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PAS Kinase and TOR, Controllers of Cell Growth and Proliferation

Brooke Jasmyn Cozzens

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

PAS Kinase and TOR, Controllers of Cell Growth and Proliferation

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Master of Science

Nutrient sensing kinases lie at the heart of cellular health and homeostasis, allowing cells to quickly adapt to changing environments. Target of Rapamycin (TOR) and PAS kinase (PASK, or PASKIN) are two such nutrient kinases, conserved from yeast to man. In yeast, these kinases each have paralogs. The two TOR paralogs in yeast mimic the mammalian TORC1 and TORC2 complexes, except both Tor1 and Tor2 may contribute to TORC1 or TORC2 function. The two PAS kinase paralogs are paired with the TOR paralogs, meaning that both Psk1 and Psk2 regulate TORC1, while Psk2 suppresses a temperature-sensitive allele of Tor2. Herein we review the evolutionary models for these paralogs, their function in yeast and mammalian cells, as well as the overlapping function of PAS kinase and TOR. We also use Rice University's Direct Coupling Analysis algorithms to analyze co-evolutionary relationships and identify potential interaction sites between PAS kinase and several of its substrates.

Keywords: PAS kinase, gene duplication, mTOR, TORC1, TORC2, glucose metabolism, signal transduction, nutrient sensing, Direct Coupling Analysis, Ugp1, Cbf1, Utr1, USF1, ATXN2

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SPECIFIC AIMS

1. Publish a review article on the co-evolution of PAS kinase and TOR.
2. Perform Direct Coupling Analysis on PAS kinase and Ugp1, Cbf1, Utr1, USF1, and ATXN2 to determine potential phosphosites and PASK-substrate interaction sites.
   A) Collect sequences for orthologs of each of these proteins in mammals and yeast species.
   B) Align sequences and perform DCA on supercomputer.
   C) Analyze data and select residues for mutation (based on highest mutual information coefficients and PASK consensus sequences).
   D) Design primers for site-directed mutagenesis, create constructs, and sequence plasmids for verification of mutation.
   E) Perform kinase assays to determine if selected residues are critical for PASK-substrate interactions, or if they are key phosphosites.

CHAPTER 1: Project Introduction

Different models exist for how proteins have evolved over time. Early scientists believed that the human genome contained a large number of base pairs that served no purpose and were evidence of "nature's failed experiments," [1] however, we now know that is much too simplistic of an explanation. It is more likely that certain proteins had side activities that conferred no selective advantage or disadvantage, but over time, cellular environments changed, and the side activity became advantageous [2]. Through amplification, divergence, and possible recombination, genes with new functions can emerge. One of the most amazing parts of nature is how different proteins/systems in the cell have evolved to work together, regulating key pathways, including: cellular metabolism, cell growth and proliferation, translational control, cellular stress, etc. This study will focus on the conserved pathways and co-evolution of two important sensory kinases – Per-Arnt-Sim kinase (PASK) and Target of Rapamycin (TOR).

1.1 Importance of PASK and TOR

Sensory protein kinases play a critical role in the health of a cell, phosphorylating many proteins and allowing them to control several critical metabolic functions, including cellular growth and proliferation. PAS kinase, a recently discovered sensory protein kinase in yeast, is one of these important sensory kinases that is highly conserved from yeast to humans, and plays an essential role in cellular metabolism [3]. PAS kinase contains both a catalytic kinase domain and a regulatory PAS domain. PAS domains are sensory domains that regulate protein activity by binding small molecules or proteins [4], while the kinase domain regulates other proteins via phosphorylation at a serine or threonine. To prevent the kinase domain from phosphorylating and activating/ inactivating substrates needlessly, the PAS domain binds the kinase domain to inhibit its catalytic activity [5]. PAS kinase regulates several important substrates, including the

serine-threonine kinase termed TOR (Target of Rapamycin), which has the effect of decreasing cell growth and proliferation [6]. TOR kinase forms two complexes in humans - mammalian TOR complex 1 (mTORC1) and mammalian TOR complex 2 (mTORC2), which are composed of several different proteins. The functions of the two TOR complexes overlap on some roles, but mTORC1 primarily controls cell expansion and proliferation [7], whereas mTORC2 controls metabolism [8] and the restructuring of the cytoskeleton [9]. Yeast also have two TOR complexes, TORC1 and TORC2, and their roles are highly conserved. Similar to PAS kinase in yeast, the genes encoding the TOR kinases in yeast are believed to be a result of a whole-genome duplication in an early ancestor. The main components of TORC1 work together to control protein synthesis in response to growth factors and nutrients [10]. The main components of TORC2 regulate cell survival, cell cycle progression, and metabolism by phosphorylating and activating other effector kinases, and it's also involved in actin organization during cell division [11].

1.2 Co-evolution of PAS Kinase and TOR

We are particularly interested in studying PAS kinase and TOR because PASK-knockout mice were hypermetabolic and more resistant to liver triglyceride accumulation than the wildtype, despite having similar exercise levels. Because of its tie to cellular metabolism, PASK could potentially serve as a therapeutic target for cancer and diabetes. When TOR is inhibited, it has also been shown to extend lifespan in *S. cerevisiae*, *C. elegans*, and *D. melanogaster* [12-14]. PAS kinase and TOR appear to have co-evolved to work together in the cell to regulate various cellular processes. PAS kinase and TOR have many overlaps in function, including their roles in regulating translation initiation. The two paralogs in yeast - PAS kinase and TOR - appear to have paired function. One of the primary functions of TORC1 in yeast is to modulate translation

initiation. TORC1 consists of either Tor1 or Tor2 together with Kog1, Lst8 and Tco89. TORC1 is inhibited by both Psk1 and Psk2p through the phosphorylation and activation of Pbp1, which in turn sequesters TORC1 to stress granules, inhibiting growth and proliferation [15]. In contrast, Psk2 regulates cell division (cytoskeleton polarization and cell wall integrity) through the activation of RhoI, suppressing lethality due to TORC2 deficiency [16]. Thus, both TOR and PAS kinase have overlapping functions for their paralogs (Tor1 and Tor2, Psk1 and Psk2) in cell growth and proliferation, and specific functions for Tor2 and Psk2 in cytoskeletal polarization and cell integrity. These roles for PAS kinase in TORC1 regulation and the suppression of TORC2 deficiency by Psk2 remain unstudied in mammalian cells.

1.3 Difficulty of Identifying Interaction Sites

Notwithstanding the vital importance of protein kinases within the cell, they can be difficult to study. Protein-kinase interactions are generally highly transient, so typical protein-protein interaction discovery methods, such as co-immunoprecipitation and mass spectrometry, rarely give the full "protein-kinase interaction picture." In addition to the transient nature of these interactions, there are often multiple sites which are phosphorylated on a target protein, often by multiple kinases, so understanding the regulation and functions of these proteins can be a difficult, but very important task because of the critical cellular processes involved. The sheer number of proteins phosphorylated in the cell – 30-50%, depending on needs in the body – also complicates the study of protein kinases [17]. The sheer quantity of kinases and their multitude of targets makes them mechanistically difficult to study. Kinases often phosphorylate multiple sites on its substrates, and PAS kinase is no exception, further complicating the identification of critical phosphorylation sites. Kinases often induce conformational changes when they phosphorylate their substrates, effectively "hiding" the phosphosite on the inside of the protein

and potentially changing how the substrate interacts with other proteins in the cell, including its location in the cell [18]. Structure affects function in proteins, so proteins are commonly crystallized to better understand how they could interact with their substrates, however, large proteins such as PAS kinase are difficult to crystallize [19].

1.4 Purpose of this Project

Because of the difficulty in studying kinase-substrate interactions, we propose a novel method of studying protein kinases and their phosphosites using Rice University's Direct Coupling Analysis (DCA) algorithms [20] with PAS kinase and five of its known substrates as a case study. Using DCA, the co-evolution of PASK with its confirmed substrates Cbf1, Ugp1, Utr1, USF1, and ATXN2 is estimated by Mutual Information, allowing us to predict the most likely interaction points or phosphosites for the proteins. We can potentially predict sites that are not captured by current kinase-study techniques, allowing us to be more thorough in our research, and then use site-directed mutagenesis of the sites predicted by DCA to confirm cellular effects caused by PAS kinase. Using DCA, we will explore the co-evolutionary relationships between PASK and TOR, as well as several other substrates in yeast and mammalian species.

