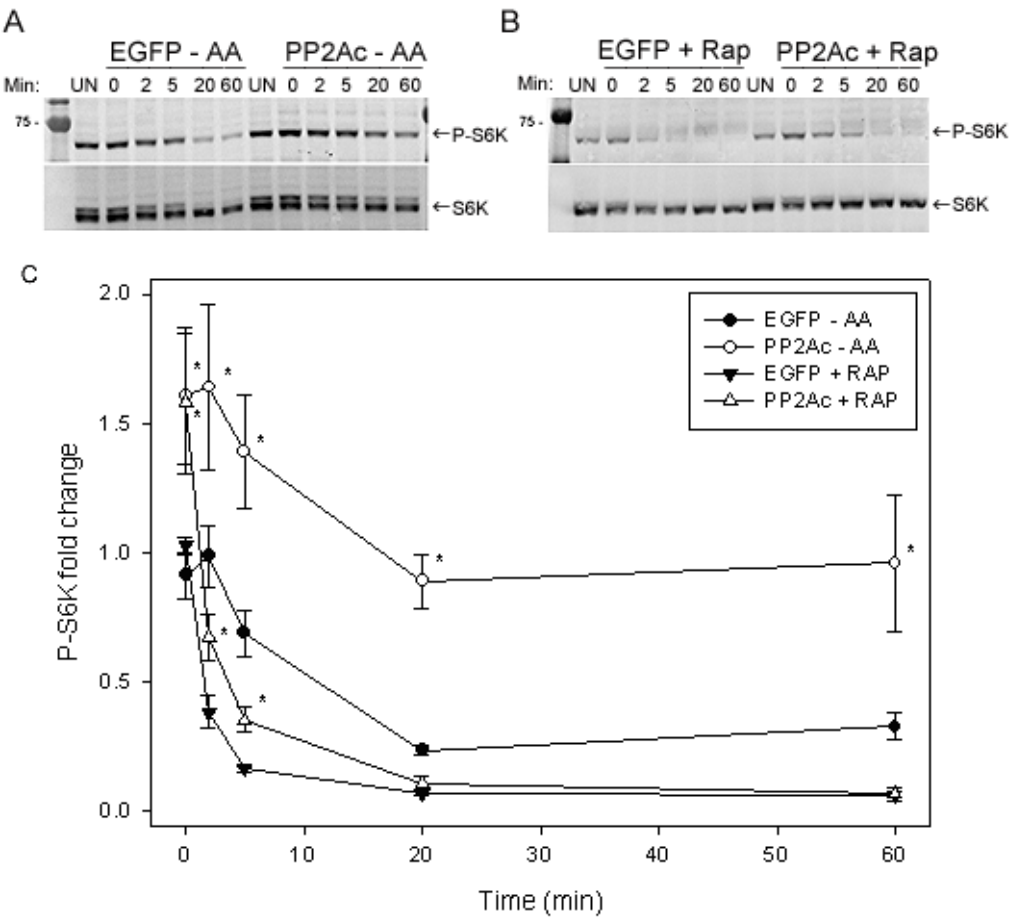

Figure 13. PP2Ac is required for T398 dephosphorylation following amino acid starvation. (A, B) Western blotting of lysates from cells treated with dsRNA against EGFP or dPP2Ac and starved of amino acids or treated with 20 nM rapamycin. Membranes were scanned and band intensity quantified using Image Master ID4 software. (C) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

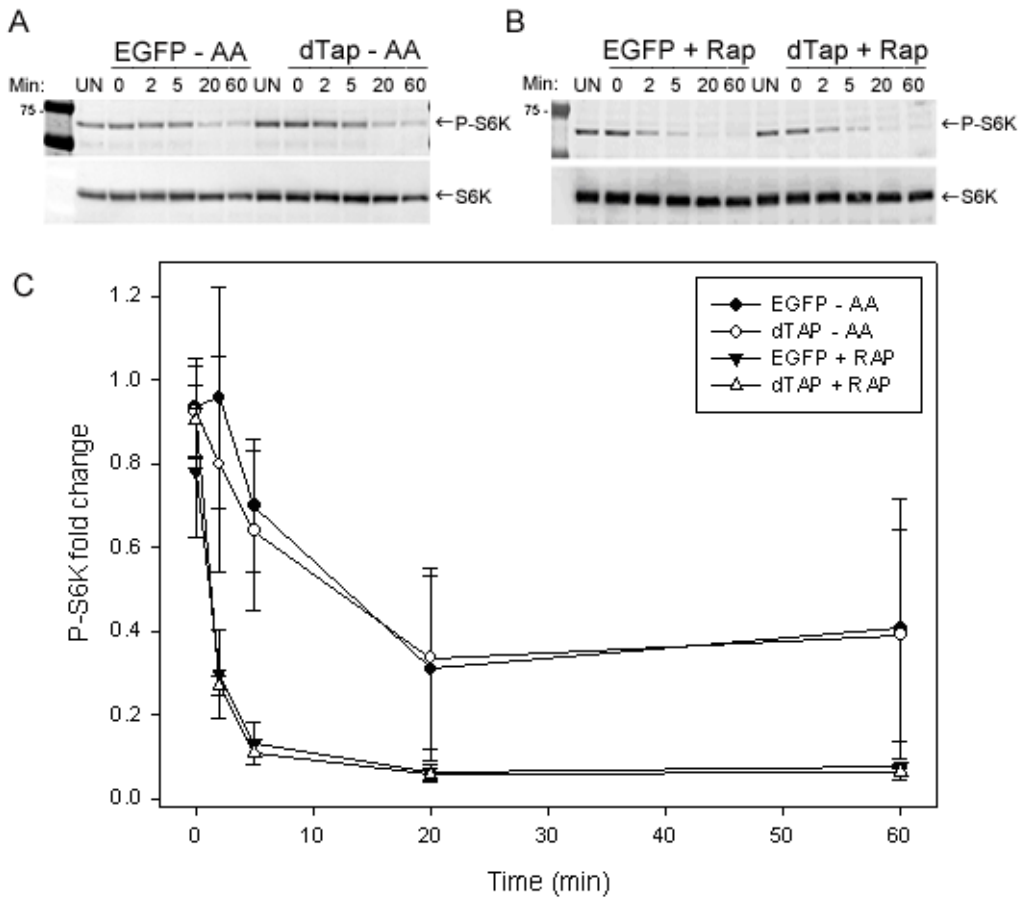

Figure 14. Knockdown of dTap does not affect dS6K phosphorylation. (A, B) Western blotting of lysates from cells treated with dsRNA against EGFP or dTap and starved of amino acids or treated with 20 nM rapamycin. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (C) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

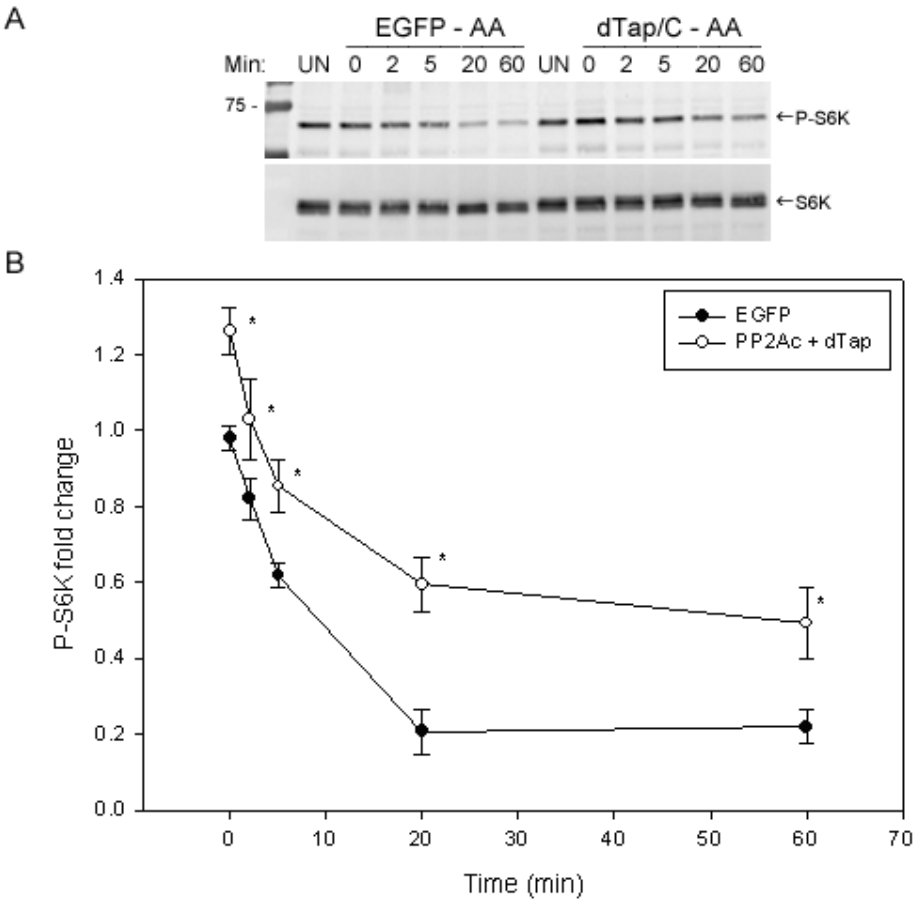

Figure 15. Simultaneous knockout of PP2Ac and dTap. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or PP2Ac + dTap in the presence of 20 nM zVAD-fmk and starved of amino acids. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

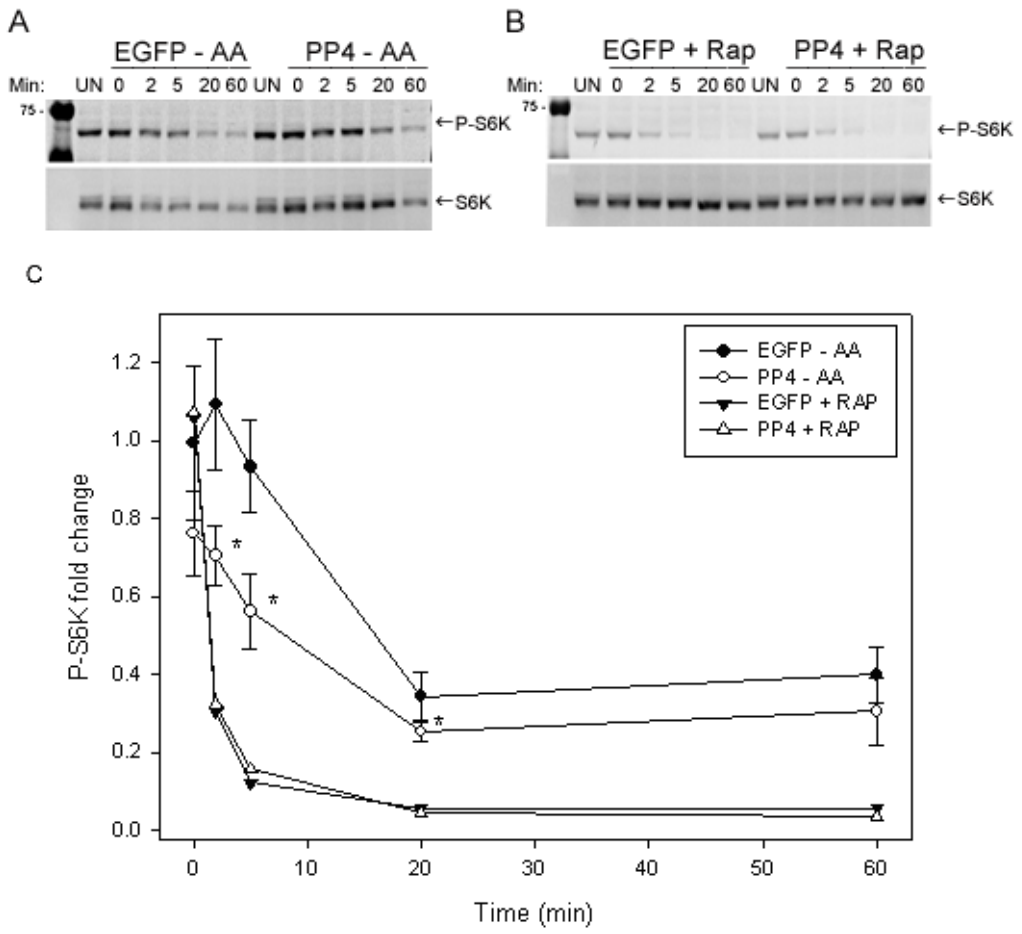

Figure 16. Loss of dPP4 results in hypophosphorylated T398. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or dPP4 and starved of amino acids or treated with 20 nM rapamycin. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

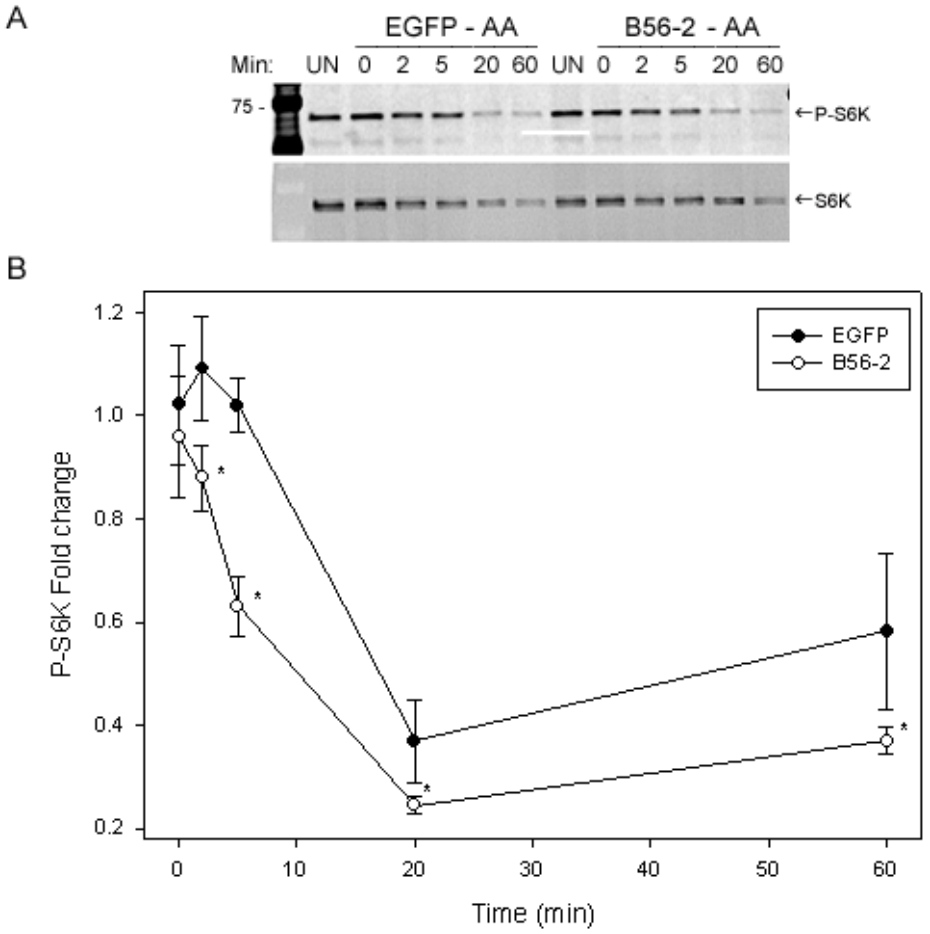

Figure 17. Loss of PP2A-B56-2 results in hypophosphorylated T398. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or B56-2 and starved of amino acids. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