CHAPTER 2: Evolution of TOR and PAS Kinase by Gene Duplication

Abstract

Nutrient sensing kinases lie at the heart of cellular health and homeostasis, allowing cells to quickly adapt to changing environments. Target of Rapamycin (TOR) and PAS kinase (PASK, or PASKIN) are two such nutrient kinases, conserved from yeast to man. In yeast, these kinases each have paralogs. The two TOR paralogs in yeast mimic the mammalian TORC1 and TORC2 complexes, except both Tor1 or Tor2 may contribute to TORC1 and TORC2 function. The two PAS kinase paralogs are paired with the TOR paralogs, meaning that both Psk1 and Psk2 regulate TORC1, while Psk2 suppresses a temperature-sensitive allele of Tor2. Herein we review the evolutionary models for these paralogs, their function in yeast and mammalian cells, as well as the overlapping function of PAS kinase and TOR.

2.1 Introduction

The complexity of life has led to the advancement of diverse evolutionary models, including models that address the molecular details for how proteins evolve over time. Early scientists believed that the human genome contained a large number of base pairs that served no purpose and were evidence of "nature's failed experiments," [1] however, this explanation is much too simplistic. It is more likely that certain proteins had side activities that conferred no selective advantage or disadvantage, but over time, cellular environments changed, and the side activity became advantageous [2]. Through amplification, divergence, and possible recombination, genes with new functions can emerge. In this review, we focus on two key nutrient-sensing kinases, Per-Arnt-Sim kinase (PASK) and Target of Rapamycin (TOR), that have paralogs which evolved by gene duplication to have unique, but related functions in the cell.

Protein kinases like PASK and TOR play critical roles in cellular health and homeostasis, particularly in response to changing environments where posttranslational control of several proteins allows for concerted adaptation of multiple pathways. Protein kinases are able to regulate other proteins in the cell through phosphorylation. This regulation can alter cellular activity by affecting the substrate's function in various ways, including modulating activity, stability, cellular localization, and/or binding partners [21]. They often have tens of substrates, allowing them to regulate multiple pathways simultaneously and potentially drastically reprogramming cellular metabolism. In fact, up to half of the eukaryotic proteome is phosphorylated, illustrating the importance of kinases in the cell [17, 22]. It is, therefore, no surprise that protein kinases are highly conserved from yeast to man and lie at the heart of many diseases such as diabetes and cancer [23]. Nutrient sensing kinases are a large class of protein kinases that respond to cellular nutrient status (such as glucose, amino acid, or ATP availability).

Not only do PASK and TOR have paralogs that have evolved to have important, unique functions in the cell, but the kinases have also evolved to work together. In other words, Psk1 and Tor2 have evolved to work together in different pathways than Psk2 and Tor1 [16], while still retaining many functional overlaps in the cell. We will discuss the evolutionary and functional similarities between PASK and TOR, and how their paralogs/complexes have evolved to work together in distinct, yet interrelated, cellular pathways.

2.2 Evolution of Proteins by Gene Duplication and Recombination

Ohno hypothesized that there was a strict upper limit for the number of working genes that could be supported in a genome. He suggested that the human genome contained evidence of "nature's failed experiments," and the extra base pairs were simply "junk DNA." Ohno claimed that excess genetic material arose in DNA when point mutations negatively affecting the

active sites of gene products accumulated and were thus eliminated by natural selection, but the inefficient or nonfunctional genes still remained as empty base pairs that did not code for any proteins. He additionally argued that in order for advantageous mutations to occur, the selective pressure of natural selection had to be temporarily removed by polyploidization or by tandem duplication [1] (see Figure 2-1). This rather basic explanation for the emergence of new genes likely does not provide the whole picture. On the route to becoming a gene with a new function, copies of the parent gene must also overcome genetic drift, mutation, recombinational segregation, gene conversion, and avoid being counter selected due to metabolic cost or a negative alteration of gene dosage [24].

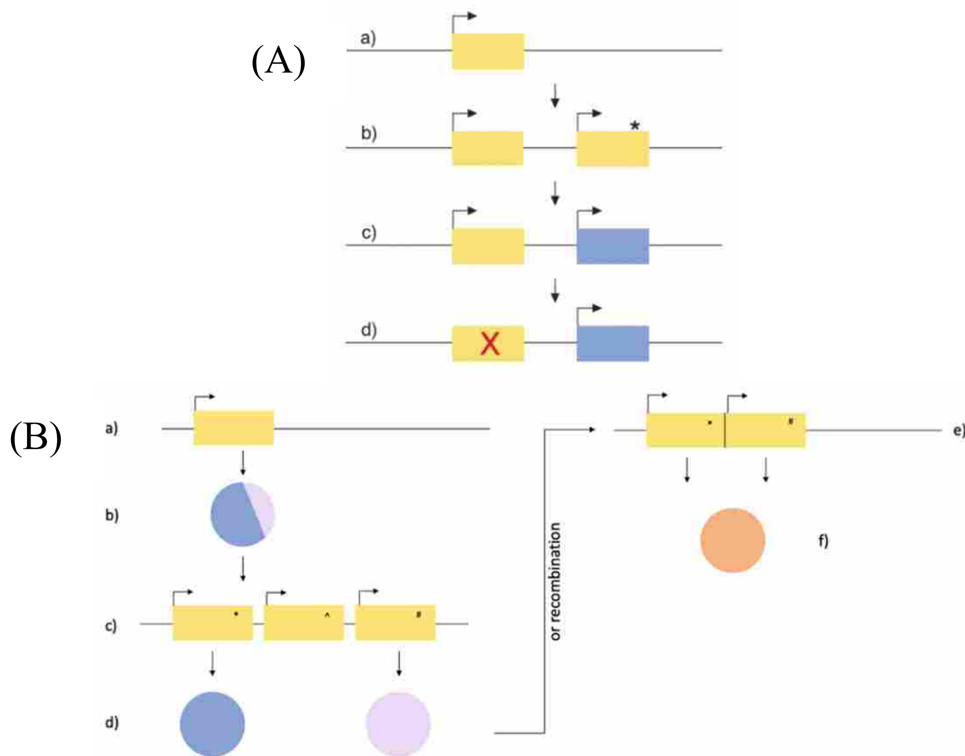

Figure 2-1. Two Models for Gene Evolution by Duplication.
(A) Ohno's Model: a) progenitor gene; b) gene duplication and divergence/mutation; c) new gene emerges with a distinct function that's advantageous; d) progenitor gene is no longer useful to the cell. (B) IAD Model: a) original gene; b) original protein with a side activity (e.g. TOR with the side activity of rapamycin resistance) that is advantageous; c) gene duplication, allowing for mutations; d) two separate proteins result – efficient original protein and an efficient side activity protein; d) recombination may then occur, producing: f) a novel protein that is efficient at both the original activity and side activity.

A more accurate model for the evolution of paralogous genes may be the innovation, amplification, and divergence (IAD) model proposed by Bergthorsson, Andersson and Roth, which allows for selection at every step of the process [2]. The main idea behind this model is that the original gene encodes a protein that provides a primary function, along with another minor activity that does not confer any selective advantage or disadvantage. Over time, conditions around the cell may change and contain a toxic compound or altered nutrient availability, and one of the side activities may then become advantageous in the new environment. The gene with enhanced side activity will then be selected for and duplicated, wherein large expanded arrays of the duplication containing multiple gene copies may form, providing more opportunities for an advantageous mutation to occur. As one of the copies is enhanced by mutation, selective pressure on the other copies is more relaxed and the gene expansions may collapse. Eventually there will be a copy that is efficient at the original activity and another copy that is efficient at the side activity. Through recombination, the best of the original activity and side activity can be combined and a new gene with a novel function emerges [2].