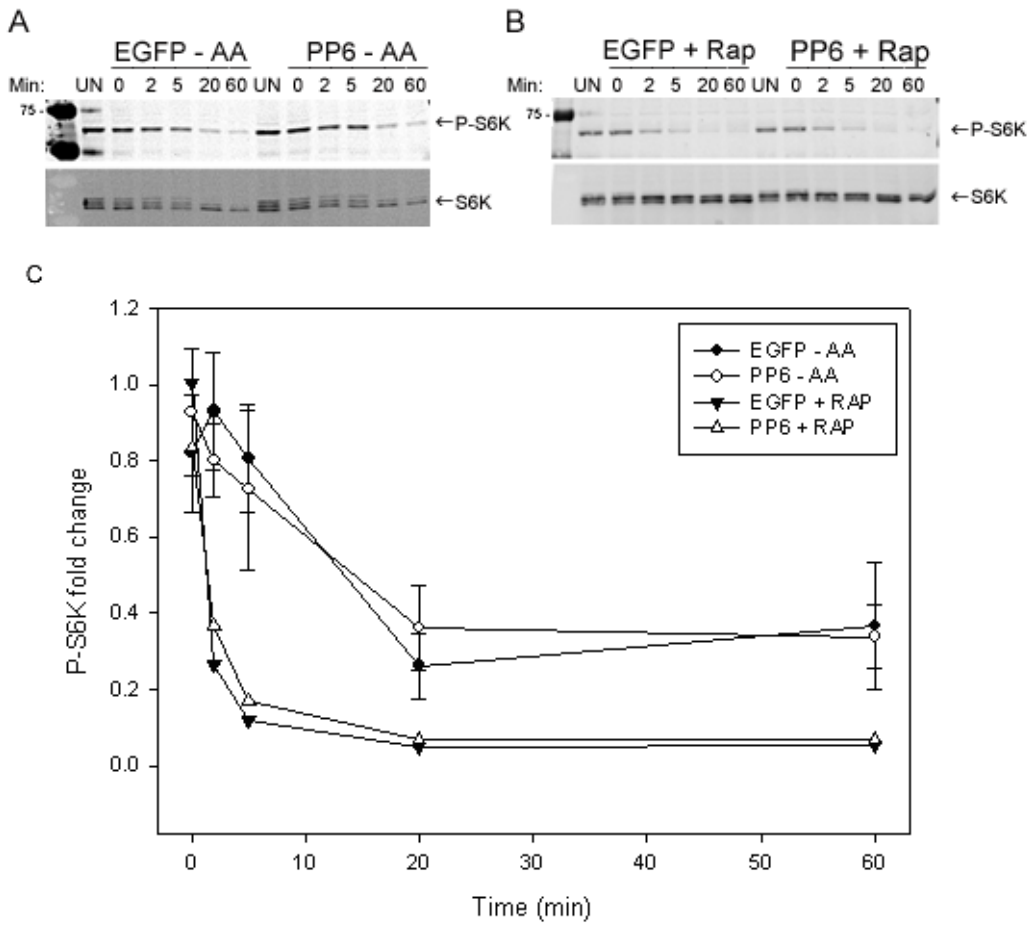

Figure 18. Ablation of dPP6 does not affect T398 dephosphorylation. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or dPP6 and starved of amino acids or treated with 20 nM rapamycin. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

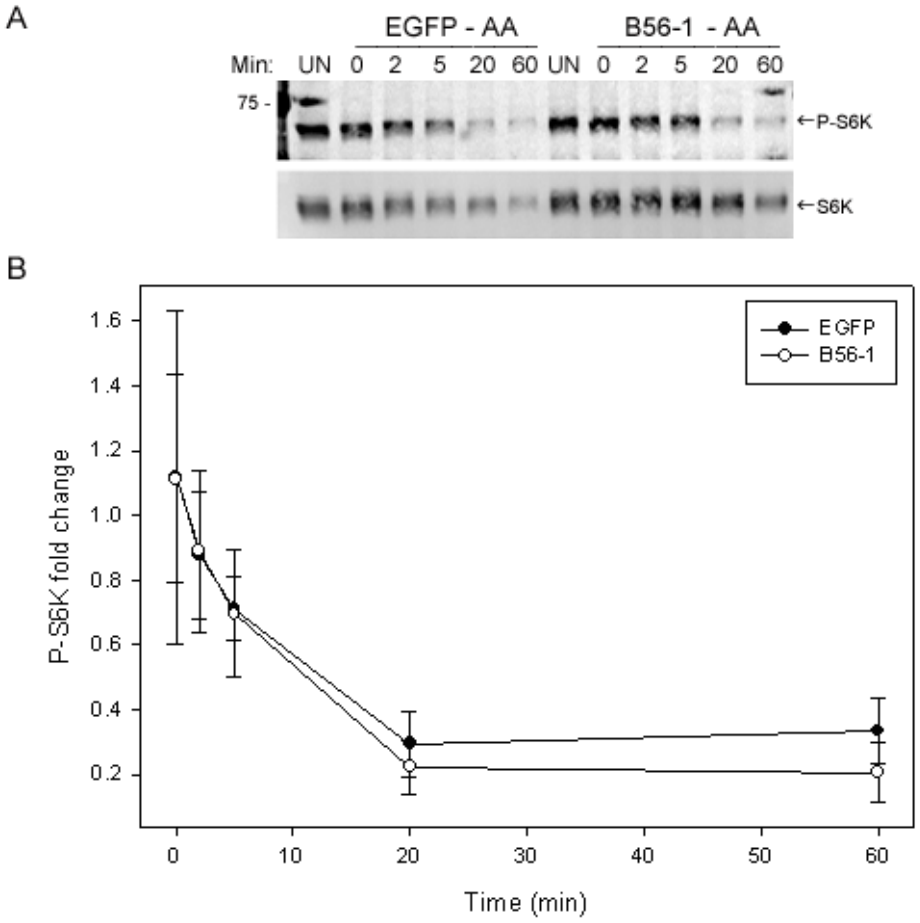

Figure 19. PP2A-B56-1 is not required for dephosphorylation of T398. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or PP2A-B56-1 and starved of amino acids. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

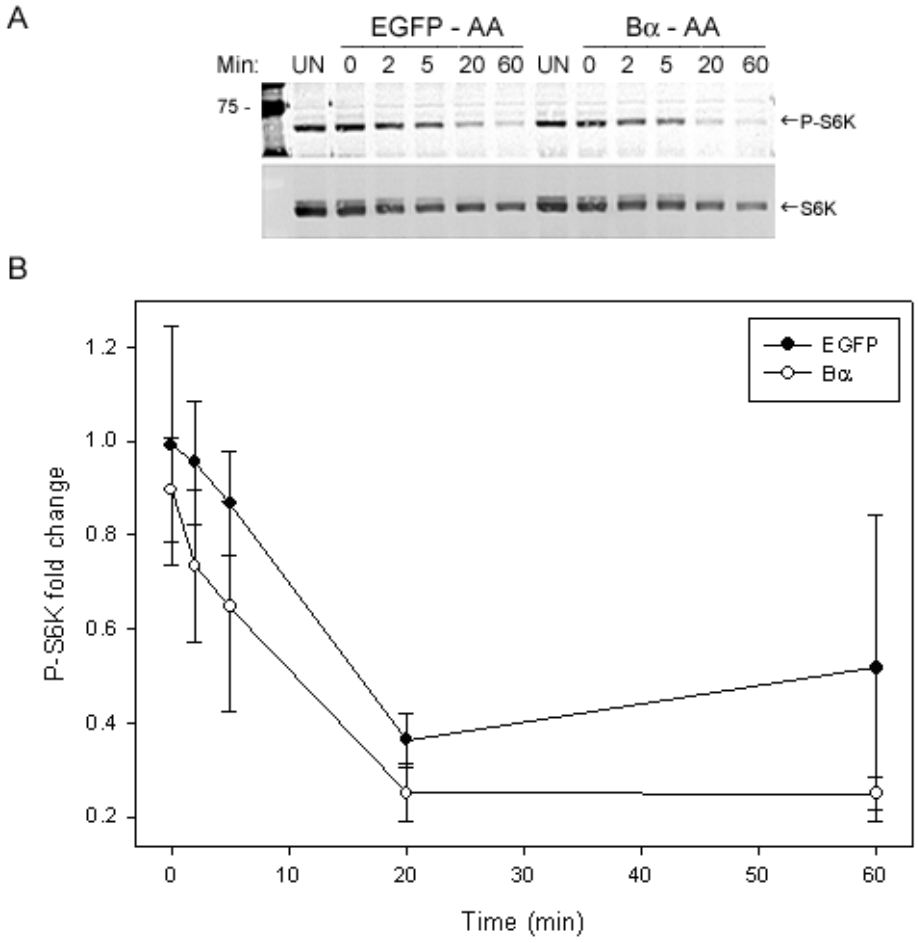

Figure 20. Loss of PP2A-Bα does not prevent T398 dephosphorylation. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or PP2A-Bα and starved of amino acids. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

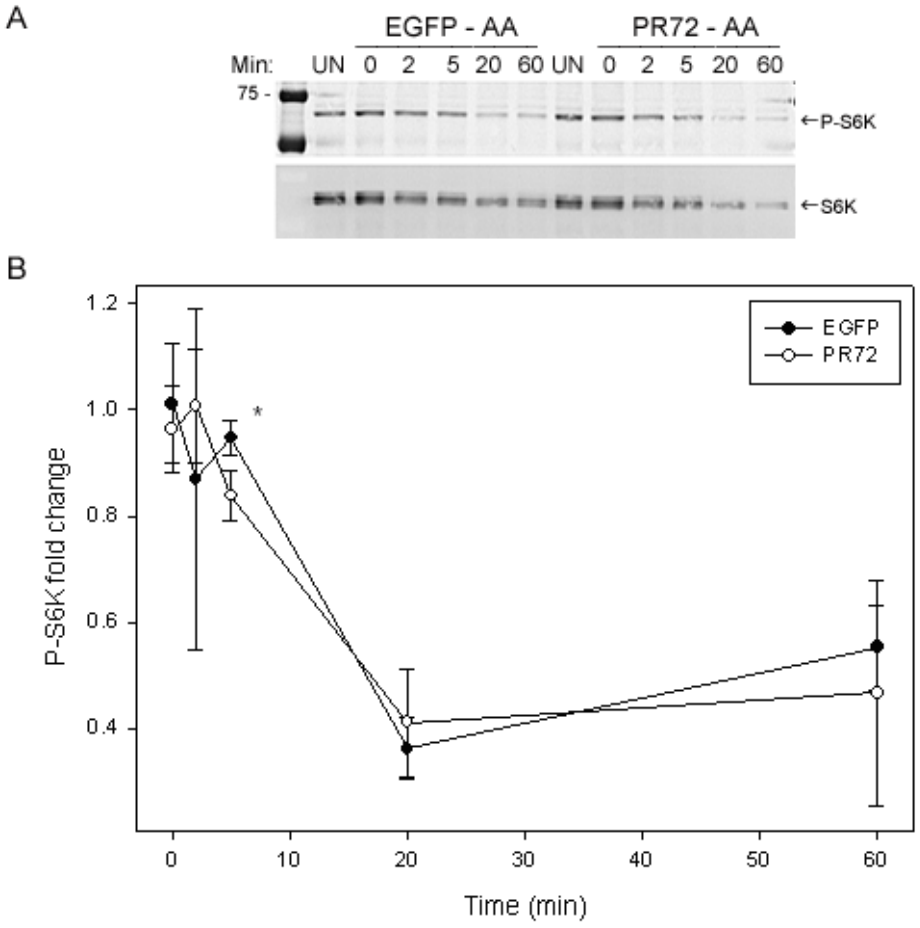

Figure 21. PP2A-PR72 is not required for T398 dephosphorylation. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or PP2A- PR72 and starved of amino acids. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

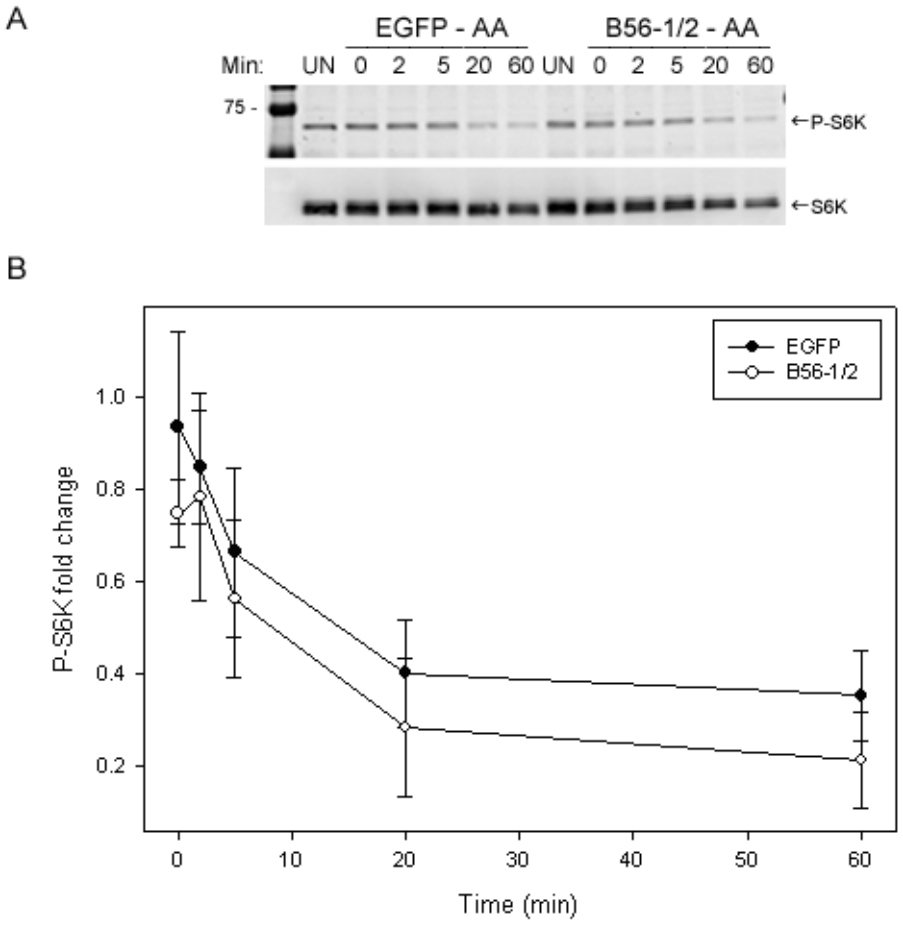

Figure 22. Double knockdown of B56-1/2 results in hypophosphorylated T398. (A) Western blotting of lysates from cells treated with dsRNA against EGFP or B56-2 and starved of amino acids. Membranes were scanned and band intensity quantified using the Image Master ID4 software. (B) Phospho-T398 intensity was normalized against dS6K intensity and fold change plotted as a percentage of the phospho-T398/dS6K ratio in lysates from untreated (UN) cells. Time points denoted with an asterik indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

TABLE 2. P VALUES FOR QUANTITATIVE STARVATION ASSAYS

					RNAi					
Time	dTap	PP2Ac	dTap + PP2Ac	PP4	PP6	Bα	B56-1	dB56-2	dB56-1 + dB56-2	PR72
0	0.908	*4.88e-03*	*2.15e-03*	0.085	0.381	0.1476	0.4148	0.4869	0.2149	0.5797
2	0.497	*0.015*	*0.040*	*0.008*	0.2007	0.8475	0.8620	*0.0114*	0.6877	0.5159
5	0.699	*2.18e-03*	*6.24e-03*	*0.002*	0.5395	0.8338	0.4558	*6.12e-05*	0.5212	*0.0281*
20	0.894	*3.98e-05*	*1.87e-03*	*0.030*	0.2051	0.2846	0.5569	*0.0242*	0.3419	0.5171
60	0.949	*6.48e-03*	*0.0107*	0.146	0.7748	0.5909	0.3788	*0.0325*	0.1669	0.5470

Statistical analysis of values observed in amino acid starvation assays as determined by the Student's t-test at each time point. Experimental values determined to be statistically different ($p \leq 0.05$) from control EGFP values are italicized.

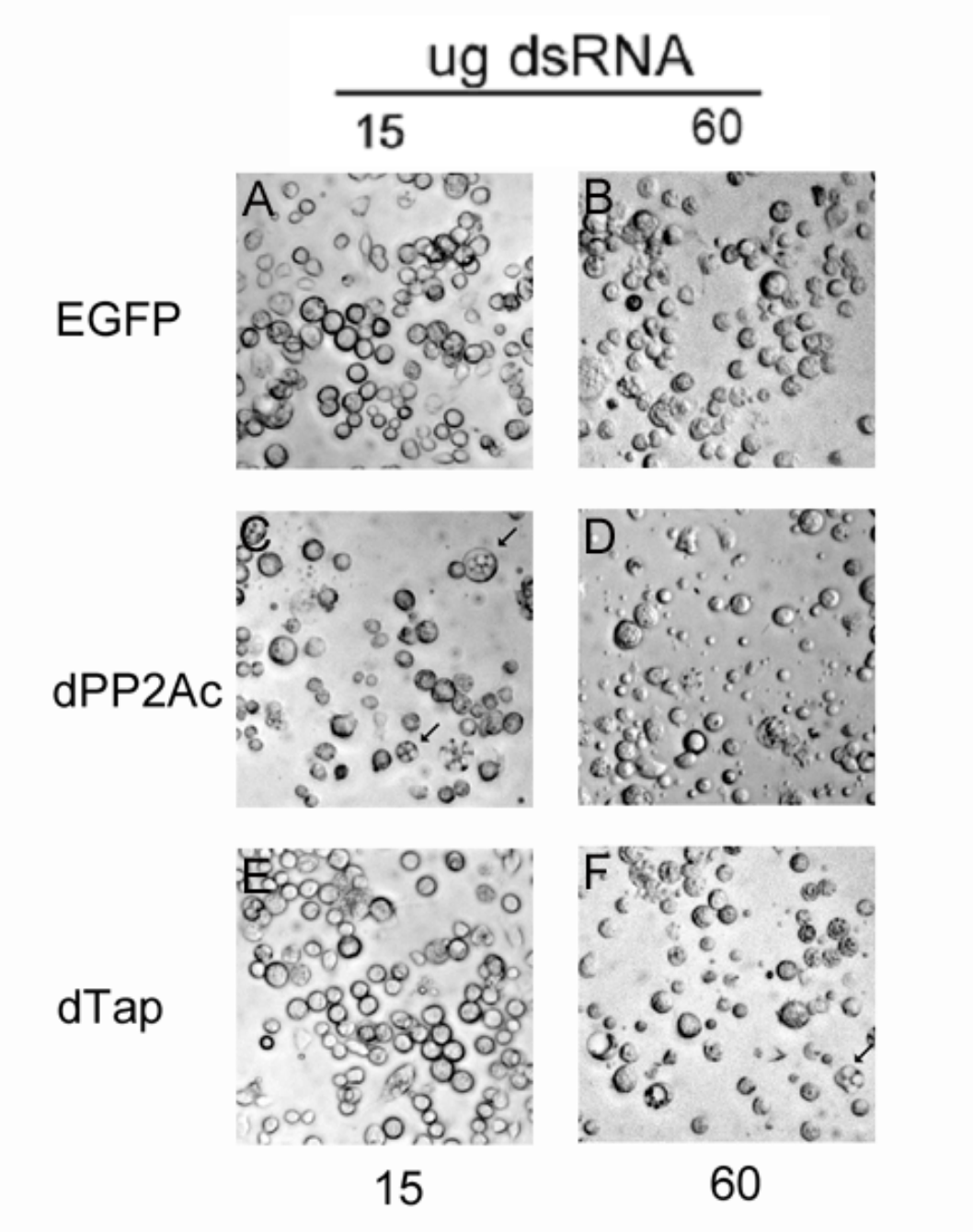

Figure 23. Depleting PP2Ac or dTap activates apoptosis in S2 cells. S2 cells were treated with 15 ug or 60 ug of dsRNA against EGFP, PP2Ac, and dTap for 96 hours. The cells were then imaged at 32x on an inverted light microscope. PP2Ac and dTAP knockdown cells displaying apoptotic features are highlighted with arrows.