The evolution of TOR and PAS kinase appear to both have been influenced by gene duplication. Two paralogs of PAS kinase and TOR exist in yeast, while many eukaryotes encode only one PAS kinase or TOR protein. Although there is only one protein, mammalian TOR (mTOR) forms two complexes, TORC1 and TORC2, which harbor analogous functions to the two yeast paralogs TOR1 and TOR2 [25].

The yeast paralogs, Psk1 and Psk2, appear to have different yet overlapping roles. The occurrence of two PAS kinase and TOR paralogs in yeast most likely arose from a whole-genome duplication of an early ancestor, and protein complexes that share subunits like TOR can

provide a selective reason for maintaining duplicate copies of the shared subunits [26-31]. Most of the duplicated genome would have been lost due to random mutation, loss of activity and deselection; however, genes which acquired new, important, functions could be selected for. Below, we will discuss the common ancestry and important, interrelated functions of the Target of Rapamycin (TOR) and Per-Arnt-Sim kinase (PASK) pathways in yeast and mammals.

2.3 Target of Rapamycin (TOR) in Yeast and Mammals

TOR is an evolutionarily conserved protein that exists in many species, including yeast, flies, and mammals. TOR was first discovered in the budding yeast *S. cerevisiae* through the characterization of mutations that allowed the cells to grow in the presence of the usually growth-inhibiting compound rapamycin. Upon entering the cell, rapamycin binds to FK506-binding protein of 12 kDa (FKBP12) and interacts with the FKBP12-rapamycin binding domain (FRB) of mTOR, therefore inhibiting mTORC1 function [32, 33]. TOR is part of a complex web of interacting proteins that work together to help cells respond to their external and internal environments. Increased TOR activity in mammalian cells has been linked to a decreased ability to deal with cellular stress and faster age progression, as well as increased cancer risk [34]. TOR is responsible for controlling cell growth in response to nutrients and growth factors [35]. As stated earlier, the presence of two TOR genes in both budding and fission yeast may have occurred by gene duplication. Eventually, the two TORs acquired specific functions. In other yeast genera and organisms including mammals, there is only one TOR gene. Nonetheless, mammalian TOR can form two distinct protein complexes, mTORC1 and mTORC2, similar to its yeast counterparts (Figure 2-2).

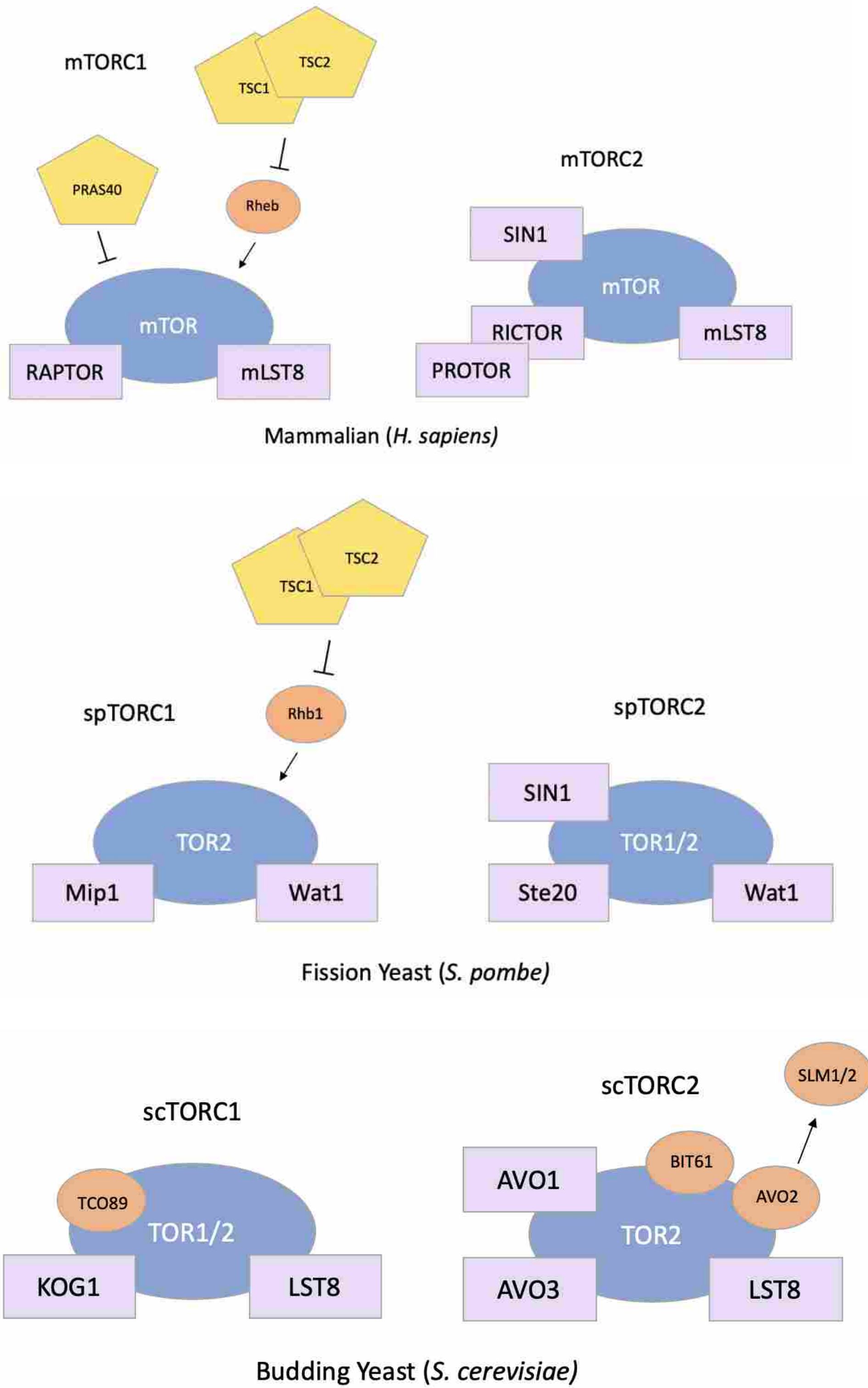

Figure 2-2. Mammalian TOR Can Form Two Distinct Protein Complexes, mTORC1 and mTORC2, Similar to its Yeast Counterparts.

2.4 Conserved Subunits of TOR Complexes in Mammals

In mammals and budding yeast, the signaling complex TORC1 regulates the cellular response to nutrients [36] and has several conserved domains important for its function. mTOR contains HEAT repeats (Huntingtin, Elongation Factor 3, A subunit of PP2A (protein phosphatase 2A), TOR1), followed by the FAT (FRAP (FKBP12-rapamycin-associated protein)/TOR, ATM (ataxia-telangiectasia), TRRAP (transactivation/transformation-domain-associated protein)) and FATC (FAT C-terminal) domains, which are important for mTOR's catalytic kinase activity. Mutations in these domains, which domains may also play a role in the folding of the kinase domain [37], eliminates mTOR-dependent phosphorylation of 4E-BP (eukaryotic initiation factor 4E-binding protein) and S6K (S6 kinase), as well as mTOR autophosphorylation activity [38-40]. The FATC domain also has a critical, conserved cysteine residue that when modified, causes decreased TOR levels [41]. Neighboring the conserved kinase domain of mTOR is the FRB (FKBP12/rapamycin-binding) domain, where rapamycin (in complex with FKBP12) binds and inhibits mTOR. Interestingly, the FRB can bind phosphatidic acid, a lipid that is speculated to mediate membrane localization of mTOR [42-45], a key feature of TOR function.