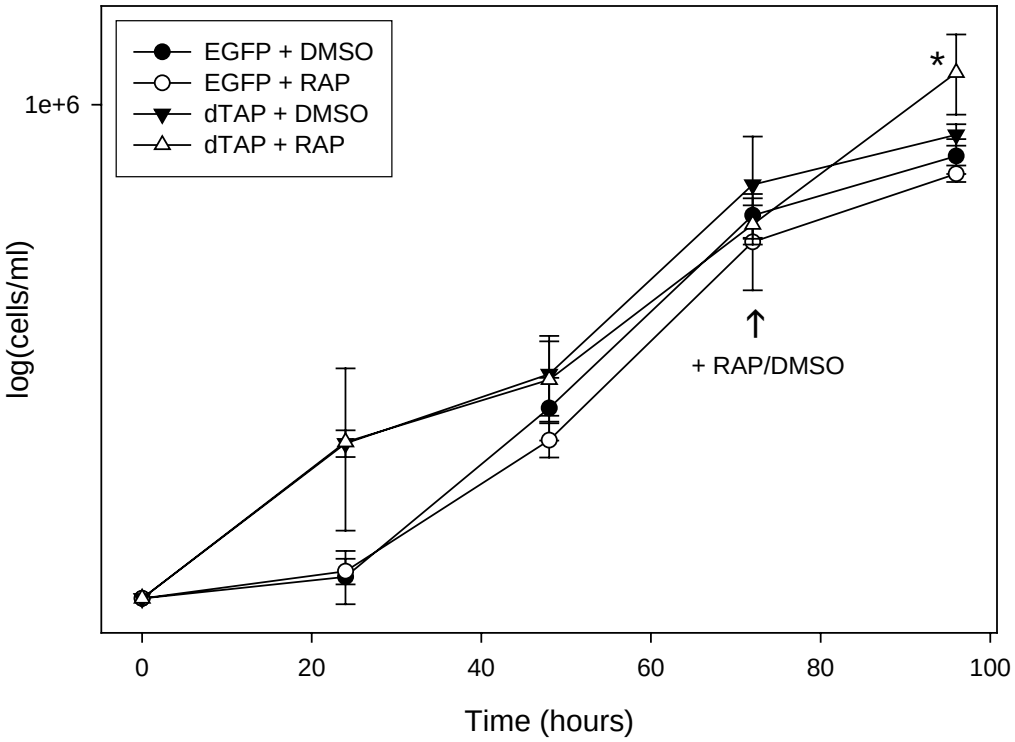

Figure 24. Loss of dTap confers rapamycin resistance to S2 cells. S2 cells were treated daily with 15 ug dTap-2 or EGFP dsRNA for 72 hours and grown at 25^{o}C. Rapamycin was then added to medium to a final concentration of 20 nM and cells incubated for an additional 24 hours, at which final counts were performed. Control cells were treated with equal volume of DMSO. Time points denoted with an asterisk indicate values statistically different from controls ($p \leq 0.05$). Experiments were performed in triplicate.

Chapter 4

Discussion

A. Regulation of Drosophila S6 Kinase by Nutrients

In agreement with previously reported results (Stewart, Berry et al. 1996; Radimerski, Montagne et al. 2002; Lizcano, Alrubaie et al. 2003), activation of dS6K occurred in the presence of free amino acids in the growth medium, as T398 phosphorylation was responsive to peptone and yestolate, both known to contain amino acids, as well as starvation medium supplemented with 1x amino acids (Fig. 4). Interestingly, phospho-T398-dS6k was observed as a band migrating near 65kDa, with several smaller bands appearing in the 50- to 55-kDa ranges. This pattern of migration is close to the predicted molecular weight of 55 kDa for dS6K. It would be prudent to assume the major band of immunoreactivity is fully phosphorylated dS6K, since phosphorylation of T398 precedes phosphorylation of T229 by dPDK1 (Pullen, Dennis et al. 1998). Also of note is the appearance of these lower bands in starved cells when treated with 1uM insulin or a combination of 1x amino acids and 1uM insulin (Figs. 5 and 6C), but not in cells restimulated with 1x amino acids alone (Fig. 6B). The immunoreactivity of the lower bands was also reduced by dTOR inhibition (Fig. 5B), suggesting that these bands may represent a pool of partially phosphorylated dS6K awaiting further modification.

In cells cultured in medium lacking amino acids, T398 phosphorylation was diminished (Figs. 4 and 6A), an effect also observed in the presence of the dTOR inhibitor rapamycin (Fig. 5B). These observations support the hypothesis

that phosphorylation of dS6K at T398 is dependent upon dTOR kinase activity, as inhibition of dTOR by both amino acid starvation and pharmacological inhibition resulted in dephosphorylation of T398. How exactly dTOR regulates T398 phosphorylation is unknown; but dTOR may sustain dS6K activity, in part, by restraining a calyculin A-sensitive phosphatase (Di Como and Arndt 1996; Rohde, Campbell et al. 2004), thereby preventing dephosphorylation and inactivation of dS6K and its downstream effectors.

Conflicting results exist concerning the ability of insulin to activate dS6K, most notably those of Thomas group (Radimerski, Montagne et al. 2002) and those of the Alessi group (Lizcano, Alrubaie et al. 2003). In our restimulation assays, insulin did not require the presence of free amino acids to induce phosphorylation of T398 (Figs. 6 and 7). Therefore, activation of dS6K by PI3K-dependent signaling pathways does not require the prior activation of dTOR by amino acids. However, dTOR activity is required for the phosphorylation of T398 and this likely occurs through the simultaneous activation of PDK1 and the ability of Akt to relieve TSC1/2-mediated repression of dTOR by phosphorylation of TSC2 (Shah, Wang et al. 2004). These results were confirmed by use of quantitative Western blotting to calculate the fold change of T398 in response to amino acids or insulin, with insulin stimulating phosphorylation of T398 to a 3-fold greater amount than amino acids alone (Fig. 7).

In agreement with previous data (Peterson, Desai et al. 1999; Petritsch, Beug et al. 2000) dephosphorylation of T398 by amino acid deprivation was prevented by the presence of the general phosphatase inhibitor calyculin A (Fig.

8). This therefore narrows down the list of possible enzymes responsible for regulating T398, as calcinuerin (PP2B) (Wang, Hashimoto et al. 1995) and PP7 (Huang and Honkanen 1998) are insensitive to CL-A. However, since calyculin A also inhibits the catalytic activities of other metallophosphatases (Honkanen and Golden 2002) and two of these phosphatases, PP4 and PP6, have been implicated in TOR signaling by virtue of their association with α4/Tap42 subunit (Kloeker, Reed et al. 2003), further studies are required to identify the protein phosphastase(s) that regulate dS6K and their responses to starvation, cellular stress and the presence of amino acids.

B. Cloning and Knockdown of dTap

Here we describe the isolation and cloning of the *Drosophila* homologue of Tap42/α4, dTap. This homologue shares 28% identity with α4 and 21% identity with Tap42 at the amino acid level. This low level of conservation is not surprising, since α4 and Tap42 only share 23% identity. Indeed, alignment of dTap with the *Arabidopsis* homologue Tap46 (Harris, Myrick et al. 1999) reveals only 21% identity between the two proteins. Alignment of dTap with α4 and Tap42 sequences does not reveal discrete domains with high levels of sequence identity. Analysis of identical residues indicate that the highest levels of conservation are found in residues with charged and bulky side chains and in semi-conserved substitutions that are found at a high frequency throughout the three sequences (Fig. 9). We therefore conclude that dTap is the *Drosophila*

homologue of Tap42/α4 in. A similar conclusion was also reached in a recent report (Cygnar, Gao et al. 2005).