The TOR complexes not only include the critical TOR protein, but also contain many other conserved subunits (see Table 2-1 for a summary of these proteins and their conservation in humans and yeast). In mTORC1, the complex includes mTOR (conserved), RAPTOR (conserved), and LST8 (conserved), as well as the close interactors, PRAS40, TSC1/2, and Rheb. In mammals, RAPTOR binds to mTOR [7, 25, 46] and is thought to serve as a scaffold to present substrates to mTOR but does not alter the catalytic activity of mTOR. RAPTOR also causes sensitivity to rapamycin, and possibly nutrients [7, 47]. RAPTOR is considered an

essential gene since no viable knockout mice were obtained when heterozygous intercrosses of RAPTOR$^{+/-}$ mice were performed (similar to mTOR$^{+/-}$ mice) [48, 49], which also provides an explanation for its conservation across species.

Table 2-1. Conservation of the TOR Complex Proteins in Select Eukaryotes.

	H. sapiens		***S. cerevisiae***		***S. pombe***	
	TORC1	**TORC2**	**TORC1**	**TORC2**	**TORC1**	**TORC2**
Conserved	mTOR	mTOR	TOR1/2	TOR2	TOR2	TOR1/2
	mLST8	mLST8	LST8	LST8	Wat1	Wat1
	RAPTOR		KOG1		Mip1	
		RICTOR		AVO3		Ste20
		SIN1		AVO1		SIN1
Non-Conserved Interactors	PRAS40					
		PROTOR (PRR5)				
			TCO89			
				AVO2		
				BIT61		
				SLM1/2		

LST8, another important conserved subunit of mTORC1, is necessary for transporting amino acid permeases from the Golgi to the cell surface [50, 51] (LST8 localizes to endosomal or Golgi membranes [52]). LST8 mutants have an abnormal growth phenotype, but this phenotype can be suppressed with the chaperonin protein CCT6 (chaperonin containing TCP-1), supporting the idea that mTORC1 is involved in protein folding and stabilization [53]. mLST8 is capable of binding to mTOR and RAPTOR when they are overexpressed [25, 54]. When bound to the kinase domain of mTOR, mLST8 stimulates its kinase activity [54]. On its own, mLST8's association with mTOR is not able to stimulate nutrient sensitivity, but it is required for the nutrient-sensitive interaction of RAPTOR to mTOR. mLST8 plays a role in the recognition and recruitment of substrates to mTOR, but it is also conjectured to be involved in the stability and folding of the mTOR kinase domain [54].

PRAS40 (proline-rich Akt substrate of 40 kDa), although not technically part of the "core proteins" of the conserved mTORC1 complex, is also important because it binds and negatively regulates mTORC1 [55-60]. PRAS40 physically interferes and directly inhibits the binding of substrates to mTOR and is phosphorylated by mTORC1 in a rapamycin-sensitive fashion [57]. Akt (also known as PKB) phosphorylates PRAS40 to negatively regulate it, therefore promoting mTORC1 activity [58].

In contrast to mTORC1, relatively little is known about mTORC2's function and biology. In mammals, growth factors and/or hormonal signals appear to regulate mTORC2 [61, 62]. mTOR forms a separate complex with unique functionality, termed mTORC2. mTORC2 is composed of mTOR (conserved), RICTOR (conserved), LST8 (conserved), SIN1 (conserved), and a close interactor protein PROTOR (also called PRR5). Although similar in structure to mTORC1, mTORC2 is considered to be resistant to rapamycin because rapamycin cannot bind to the mTORC2 complex. However, this resistance model may be an oversimplification since continued exposure to rapamycin has been shown to inhibit the assembly of the mTORC2 complex [63]. Despite a lack of understanding, mTORC2 is no less important than mTORC1; mTORC2 plays a key part in various biological processes, including: cellular respiration and lifespan, cytoskeleton polarization, cellular stress, and salt balance.

PROTOR (protein observed with RICTOR), also called PRR5 (proline-rich protein 5), is an interacting partner of mTORC2. It binds to RICTOR independently of mTOR, even while RICTOR is actively bound to mTOR [64, 65]. The function of PROTOR is unknown, but it may be involved in PDGFR (platelet-derived-growth-factor receptor) signaling [64]. A PROTOR-like protein which regulates apoptosis, was also found to bind mTORC2 [60]. However, PROTOR is not required for the binding of other mTORC2 subunits, including RICTOR and

SIN1, but may mediate other unknown mTORC2 functions. Also, PROTOR knockdown does not significantly reduce phosphorylation of Akt at Ser473 [64], further suggesting that PROTOR may not be necessary for mTORC2 function. SIN1 is essential for phosphorylation and activation of certain AGC-family kinases but is not required for TOR's catalytic activity (TOR may phosphorylate substrates *in vitro*, despite a lack of SIN1). In the Shiozaki lab, it was recently shown that the substrate specificity of TORC2 is determined by the ubiquitin-fold domain of the SIN1 subunit [66].

2.5 The Regulation of TORC1 and TORC2 in Mammals

Because TOR and many other subunits of the TOR complexes are highly conserved across species and act in a wide variety of pathways to control cellular metabolism, it comes as no surprise that diseases often result when they are dysregulated. Below, we will discuss several regulators of TOR (summarized in Table 2-2), followed by cellular pathways/ functions affected by TOR (summarized in Table 2-3).

Table 2-2. A Summary of the Known Pathways/Proteins that Regulate TOR in the Mammalian and Yeast Cells.

Regulator	**TOR***	**Mechanism/ Function**	**Ref.**
Rapamycin	mTORC1/TORC1, mTORC2/TORC2	Inhibitory, but affects TORC1 more than TORC2	[32, 33, 63]
Growth Factors (amino acids, glucose, etc); Hormonal Signals (insulin, etc.); High ATP:AMP ratio	mTORC2	mTORC2 responds to different signals to regulate many cellular processes	[62, 67]
PROTOR (PRR5)	mTORC2	Unknown function and not required, but interacts with mTORC2 and may be involved with PDGFR signaling	[64]

SIN1/AVO1	mTORC2/TORC2	Regulates substrate specificity; Essential for phosphorylation of certain AGC kinases; Actin polarization	[25, 66]
Rheb/Rhb1	mTORC1/TORC1	Directly binds TOR (in mTORC1) and TOR2 (in TORC1) to promote its activity	[58, 68]
TSC1/2	mTORC1/spTORC1	Negative regulator; Conveys the presence of insulin; GTPase inactivator of Rheb/Rhb1	[58, 68-71]
Akt (PKB)	mTORC1	Negatively regulates PRAS40 to promote mTORC1 activity	[58]
PRAS40	mTORC1	Binds and negatively regulates mTORC1 by interfering with substrate binding	[55-60]
RAPTOR; KOG1/Mip1	mTORC1	Essential; Serves as a scaffold to present substrates; Sensitive to rapamycin; KOG1/Mip1 function has not been shown *in vivo*, but they are orthologous to RAPTOR	[7, 25, 46, 48, 49]
LST8/Wat1	mTORC1/TORC1, mTORC2/TORC2	Important for recognition and recruitment of substrates; Stimulates transportation of amino acid permeases to cell surface; Stimulates kinase activity; Required for nutrient-sensitive interaction of RAPTOR+mTOR; Proper localization of F-actin	[50, 51, 54, 72]
Rag Proteins	mTORC1	Necessary and sufficient for sensing cellular nutrients and moving mTORC1 to lysosomal surface; Promotes localization of mTORC1 to areas containing its activator Rheb	[73, 74]
DNA Damage/ p53	mTORC1/spTORC1	Activation of p53 activates AMPK, which activates TSC2 (see above)	[75]
RICTOR/AVO3	mTORC2/scTORC2	Required for TORC2 structural integrity	[76]
TCO89	scTORC1	Associates with TORC1, but may operate independently	[10, 77]

* The prefix “sc” or “sp” means the protein only exists or has only been studied in *S. cerevisiae* or *S. pombe*. If the protein is mammalian, it has the prefix “m.”

In mammals, mTOR is part of the complexes TORC1 and TORC2. Because mTORC1 is considered a nutrient-sensing kinase, it is controlled and activated by a high ATP:AMP cellular energy level, growth factors such as insulin, and nutrients such as amino acids and glucose. In mammals, several pathways convey the presence of growth signals, such as insulin, to mTORC1 via regulation of TSC1/TSC2 [69]. The tuberous sclerosis complex (TSC), which is composed of TSC1 and TSC2, is one of the most important regulators of mTORC1. The TSC complex functions as a GTPase-activating protein (GAP) for the GTPase Rheb (see Table 2-2). The active form of Rheb directly interacts with mTORC1 to promote its activity [68, 78], while TSC1/2 serve as negative regulators of mTORC1 by converting Rheb into its inactive GDP-bound state [70, 71]. Stimulation of this TSC pathway increases the phosphorylation of TSC2 by Akt [79, 80] and leads to the inactivation of the TSC complex, and consequently to the activation of mTORC1. Also, Akt activation by growth factors can activate mTORC1 by promoting the phosphorylation and dissociation of PRAS40 from mTORC1 [56, 78, 81]. A very important role of mTORC1 is sensing the energy status and needs of the cell. In response to energy depletion, AMPK is activated and phosphorylates TSC2, which increases its activity toward Rheb, reducing mTORC1 activation [79]. AMPK can also reduce mTORC1 activity directly by phosphorylating RAPTOR when there's a low ATP:AMP ratio [82]. Mild hypoxia is also capable of activating AMPK and inhibiting mTORC1 [83, 84]. Inflammatory mediators also communicate with mTORC1 via the TSC1/2 complex. Pro-inflammatory cytokines (such as TNFα) activate IkB kinase-b (IKKb), which interacts with and inactivates TSC1, leading to mTORC1 activation [85]. This relationship between inflammation and mTORC1 activation is thought to be correlated with tumor angiogenesis [85], as well as the development of insulin resistance [86]. mTORC1 is also

capable of responding to amino acids. In the presence of amino acids, Rag proteins bind to RAPTOR and promote mTORC1's localization to areas containing its activator Rheb [73].

Until the work of Yasemin Sancak, it was unknown how mTORC1 was able to sense nutrients, such as growth factors, intracellular energy levels, and amino acid availability; she found that heterodimeric Rag GTPases interacted with mTORC1 when amino acids and other nutrients stimulated the cell [73]. In fact, Rag GTPases were found to be necessary and sufficient for mTORC1 to sense amino acids [73, 74]. To further understand the function of mTORC1, knock-in mice with a constitutively active RagA allele were generated, but this mutation was found to cause the mice to die during periods of fasting because they were unable to switch from an anabolic to a catabolic state (due to mTORC1 remaining active despite nutritional starvation) [87]. Using immunofluorescent antibodies, it was determined that mTORC1 localized to lysosomes, but nutrient availability (made known by the Rag GTPases) signaled mTORC1 to move to the surface of the lysosome [73, 74, 87, 88].

Mammalian TORC1 is also capable of responding to amino acids. In the presence of elevated amino acid levels, Rag proteins bind to RAPTOR and promote mTORC1's localization to areas containing its activator Rheb [73]. LST8, another important conserved subunit of mTORC1, is necessary for transporting amino acid permeases from the Golgi to the cell surface [50, 51] (LST8 localizes to endosomal or Golgi membranes [52]). LST8 mutants have an abnormal growth phenotype, but this phenotype can be suppressed by overexpressing the chaperonin protein CCT6 (chaperonin containing TCP-1), supporting the idea that mTORC1 is involved in protein folding and stabilization [53]. mLST8 is capable of binding to mTOR and RAPTOR when they are overexpressed [25, 54]. When bound to the kinase domain of mTOR, mLST8 stimulates its kinase activity [54]. On its own, the association of mLST8 with mTOR is

not able to stimulate nutrient sensitivity, but it is required for the nutrient-sensitive interaction of RAPTOR to mTOR. mLST8 plays a role in the recognition and recruitment of substrates to mTOR, but it is also conjectured to be involved in the stability and folding of the mTOR kinase domain [54].

DNA damage also stimulates mTORC1 activation. For instance, the activation of p53 in response to DNA damage rapidly activates AMPK through an unknown process, which in turn phosphorylates and thereby activates TSC2 [75].

Table 2-3. A Summary of the Known In Vivo Effects of TOR in Yeast and Mammalian Cells.

Protein*	TOR	Mech.*	Apparent effect	Ref.
Wide-Range Effects				
mS6K	mTORC1	DP	protein synthesis/translation, mRNA processing, glucose homeostasis, cell growth, and survival	[89]
mAKT (PKB)	mTORC2	UK	cell survival, substrate specificity	[61, 62]
mJNK	mTORC2	DB	cell survival, T-cell differentiation	[90-92]
scYpk1/2	scTORC1/2	DP	cell growth, translation initiation, actin organization, cell integrity	[93, 94]
scSch9	scTORC1	DP	lifespan, cell growth, stress response	[95-98]
Pancreatic Function				
mIRS1	mTORC1	DP	decreased stability when phosphorylated	[99, 100]
Translational Control				
m4E-BP	mTORC1	DP	promotes cap-dependent translation	[101]
scSfp1	scTORC1	DP	activation; increased ribosome biosynthesis	[102]
Respiration Control				
mPKM2	mTORC2	DP	upregulation	[103, 104]
mcPKC	mTORC2	UK	glycogenolysis, gluconeogenesis	[105]
Stress Response				
spSAPK	spTORC2	UK	activation of stress response to multiple stressors	[90-92]
Lifespan				
scRim15	scTORC1	DP	increases lifespan	[106]

Cellular Signaling				
mMEKK2	mTORC2	DB	cellular signaling	[91]

*The abbreviation "sc" is for *Saccharomyces cerevisiae* and "sp" is for *Schizosaccharomyces pombe*, meaning that the data comes from studies with yeast proteins in yeast, while "m" is for mammalian proteins and "x" is for both yeast and mammalian proteins. DP is for direct phosphorylation, DB is for direct binding, UK is unknown. This table excludes *in vitro* substrates that have been identified but may lack *in vivo* evidence.

2.6 The Functions of TORC1 in Mammals

2.6.1 Pancreatic Function. It has recently been shown that mTORC1 is closely linked to β-cell development *in utero* and during the neonatal period, and thus may be tied to Type 2 Diabetes (T2D) and obesity in adulthood [107-109]. In fact, mTOR inhibitors are already on the market for the treatment of certain types of cancers [110], where they were found to cause a high incidence of new-onset diabetes, ranging from 13 to 50% [111]. mTORC1 is also capable of activating ribosomal protein S6 kinase β-1 (S6K1) by phosphorylating insulin receptor substrate 1 (IRS1) and reducing its stability, which has important implications for metabolic diseases [99, 100].

2.6.2 Cellular Respiration and Lifespan. Although mTOR inhibitors can have the negative side effect of increasing the likelihood of diabetes, decreased mTOR activity has been shown to increase lifespan in *S. cerevisiae, C. elegans,* and *D. melanogaster* [12-14]. The mTOR inhibitor rapamycin has also been shown to increase lifespan in mice [112, 113]. It is proposed that mTOR signaling may increase with age especially in adipose tissue, but if it is inhibited by rapamycin, lifespans may lengthen [114]. The free radical concept of aging [115] says that reactive oxygen species damage mitochondria, and therefore reduce ATP output, which in turn inhibits the mTOR pathway [116]. Decreased mTOR upregulates glycolysis [117] and downregulates autophagy [115]. This downregulation of autophagy can also indirectly support tumor growth.