A full-length dTap cDNA was cloned and expressed as an epitope-tagged protein in S2 cells. Exogenous expression of V5-tagged dTap does not result in gross morphological changes in S2 cells. Western blots of the lysates from cells transfected with dTap-V5 revealed three major bands of anti-V5 immunoreactivity (Fig. 10). These bands migrated with apparent molecular weights between 42- and 48-kDa on SDS-polyacrylamide gels. These results are close to the predicted molecular weight of dTap-V5 (55-kDa). The three bands visualized by Western blotting were unexpected. One explanation is that overexpressed dTap-V5 is unstable and subject to proteolytic degradation. In this case, cleavage must occur within the N-terminal region since the C-terminal V5 tag is intact. Alternatively, dTap-V5 may be subjected to post-translational modifications. This latter possibility is in agreement with the findings that both α4 and Tap42 are phosphoproteins. The slowest migrating band could be a phosphorylated form of dTap-V5 and the two lower bands less phosphorylated forms, resulting in the different migration patterns observed on SDS polyacrylamide gels. The sequestration model for dTap predicts that at least two differentially phosphorylated forms of dTap are present in the cell (Fig. 3). Further studies are needed to elucidate whether dTap is indeed a phosphoprotein and if phosphorylation is regulated in a dTOR-dependent manner.

To determine if dTap expression could be selectively ablated with RNAi, quantitative PCR was carried out on samples from cells treated with dTap

dsRNA. We found that dTap mRNA levels could be significantly reduced by both dTap-directed dsRNAs, with the dTap-2 dsRNA resulting in a 90% knockdown when compared to untreated S2 cells (Fig. 11). The addition of non-specific dsRNA encoding a region of the EGFP gene does not affect dTap mRNA levels, confirming that loss of dTap mRNA was due to treatment of cells with dTap-specific dsRNAs. These results demonstrated that dTap mRNA can be efficiently reduced by RNAi.

C. Effects of RNAi on dS6K Phosphorylation

1. dTap and PP2Ac

In order to identify the phosphatase complex responsible for regulation of dS6K, cells were depleted of specific phosphatase subunits and then subjected to amino acid starvation. Presumably, the loss of the catalytic or regulatory subunit catalyzing T398 dephosphorylation will disrupt phosphatase activity and prevent dephosphorylation of dS6K under conditions that inhibit cell growth. Loss of a dS6K phosphatase would result in sustained increase in phospho-T398 levels during starvation compared to wild-type cells.

The sequestration model of dTap activity predicts that loss of dTap would result in increased PP2A activity and hypophosphorylation of T398 even during periods conducive to growth. The results presented here show that dTap knockdown actually causes a slight increase in phospho-T398 levels when compared to control cells. This observation is in agreement with a recent report (Cygnar, Gao et al. 2005). This data does not support the sequestration model in

which dTap inhibits PP2Ac by sequestering phosphatase activity away from dS6K. While the results do not eliminate a possible role for dTap in regulation of dS6K, they clearly indicate that the sequestration model is not correct.

Selective ablation of the PP2A catalytic subunits by RNAi showed that loss of PP2Ac resulted in increased basal levels of phosphorylated T398 in the presence of amino acids and serum (Fig. 13). Importantly, cells lacking PP2Ac were unable to efficiently dephosphorylate T398 during amino acid starvation (Fig. 13A and C). Quantitative analysis of whole cell lysates from cells treated with PP2Ac dsRNA showed a 2-fold increase in basal phospho-T398 levels (Fig. 13C). In control cells, phospho-T398 intensity is diminished considerably by 20 minutes of amino acid withdrawal and remains very low 60 minutes into starvation. The small change in phospho-T398 signal between 20 and 60 minutes suggests that a majority of T398 dephosphorylation occurs within a few minutes of amino acid withdrawal. PP2Ac RNAi cells maintained elevated levels of T398 phosphorylation 60 minutes after withdrawal of amino acids. Interestingly, the shape the of the PP2Ac RNAi dephosphorylation curve mirrors that of control lysates (Fig. 13C), but the level of phospho-T398 in PP2Ac RNAi cells remains roughly 50% higher than control cells. This decrease in phospho-T398 levels in PP2Ac RNAi cells is likely due to incomplete knockdown of catalytic subunit. PP2Ac protein levels appear to be greatly reduced in RNAi cells but enough PP2Ac is present to be detected by Western blotting. There is likely a small amount of remaining PP2Ac that can dephosphorylate a portion of the phospho-T398 pool, accounting for the decrease in phospho-S6K intensity during

starvation. The increased levels of phospho-T398 indicate that PP2Ac is a major activity catalyzing dephosphorylation of T398. This data is also in agreement with earlier inhibitor studies suggesting T398 is dephosphorylated by a calyculin A-sensitive phosphatase (Peterson, Desai et al. 1999) and a study reporting that loss of dPP2Ac increased basal phospho-T398 levels (Cygnar, Gao et al. 2005).

To test if dTap depletion affects the activity of dPP2Ac towards dS6K, RNAi was employed to deplete both genes simultaneously (Fig. 15). Analysis of phospho-S6K in cell lysates depleted of both dPP2Ac and dTap and subjected to amino acid starvation did not appear to be distinct from the lysates of cells depleted of dPP2Ac alone (compare Figs. 13 and 15). These results suggest that the hyperphosphorylation of T398 in the absence of dPP2Ac does not depend upon the presence of dTap, arguing against the hypothesis that these two proteins catalyze dephosphorylation of T398 as part of a novel heterodimeric phosphatase. However, it is of interest to note that the shape of the dephosphorylation curve calculated from lysates of dTap and dPP2Ac double knockdown cells appears to differ from that of the dPP2Ac RNAi lysates in a steeper initial slope in the early time points. This apparent effect of dTap depletion on the phosphorylation state of T398 is intriguing, but other roles for dTap in growth pathways could contribute to this effect.

One possible explanation for the lack of an observed effect by dTap depletion could be that withdrawal of both amino acids and serum activate stress pathways that down-regulate dS6K. These stress pathways may over-compensate for the reduction in dTap and may provide the cell an alternative way to turn off

dS6K other than the dTOR-dependent mechanism examined here. In order to eliminate any global effects of amino acid and serum starvation, cells were treated with rapamycin in complete growth medium. Rapamycin results in dephosphorylation of T398, but is a much more specific method of dTOR inhibition. However, depleting dTap did not prevent the dephosphorylation of T398 by rapamycin (Fig. 14), suggesting that dTap does not exert an effect on nutrient signaling pathways in *Drosophila* downstream of dTOR.

2. Knockdown of PP2A Regulatory Subunits and Effects on dS6K

The lack of evidence that dTap acts as a PP2A targeting protein for dS6K leads to the conclusion that the phosphatase that catalyzes T398 dephosphorylation contains PP2Ac. Based on previous data in other systems, we would assume that the dS6K phosphatase is likely a heterotrimeric complex. Accordingly, ablation of the PP2A B subunit responsible for targeting the complex to dS6K should decrease dephosphorylation of T398. Surprisingly, knockdown of each of four B subunits genes (Bα/B56-1/B56-2/PR72) individually did not prevent dephosphorylation of T398 during starvation or increase basal phospho-T398 levels in lysates (Figs. 17, 19-21). These results suggest that the T398 phosphatase does not require any of the four known *Drosophila* PP2A regulatory subunits. This observation is unexpected, but raises the possibility that PP2Ac is directed to dS6K by an unidentified targeting subunit. This implies that *Drosophila* PP2Ac may interact with more proteins

than previously thought and supports the existence of novel PP2Ac-associated phosphatase complexes.

When the fold change in P-S6K intensity for PP2A B subunit knockdown cells was calculated, an interesting effect was observed in cells depleted of the dB56-2 subunit (Fig. 17). The absence dB56-2 results in a more rapid dephosphorylation of T398 during amino acid withdrawal and phospho-T398 intensity is significantly lower than lysates from control RNAi cells (Table 2) throughout the time period examined. These results all but eliminate dB56-2 as a component of the T398 phosphatase complex. However, the significant decrease in phospho-T398 levels during starvation may be reflective of a requirement for a dB56-2-associated PP2A complex to maintain dTOR activity. The depletion of this dB56-2-containing complex would decrease basal dTOR activity levels and facilitate inactivation of dTOR, leading to a more rapid dephosphorylation of T398 during starvation. However, it must be note that basal phospho-T398 levels in dB56-2 RNAi lysates do not differ significantly from control lysates. This does not support the notion of dB56-2 being required for basal dTOR activity; however, since the hypophosphorylation of T398 is observed during starvation, it is more likely that a dB56-2-associated heterotrimer is a limiting factor antagonizing the inactivation of dTOR. Further studies will be required to characterize the effects of dB56-2 depletion on dTOR kinase activity, as well as the effects of loss of dB56-2 on dephosphorylation of T398 by rapamycin.