2.6.3 Translational Control. The central function of mTORC1 is to regulate the intracellular metabolic state by shifting from catabolism to promoting the synthesis of proteins, lipids, and nucleotides [118, 119]. mTORC1 is capable of regulating its substrate S6 kinase (S6K). S6K has been linked to diverse cellular processes, including: protein synthesis/translation, mRNA processing, glucose homeostasis, cell growth, and survival. Interestingly, S6K contains a TOR-signaling (TOS) motif [89], suggesting a co-evolutionary relationship between the TORCs and S6K. Depending on the activation state of S6K (a complex process, involving multiple proteins), it can associate with different regulatory complexes. In its inactive state, it binds eukaryotic initiation factor 3 (eIF3) [120]. However, upon cellular stimulation, mTOR/RAPTOR binds to eIF3, then subsequently phosphorylates S6K at Thr389. When S6K is phosphorylated by mTOR, it dissociates from the eIF3– mTORC1 complex, thus becoming available to phosphorylate its downstream targets. These findings raise the possibility that the activity of mTOR/RAPTOR is dependent on the signaling complex it associates with and on the cellular compartment it localizes to. mTOR can also phosphorylate the translational regulator 4E-BP [101]. 4E-BP has no enzymatic activity, but the small regulatory protein can be phosphorylated at multiple residues in a rapamycin-sensitive fashion to promote cap-dependent translation. 4E-BP contains a common recognition motif, TOS (TOR signaling), which is also found in S6K [89], which could explain why mTOR is capable of phosphorylating such structurally distinct proteins.

Through these conserved proteins/pathways, mTORC1 is able to positively regulate cell growth and proliferation by promoting many anabolic processes (i.e. biosynthesis of proteins, lipids, and organelles) and by limiting catabolic processes (such as autophagy). mTOR is highly

conserved (Table 2-1), and even the functions of the pathway are highly similar between yeast and man (see Table 2-3 summary).

2.7 The Functions of TORC2 in Mammals

2.7.1 Cellular Respiration and Lifespan. TOR is also able to influence cell survival, growth, proliferation, and migration through the activity of Akt. Three Akt genes have been identified in mammals: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) [121]. mTOR, along with several other kinases (DNA protein kinase (DNA-PK), integrin-linked kinase (ILK), ATM, cPKC, and Akt itself), has been identified as a PDK2 (the term for a kinase that phosphorylates the hydrophobic motif site of Akt) [122-126] that phosphorylates the hydrophobic motif site of Akt [127]. Phosphorylation of the hydrophobic motif by mTOR (as well as other PDK2 kinases) is induced by growth factors such as insulin and is sensitive to PI3K inhibitors such as wortmannin. mTORC2 promotes phosphorylation of Ser473 *in vitro* in a serum-inducible and wortmannin-sensitive manner [61, 128], though there are several ideas for how Akt is regulated in the cell [128-130]. Interestingly, Ser473 phosphorylation is highly inducible *in vivo* by growth-factor or serum stimulation, but its induction *in vitro* using the immunoprecipitated mTOR complex is not as apparent. There could be several reasons for this discrepancy, however, it is possible that negative regulators of the mTOR complex were inhibiting mTOR activity in the assays [58]. Additional *in vivo* evidence for the TOR regulation of Akt is that the knockdown of the mTORC2 component RICTOR, but not the mTORC1 component RAPTOR, led to defective phosphorylation of Akt at Ser473 [61]. Genetic studies of MEFs with deficient mTORC components confirmed that mTORC2 is required for hydrophobic motif site phosphorylation. In RICTOR^{-}, mLST8- or SIN1-knockout cells, Ser473 phosphorylation was completely eliminated. Akt remained partly active in mTORC2-disrupted cells and

phosphorylated a subset of its known substrates but was defective in the phosphorylation of Foxo1/3a (apoptosis-promoting proteins) [62]. These findings suggest that the phosphorylation of Ser473 may play a role in cell survival and that hydrophobic motif site phosphorylation may regulate substrate specificity in Akt. mTOR may not directly phosphorylate Akt, but mTORC2 may facilitate autophosphorylation, activate other kinases, mediate cellular compartmentalization, or even inhibit phosphatases (as has been shown in other yeast studies [131]) to control these complex and inter-dependent regulatory pathways.

It has also been suggested that mTOR may play a role in the phosphorylation of cPKC, a kinase important for gluconeogenesis, glycogenolysis [105], and endothelial permeability [132]. In the Sarbassov study, knockdown of RICTOR and mTOR expression revealed diminished phosphorylation of cPKCα [9]. In RICTOR-, SIN1-, and mLST8-knockout cells, phosphorylation of cPKCα at the hydrophobic motif site (Ser657) was eliminated, but it was also accompanied by a dramatic reduction in cPKCα protein expression [48]. However, the decrease in cPKCα expression was not evident in RICTOR-knockdown cells [9].

Interestingly, mTORC2 can also contribute to the Warburg effect seen in cancerous cells, where they tend to prefer metabolism via aerobic glycolysis rather than the more efficient oxidative phosphorylation, because mTORC2 upregulates the glycolytic enzyme PKM2 [103].

2.7.2 Cytoskeleton Polarization. One of the central components of mTORC2 is RICTOR. In mammals, knocking down the highly conserved RICTOR protein leads to defects in both actin cytoskeleton organization and in the phosphorylation of two AGC kinases - cPKCα (conventional protein kinase Cα) and Akt (PKB) - at their hydrophobic motif sites [9, 61, 72]. Predictably, RICTOR knockout is embryonic lethal [48, 133, 134]. However, MEFs (murine embryonic fibroblasts) isolated from null embryos did not show any noteworthy defects in actin

cytoskeleton nor morphology, but the growth rate of RICTOR$^-$ cells was slower than wildtype MEFs [133].

An important conserved protein included in mTORC2 is LST8. mLST8, in addition to its nutrient-sensing and amino acid transporting functions in mTORC1, also functions as part of mTORC2 to support the reorganization of the actin cytoskeleton. Knockdown of mLST8 leads to defects in actin cytoskeleton reorganization when fibroblasts are stimulated with serum [72]. Additionally, more recent studies using mLST8-knockout cells have shown that mLST8 is required for mTORC2, but not mTORC1 functions [48].

2.7.3 Cellular Stress. Mammalian SIN1 is another important component of mTORC2 that is conserved [62, 133]. It has been shown that knocking out mSIN1 leads to embryonic lethality, loss of TORC2 complex formation, as well as loss of kinase activity [62, 134]. In mammals, at least five alternatively spliced isoforms of SIN1 could be generated [91, 135], three of which are capable of forming distinct mTORC2 complexes [136]. Most SIN1 orthologs contain a conserved Raf-like RBD (Ras-binding domain) and Pleckstrin Homology (PH) domain, but the exact functions of these domains *in vivo* is unknown [137]. In mammals, Sin1 binds JNK (c-Jun N-terminal kinase) and MEKK2 (MAPK -mitogen-activated protein kinase/ERK - extracellular-regulated-protein kinase- kinase kinase 2). JNK is responsive to many different types of stimuli, including cytokines, ultraviolet radiation, osmotic shock, and heat shock. Once activated, JNK plays a role in T-cell differentiation and apoptosis, possibly implying new functions of mTORC2 signaling. SIN1 binding MEKK2 is also important in cellular signaling [90-92].

2.7.4 Salt Balance. A direct-interaction relationship between SGK and mTOR has not been confirmed yet, but it's likely that an interaction occurs since SGK's structure is highly

similar to that of Akt [138]. Three SGK paralogs exist in mammals and mainly function to regulate salt balance by activating ion channels, ion carriers, and the Na^{+} /K^{+} - ATPase. SGK is highly regulated on multiple levels, including its expression, its kinase activity, and its subcellular localization [139]. SGK also phosphorylates other substrates involved in stress responses, with some of the targets overlapping with Akt substrates. Interestingly, rapamycin treatment does not block the hyperphosphorylation of SGK upon serum stimulation [140], suggesting that mTORC2 may mediate phosphorylation.

2.8 Conserved Subunits of TOR Complexes in Yeast

Similar to mammals, yeast TOR complexes are controlled by different nutrients, stressors, and amino acids within the cell. Below, we will discuss several pathways affected by TOR in yeast and the important cellular processes that they control. The conservation of TOR complexes (that exist in both budding and fission yeast) will also be reviewed. In budding yeast, TOR is encoded by two different genes, TOR1 and TOR2, distinguishing it from mammalian TOR complexes. TOR1 is part of TORC1, while TOR2 is a component of both TORC1 and TORC2 in budding yeast. In the absence of TOR1, TOR2 can substitute in TORC1, which explains why deletion of TOR1 is nonlethal but a deletion of TOR2 is lethal [36].

In addition to TOR, TORC1 in budding yeast such as *S. cerevisiae*, is composed of LST8 (orthologous to Wat1), KOG1 (orthologous to RAPTOR and Mip1), and an interactor protein called TCO89 (see Figure 2-2). In fission yeast such as *S. pombe*, TORC1 includes TOR2, while TOR1 or TOR2 can be part of TORC2. TORC1 in fission yeast also includes Mip1 (orthologous to KOG1 and RAPTOR) and Wat1 (orthologous to LST8). Consistent with the conserved nature of these proteins, TORC1 in mammals, budding yeast, and fission yeast, are all responsive to nutrient conditions [141, 142]. The similarities to mammals don't stop there - fission yeast in

TORC1 is controlled by Tsc1/Tsc2 (where Tsc is tuberous sclerosis complex) via negative regulation of the GTPase Rhb1 [143, 144]. The tuberous sclerosis complexes do not have orthologs in budding yeast, however, a Rheb ortholog (RHB1) that is capable of promoting TORC1 activity exists [145, 146]. Whether RHB1 still regulates TOR in budding yeasts, even with a lack of TSCs, remains to be investigated. Differently from humans, rapamycin does not inhibit TORC1 in fission yeast during vegetative growth but appears to affect some of the TORC2-dependent functions [147, 148].

KOG1 (an essential gene product containing four internal HEAT repeats and seven C-terminal WD40 repeats) copurifies with TOR1 [25]. In fact, it was shown in *S. cerevisiae* that the HEAT repeats that are part of TOR form a curved tubular-shaped domain that associates with the C-terminal WD40 repeat domain of KOG1 (kontroller of growth 1), a “core protein” of budding yeast’s TORC1 [38]. KOG1’s counterpart in fission yeast is Mip1 in TORC1 [141, 142].

In budding yeast, AVO3 (orthologous to RICTOR and Ste20) is required for TORC2 structural integrity but is nonessential for the kinase activity of TOR2 *in vitro* [76]. AVO3 mutations suppress csg2 mutants which excessively accumulate sphingolipids [149]. Because of this fact, TORC2 may be involved in sphingolipid metabolism or signaling, since the TORC2 effectors SLM1/2 (synthetic lethal with MSS4 1/2) are targets of sphingolipid signaling during heat stress [150-153]. *S. pombe* Ste20 (orthologous to RICTOR and AVO3, but different from *S. cerevisiae* STE20) binds Tor1 more strongly than Tor2 when it’s overexpressed [141], but in *S. cerevisiae,* Tor1 is a subunit of TORC2, which illustrates conservation of function across species.

LST8, a subunit which is conserved from humans to yeast, is a critically important part of both TORC1 and TORC2 and is capable of binding the kinase domain of TOR2 [76]. In fission yeast, the LST8 ortholog Wat1 binds both Tor1 and Tor2 [141, 142] and is required for proper localization of F-actin [154], although another study found that neither TORC1 nor TORC2 play a significant role in actin organization in fission yeast [141].

TCO89 (TOR complex one 89 kDa subunit) is the product of a yeast-specific non-essential gene in *S. cerevisiae*. Although it is not conserved across species, TCO89 is still interesting because it shows the flexibility of the TOR complexes, while still maintaining the critical "core proteins" that are conserved across species throughout evolutionary history. TCO89 associates with TORC1, but it also localizes to distinct vacuolar structures and is believed to also operate independently of TORC1 [10, 77].

TORC2 is also highly conserved and shares similar structure and proposed functions to mammalian TORC2. In fission yeast, TORC2 is required for responses to starvation, sexual development, and stress conditions [155, 156], but is also required for cell integrity under different stressors [150, 157]. Interestingly, in a two-hybrid screen, FKBP12 was shown to be capable of binding Tor1 (a component of TORC2) in the presence of rapamycin [148]. However, similarly to mammals, TORC2 in budding yeast in not very sensitive to rapamycin [147].

TORC2 in budding yeast is composed of TOR (conserved), LST8 (orthologous to Wat1), AVO1 (orthologous to SIN1), and AVO3 (orthologous to RICTOR and Ste20), with protein interactors, including: AVO2, BIT61, BIT2, and SLM 1/2. The TORC2 complex of fission yeast includes TOR (conserved), Wat1 (orthologous to LST8), Sin1 (orthologous to AVO1), and Ste20 (orthologous to RICTOR and AVO3) [141]. In yeast, TOR2 performs a crucial function that

cannot be replaced by TOR1, since TOR2 can replace TOR1 in the complexes if necessary, but the opposite is not true [36].

2.9 The Regulation of TORC1 and TORC2 in Yeast

Because the TOR complex sequences are highly conserved from yeast to man, many of the functions are consistent as well. Rapamycin is still capable of inhibiting the TOR complexes, and similarly to humans, TORC1 is more sensitive to the compound than TORC2 [32, 33, 63]. LST8 in *S. cerevisiae* (conserved, called Wat1 in *S. pombe*) regulates both TORC1 and TORC2 and is important for recognition and recruitment of substrates, stimulates transportation of amino acid permeases to the cell's surface, stimulates the kinase activity of TOR, and influences the proper localization of F-actin in the cytoskeleton. Although LST8/Wat1 is not required for the phosphorylation ability of the TOR complexes, it is required for the nutrient-sensitive interaction of RAPTOR (yeast orthologs are KOG1 and Mip1) and mTOR [50, 51, 54, 72]. Rhb1, the ortholog of human Rheb, is responsible for binding TORC1 and promoting its activity [58, 68]. TSC1/2 exists in humans and *S. pombe*; it serves as a negative regulator of spTORC1 [58, 68-71]. It has also been shown that DNA damage, specifically p53, is capable of activating AMPK, which then activates TSC2 and negatively regulates spTORC1 [75]. TCO89 associates with scTORC1, so it may be involved with its regulation, however, it may also be able to operate independently [10, 77].

AVO1, a non-conserved interactor protein of TORC2 in *S. cerevisiae*, is capable of regulating substrate specificity of the complex, is essential for the phosphorylation of certain AGC kinases by TOR, and influences actin polarization in the cell [25, 66]. It was also shown that AVO3 in *S. cerevisiae* is important for regulating the structural integrity of scTORC2 [76].

2.10 The Functions of TORC1 in Yeast

2.10.1 Cellular Respiration and Lifespan. SCH9 is required for longevity and cell size in budding yeast [97, 98]. The closest mammalian ortholog of SCH9 is Akt, but is has also been shown to be functionally similar to S6K [95]. Six of the seven phosphorylated residues at the C-terminus of SCH9 that occur *in vivo*, are nutrient- and rapamycin- sensitive, suggesting they are regulated by TORC1 [95] (it has also been shown that TORC1 directly phosphorylates at least five of the phosphosites *in vitro*). The phosphorylation of these residues is essential for SCH9 function, since mutation of these sites abolishes *in vitro* kinase activity of SCH9 [95]. When TORC1 activates SCH9 by direct phosphorylation, it can then inhibit Rim15 by direct phosphorylation [106]. They found that treatment of yeast cells with caffeine releases Rim15 from TORC1-SCH9-mediated inhibition and consequently increases lifespan. Because this kinase cascade is evolutionarily conserved, it suggests that caffeine may extend lifespan in other eukaryotes, including humans [106]. SCH9 is predominantly located at the vacuolar membrane [158], and since the vacuole is an important reservoir of nutrients in yeast, TORC1 and/or SCH9 may play a role in sensing and/or remobilization of intracellular nutrients as well [95].

2.10.2 Cellular Stress. SCH9 may also play a role in the transcriptional activation of genes that are essential for osmotic stress responses [96]. It has been shown that SCH9 phosphorylation is reduced following osmotic, oxidative or thermal stress [95]. This stress response function of SCH9 is dependent on the MAPK HOG1 (high osmolarity glycerol response 1) and the ATF (activating transcription factor)/CREB (cAMP-response-element-binding protein) transcription factor SKO1 [96].