3. PP2A-like Phosphatases

If PP2Ac catalyzes dephosphorylation of T398 in a novel manner without the use of the known regulatory B subunits, it is possible that the protein phosphatase 2A-like family members dPP4 and dPP6 may also be targeted to dS6K. The catalytic subunits of both phosphatases are highly similar to dPP2Ac and reduction of phospho-T398 signal in PP2Ac RNAi cells could be due to compensatory dephosphorylation carried out by dPP4 or dPP6. However, depletion of either gene had no effect on dephosphorylation of T398 (Figs. 16 and 18). Similarly, loss of either dPP4 or dPP6 did not result in the increase in basal T398 phosphorylation observed in dPP2Ac RNAi cell lysates.

Quantitation of phospho-T398 intensity from dPP4 RNAi cells revealed depletion of dPP4 leads to a 20% reduction in basal T398 phosphorylation (Fig. 16). Loss of dPP4 results in effects similar to those observed in cells depleted of dB56-2 (Fig. 17). Amino acid starvation caused a greater extent of T398 dephosphorylation in cells treated with dPP4 dsRNA. These results suggest a function for dPP4 upstream of dTOR and that the loss of dPP4 results in down-regulation of dTOR activity. This result is in agreement with previous studies reporting that dPP4 ablation reduced S2 cell growth by 20% (Silverstein, Barrow et al. 2002). Human PP4 has been reported to interact with insulin receptor substrate 4 (IRS-4) and regulate the phosphorylation state and half-life of this insulin receptor adaptor protein (Mihindukulasuriya, Zhou et al. 2004). Deletion of PP4 results in a substantial increase in IRS-4 degradation, impairing the ability of the insulin receptor to activate downstream effectors. *Drosophila* PP4 could

regulate cell growth by modulating growth factor input into the dTOR pathway via effects on IRS proteins. Importantly, these results show that dPP4 and dPP6 do not directly catalyze dephosphorylation of T398.

4. Modified Sequestration Model of dTap Activity

In summation, we present evidence supporting the conclusion that dPP2Ac is the phosphatase responsible for catalyzing dephosphorylation of T398 in *Drosophila* during periods of dTOR inhibition. However, dPP2Ac does not appear to require the presence of any of the four known dPP2A targeting subunits and T398 is not dephosphorylated by the PP2A-like phosphatases dPP4 and dPP6. Dephosphorylation of T398 is not affected by the absence of dTap, thereby rendering the sequestration model inaccurate. Also, we present evidence that dTap does not act as targeting subunit of a dTap/dPP2Ac heterodimeric phosphatase. However, loss of dTap makes S2 cells resistant to the growth inhibitory effects of rapamycin, indicating that dTap has a function in dTOR-mediated growth of *Drosophila* S2 cells.

One possible model to explain these results is a modified version of the sequestration model. In this model, dPP2Ac is bound to an unidentified targeting subunit as a part of a ternary complex with dTap under periods of growth (Fig. 25). When active, dS6K is localized away from phosphatase through interactions with substrate and possibly binding of dPDK1 to T398. This allows for repression of phosphatase by a dTOR-dependent mechanism, as well as providing an extra level of control by occlusion of the phosphatase from T398. During

periods of low nutrient availability, dTap is dephosphorylated and the dPP2Ac-containing phosphatase complex is released due to reduced affinity for dTap. This model requires dissociation of dS6K from substrate and dPDK1, which allows dPP2Ac to dephosphorylate T398. Indeed, in the course of these studies it was reported that both mTOR and p70-S6K associate with the eukaryotic initiation factor 3 (eIF3) translational pre-initiation complex during growth and maneuver on and off of eIF3 in a signal-dependent fashion (Holz, Ballif et al. 2005). If dS6K behaves in a similar manner, dissociation of dS6K from pre-initiation complex could be a pre-requisite, and the rate-limiting step, for dephosphorylation of T398 by dPP2Ac. Once T398 is dephosphorylated, the dPP2Ac-associated phosphatase dissociates from dS6K and reforms the sequestration complex with dTap when dTOR is again activated.

This model also accounts for the lack of effect observed in cells depleted of dTap. Loss of dTap would release the dPP2Ac-associated phosphatase from sequestration; however, interaction of dS6K with substrate is the rate-limiting step of inactivation and would prevent dephosphorylation of T398 even in the presence of higher levels of free phosphatase. This model suggests that T398 dephosphorylation is a two-step process requiring dissociation of at least two different protein complexes (dTap/phosphatase and dS6K/substrate). The slight increase of basal T398 phosphorylation seen with loss of dTap could be accounted for by inappropriate localization of dPP2Ac, as loss of dTap would result in mis-localization of dPP2Ac. Cygnar and coworkers reported an increase in the amount of nuclear dPP2Ac in the absence of dTap (Cygnar, Gao et al. 2005); however, this

group used a fluorescence-based assay to measure dPP2Ac levels, and this result needs to be confirmed. A reduction in the amount of properly localized dPP2Ac-associated phosphatase could lead to reduced T398 dephosphorylation, resulting in a slight increased in basal dS6K phosphorylation.

D. Effects of RNAi on Cell Growth and Viability

1. Loss of dTap or PP2Ac Causes Apoptosis in S2 Cells

Previous studies have shown that PP2A is required for cell growth and viability. Generation of knockout mice and flies lacking PP2A subunits has resulted in embryonic lethality (Gotz, Probst et al. 1998) or defects in growth and development (Uemura, Shiomi et al. 1993; Hannus, Feiguin et al. 2002). Pharmacological inhibition of PP2A activity results in cell cycle arrest (Zheng, Woo et al. 1991) and cell death (Inomata, Saijo et al. 1995) in all organisms tested thus far. Knockdown of PP2A subunits in *Drosophila* S2 cells activates apoptosis (Li, Scuderi et al. 2002; Silverstein, Barrow et al. 2002), with the most severe phenotype resulting from loss of PP2A catalytic subunit. The data presented here confirms and extends previous information regarding the role of PP2A in cell survival.

The presence of the caspase-3 inhibitor zVAD-fmk in culture medium during dsRNA treatment prevents PP2Ac RNAi-induced cell death and the appearance of apoptotic bodies (Lu, in preparation). This supports evidence that loss of PP2Ac activates apoptosis in a caspase-3-dependent manner. However, PP2Ac RNAi cells cultured in the presence of ZVAD-fmk displayed a severe

defect in cell growth. PP2Ac RNAi cells cultured in the presence of 20 nM zVAD-fmk grew to a density of 800,000 cells/mL after 96 hours; substantially lower than EGFP control cells (2-2.3x10^6 cells/mL). The data presented here indicates that the reduced growth of dPP2Ac knockdown cells is not due to a loss of dTOR activity, since dS6K is hyperphosphorylated. The growth defect is likely caused by disruption of an essential PP2Ac-associated activity or cell cycle arrest. However, with the multiple roles for PP2Ac throughout the cell, the reduction in cell growth could result from disruption of multiple processes. Further studies will be required to pin down the direct effects of PP2Ac knockdown and activation of apoptosis.

These results suggest one possible mechanism of cell death induced by loss of PP2Ac is that depletion results in inappropriate activation of dS6K under non-permissive growth conditions. The inability to turn off dS6K would lead to unregulated biogenesis, exhausting intracellular energy and amino acid stores. In humans, there is evidence that prolonged activation of p70-S6K may lead to insulin resistance through feedback mechanisms that down-regulate signaling (Tremblay, Krebs et al. 2005). At least one study has reported that mTOR is phosphorylated by p70-S6K in a repressor domain (Holz and Blenis, 2005), suggesting that inappropriate inactivation of p70-S6K could lead to inactivation of dTOR. This prolonged inactivation of essential growth pathways could be a contributing factor to dPP2Ac-induced cell death, but further studies will be required in this area.

Considering the small amount of amino acid identity shared between α4 and dTap, evidence for functional homology between these proteins could be provided by finding similar phenotypes when the genes are knocked down. In mammals, loss of the α4 allele in embryonic stem cells resulted in apoptosis, suggesting an essential role for α4 in development (Kong, Fox et al. 2004). Interestingly, treatment of S2 cells with dsRNA directed against dTap also appeared to activate apoptosis, but not to the extent seen with ablation of PP2Ac (Fig 2C). The appearance of apoptotic bodies in culture medium coincided with an observed slowing of growth in dTap RNAi cells. Loss of dTap also resulted in morphological changes to a small number of cells; some resembling the "honeycomb" appearance seen in the PP2Ac knockout (Fig. 23). This data provides *in vivo* evidence that dTap is required for cell viability, but the mechanism by which loss of dTap triggers apoptosis in *Drosophila* and mammalian cells remains unclear. It is also important to mention that dTap, through potential interactions with dPP4c and dPP6c, has other functions, as demonstrated for yeast Tap42. Thus, loss of dTap protein product would impact a number of cellular processes, possibly culminating in cell death.

2. Knockdown of dTap Confers Rapamycin Resistance to S2 Cells

Loss of *TAP42* in *S. cerevisiae* allows cell growth in the presence of rapamycin (Di Como and Arndt 1996). If dTap is indeed a functional homolog of Tap42, reduction of dTap mRNA levels should confer rapamycin resistance to S2 cells. Results from cell growth assays showed that cells treated with dTap-2

dsRNA grew to a higher density than EGFP control cells in the presence of 20 nM rapamycin (Fig. 24). These results suggest that loss of dTap makes S2 cells resistant to rapamycin, in agreement with previous reports on yeast Tap42 (DiComo and Ardnt 1996).

There are multiple mechanisms that could be involved in the rapamycin insensitivity of cells depleted of dTap. According to the sequestration model of dTap-mediated phosphatase repression, addition of rapamycin would result in dephosphorylation of dTap and reduction in affinity for the PP2A catalytic subunit. In the presence of drug, phosphatase activity would increase, resulting in the dephosphorylation of dS6K, and potentially other substrates, and a reduction of ribosomal biogenesis. A loss of dTap would be expected to further decrease cell growth by increasing phosphatase activity towards dS6K even under conditions favorable to growth. The observation that the loss of dTap confers rapamycin resistance to S2 cells differs from this prediction and suggests two possibilities. 1) dTap is the functional homolog of Tap42 and is required for the growth inhibitory effects of rapamycin or 2) loss of dTap negatively affects the rapamycin sensitivity of S2 cells. This reduction of rapamycin sensitivity could be mediated by a dTap/phosphatase complex (possibly dPP4 or dPP6) upstream of dTOR and required for proper regulation of the pathway. Indeed, several processes requiring both Tap42 and Sit4 have been described (Rohde, Campbell et al. 2004), suggesting a role for positive regulation of phosphatases by Tap42 in yeast.

E. Future Directions

1. Other dTap Binding Partners

Cygnar and collegues reported that exogenously expressed dTap associated with PP2Ac (Cygnar, Gao et al. 2005), but did not investigate any other potential dTap-interacting proteins (i.e., dPP4 and dPP6). In fact, there is no evidence at the time of this writing that dTap interacts with PP2Ac independently of the PP2A A subunit, as reported for Tap42 and α4. The possibility remains that dTap also associates with proteins other than phosphatase catalytic subunits, therefore it would be useful to utilize a proteomics-based approach to identify other dTap-binding proteins, either by immunoprecipitation of exogenously expressed dTap-V5 or challenging S2 cell lysates with a recombinant GST-dTap fusion protein. It would also be worthwhile to examine if the association dTap with binding partners is sensitive to dTOR inhibition.

2. Other Functions of dTap in S2 Cells

The results presented here suggest that the sequestration model of dTap activity is inaccurate. However, loss of dTap conferred rapamycin resistance to S2 cells (Fig. 24) and knockout of dTap and PP2Ac in combination produced a dephosphorylation curve that differed from the curve observed in PP2Ac knockout cells (compare Figs. 13 and 15). Loss of another putative dTap-interacting phosphatase (dPP4) resulted in hypophosphorylation of dS6K during starvation. These observations suggest that dTap functions in the regulation of nutrient signaling, but may not be directly involved in the regulation of dS6K

phosphorylation. Future studies examining the activity and phosphorylation state of other components of *Drosophila* nutrient signaling pathways (dTOR, PDK1 and the TSC complex) in the absence of dTap could reveal a physiological role for dTap in S2 cells during amino acid starvation.

3. Novel PP2Ac Targeting Subunits

The observation that dephosphorylation of T398 during starvation does not require any of the four known *Drosophila* PP2A B subunits was unexpected and intriguing. These results suggest that PP2Ac regulates dS6K phosphorylation through interaction with an unknown targeting subunit. Protein interaction studies of dS6K binding partners could reveal the identity of this novel PP2Ac targeting subunit. It would be predicted that the associations of PP2Ac and the targeting subunit with dS6K would be low during growth and increase during periods of dTOR inactivation.

Although it appears that the genes comprising the canonical PP2A B subunit families have been identified in *Drosophila*, it should be noted that in mammals, the PP2A core A and C dimer complexes with other proteins, notably striatin/SG2NA (Moreno, Park et al. 2000). Striatin is expressed predominantly in the brain and is involved in regulation of lipid raft formation (Gaillard, Bartoli et al. 2001). SG2NA is nearly identical to striatin, but is expressed in both brain and muscle (Castets, Rakitina et al. 2000). BLAST searches of the *D. melanogaster* genome with the amino acid sequence of human striatin identified *CG7392-PA* (e^{-128}), a predicted gene sharing 61% identity at the amino acid level.

It is interesting to point out that striatin contains many WD repeats, a protein-protein interaction domain (Neer, Schmidt et al. 1994). It is currently unknown if *CG7392-PA* is the functional homolog of striatin/SGN2A, however, this observation does raise the possibility that the complete set of PP2A-interacting genes in *Drosophila* has not been described to date. It would prudent to determine if S2 cells express *CG7392-PA* and, if so, determine if knockout of this gene affects dS6K phosphorylation.

4. Knockdown of dTap and Apoptosis

In these studies, loss of dTap in S2 cells resulted in an apoptotic phenotype characterized by small amounts of cell blebbing and an overall reduction in cell numbers, in agreement with previously reported observations (Cygnar, Gao et al. 2005). However, it is unknown if this cell death is due to loss of a specific dTap function or rather an indirect effect induced by ablation of dTap. It would be worthwhile to characterize the cell death activated during dTap knockout by assaying the activation of apoptotic pathway components and the appearance of apoptosis-specific cells markers (i.e., presentation of phosphatidylserine on the cell surface and cleavage of PARP). Depletion of components of the *D. melanogaster* apoptotic pathways by RNAi could also be employed to determine if dTap-induced cell death is activated through caspase-mediated processes.

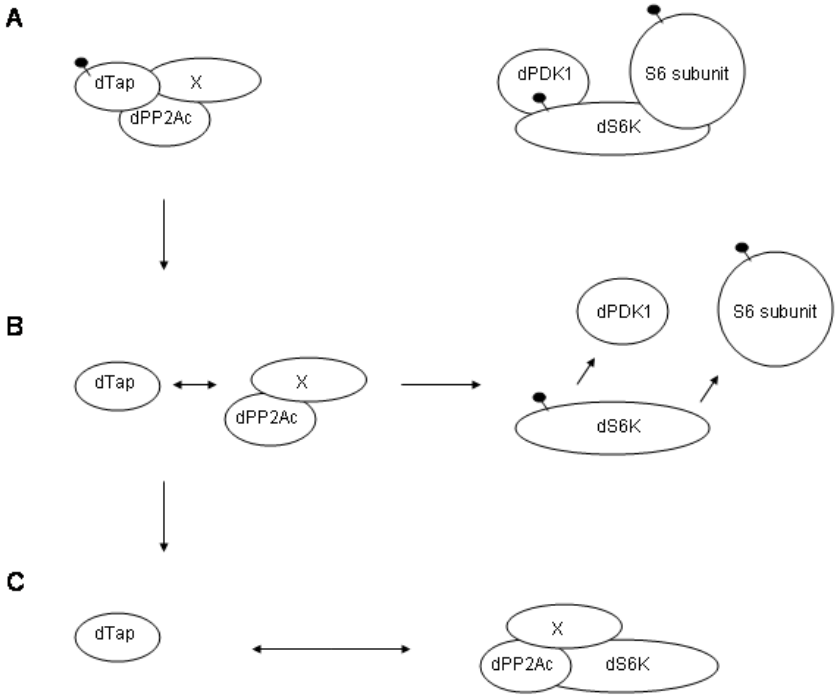

Figure 25. Modified sequestration model of dTap activity. (A) dTap forms an inhibitory complex with dPP2Ac and an unidentified targeting subunit (X) during periods of nutrient sufficiency. dS6K interacts with substrate (S6) and dPDK1 through binding of dPDK1 to phospho-T398. (B) Inactivation of dTOR results in dephosphorylation of dTap, releasing dPP2Ac-associated phosphatase. Interaction of dS6K with substrate and dPDK1 are disrupted through an unknown mechanism. (C) The dPP2Ac-associated phosphatase interacts with dS6K and catalyzes dephosphorylation of T398.

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VITAE

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