There is evidence that TORC1 controls the response to saline stress in the cell. The Hall lab showed that transcription of *ENA1,* a gene encoding a lithium and sodium ion transporter, is essential for salt tolerance in yeast and is controlled by the TOR signaling cascade [159]. Under

TOR-activating conditions, *ENA1* expression is strongly induced. The absence of the TOR-controlled GATA transcription factors GLN3 and GAT1 also results in reduced basal and salt-induced expression of *ENA1*. In the same study, the Hall lab also showed that a *gln3 gat1* mutant displayed a marked sensitivity to high concentrations of lithium and sodium. TOR1, similar to ENA1, is required for growth under saline stress conditions. Because of this, it is believed that TOR plays a role in the general response to saline stress by regulating the transcription of *ENA1* via GLN3 and GAT1 [159].

YPK1/2 (yeast protein kinase) have close homology with mammalian SGK (serum- and glucocorticoid inducible kinase) and Akt. YPK1/2 can be functionally replaced by SGK, and partially by Akt1 [160]. When *YPK1* was deleted, it resulted in a slow growth phenotype and rapamycin hypersensitivity, while the loss of *YPK2* showed no obvious defect [93, 161]. When wildtype cells were starved for nitrogen, YPK1 itself is rapidly degraded, and *YPK*-deficient cells exhibit translation initiation arrest. Deletion of both *YPK1/2* is lethal, suggesting they may have overlapping functions in the cell [93]. All of this together suggests that YPK may be part of a nutrient-sensing pathway that regulates translation initiation and that TORC1 (which is rapamycin-sensitive) is a regulator of YPK; however, administering rapamycin does not affect the kinase activity and expression levels of YPK [93]. It is likely that there is significant crosstalk between TORC1 and TORC2, because other studies suggest TORC2 is the regulator of YPK. In a TOR2-deficient cell line, a 5′-truncated version of *YPK2* was capable of rescuing the cells [94]. Interestingly, mutation of a conserved sequence in the N-terminal region of *YPK2* suppressed the defective actin organization of a *tor2* mutant and the impaired phosphorylation of MPK1, a downstream effector of TOR2 in the cell-integrity pathway (the N-terminal sequence negatively regulates YPK activity). Furthermore, TORC2 can phosphorylate YPK2 *in vitro* and

this phosphorylation is decreased when suspected interaction sites are mutated, suggesting that these sites are targeted by TORC2 [94]. We also know that YPK1 and TORC2 act in the same regulatory pathway, because overexpression of the RHO1-GEF (RHO1-guanine-nucleotide-exchange factor), TUS1, suppresses *YPK* or *tor2* mutants [162, 163].

2.10.3 Ribosomal Biogenesis and Translational Control. TORC1 binds and directly phosphorylates the Split Zn-finger transcription factor Sfp1 to promote its binding to a subset of ribosomal protein gene promoters. This protein cascade presumably regulates the nuclear localization and/or binding to ribosomal proteins and possibly gene promoters to stimulate their expression. Interestingly, unlike Sch9, Sfp1 phosphorylation by TORC1 appears to be unaffected by osmotic or nutritional stress, suggesting that TORC1 regulates these substrates by very different mechanisms [102].

2.10.4 Amino Acid Synthesis and the Retrograde Response. TORC1 is also known to control the transcription factors GLN3, RTG1, and RTG3, all of which are known to mediate glutamine synthesis. When glutamine synthetase inhibitor L-methionine sulfoximine (MSX) was added, it provoked glutamine depletion in the yeast cells, causing nuclear localization and activation of GLN3, RTG1, and RTG3, which are normally inhibited by TOR [164].

TORC1 also regulates the subcellular localization of the Rtg1/Rtg3 transcription factor complex through an indirect mechanism. Results of an epistasis analyses suggest that Rtg2 and Mks1 act downstream of TOR and upstream of Rtg1 and Rtg3 [165]. The Powers lab also demonstrated that TOR negatively regulates a concise cluster of genes (termed *RTG* target genes) that encode mitochondrial and peroxisomal enzymes required for *de novo* amino acid biosynthesis. In order to localize to the nucleus, it requires the cytoplasmic protein Rtg2. It is

believed that the likely role of Rtg2 is to antagonize the activity of Mks1, which is a negative regulator of *RTG* target gene activation [165].

2.11 The Functions of TORC2 in Yeast

2.11.1 Cytoskeleton Polarization. In budding yeast, AVO1 (orthologous to Sin1) binds to yeast TORC2. Lowering expression of AVO1 in yeast leads to actin depolarization, a phenotype that is also seen in Tor2 mutants [25]. Sin1 binds *S. pombe* SAPK (stress-activated protein kinase) [90-92], which may explain a few of its downstream effects on regulating the cell's response to different stressors.

Although nonessential, TORC2 also physically interacts with AVO2 and BIT61 [10, 25]. The complete function of the interaction is unknown, but BIT61 can associate with SLM1 and SLM2, two proteins that mediate TORC2 responses to stressors such as heat, oxidation, and actin cytoskeleton formation/organization [151-153, 157, 166]. TOR2 has been linked to the phosphorylation of the SLMs *in vivo* and *in vitro*, however, it may not be a direct regulatory effect [151, 157]. By describing the known structures and functions of these interacting proteins, we can see how critically important the conservation of this system is to the health and viability of cells across many distinct species.

2.11.2 Lipid Biosynthesis. In budding yeast, AVO3 (orthologous to RICTOR and Ste20) is required for TORC2 structural integrity but is nonessential for the kinase activity of TOR2 *in vitro* [76]. AVO3 mutations suppress csg2 mutants which excessively accumulate sphingolipids [149]. Because of this fact, TORC2 may be involved in sphingolipid metabolism or signaling, since the TORC2 effectors SLM1/2 (synthetic lethal with MSS4 1/2) are targets of sphingolipid signaling during heat stress [150-153]. *S. pombe* Ste20 (orthologous to RICTOR and AVO3, but different from *S. cerevisiae* STE20) binds Tor1 more strongly than Tor2 when it's overexpressed

[141], but in *S. cerevisiae*, Tor1 is a subunit of TORC2, which illustrates conservation of function across species.

2.12 PAS Kinase in Yeast and Mammals

In 2001, two laboratories first recognized PAS kinase as a serine/threonine kinase with a Per-Arnt-Sim (PAS) sensory domain that is similar to the sensory PAS domains commonly found in bacteria, archaea, and plants (such as FixL, a bacterial oxygen sensor). They hypothesized the importance of a protein kinase that could regulate many proteins and pathways in response to cellular conditions. Both laboratories simultaneously cloned the PAS kinase gene and performed initial characterization. Roland Wenger's laboratory demonstrated ubiquitous expression in mouse tissue, while Steven McKnight's laboratory demonstrated regulation of the kinase domain by the sensory N-terminal PAS domain [3]. This regulation in *cis* was further characterized by Amazecua laboratory, who demonstrated the ability of the PAS domain to bind specific ligands, which then removed the inhibition [5]. Unlike TOR (which is known to be found in complexes), complexes required for PAS kinase function have yet to be discovered.

Table 2-4. A Summary of the Known In Vivo Effects of PAS Kinase in Yeast and Mammalian Cells.

Protein*	**PASK**	**Mech.**	**Apparent effect**	**Ref.**
Pancreatic Function				
Proinsulin, Insulin	mPASK	UK	Ins2, Pdx2 decrease; Abnormal insulin secretion	[167]
mGSK3ß	hPASK	DP	inactivation of mGSK3ß, decreased insulin expression	[168]
Liver Function				
mSREB1c	mPASK	UK	decreased maturation and function; decreased fatty acid synthesis	[169]
Respiration				
scCbf1 mUSF1	Psk1		inhibition of Cbf1 and increased respiration	[170, 171]

Cell Differentiation				
Wdr5	mPASK	DP	promotes myogenesis	[172]
Cell Wall Biosynthesis				
scUgp1	Psk1/2	DP	increased UDP-glucose partitioning to cell wall	[173, 174]
scRho1	Psk2	UK	Rho1 activation	[16]
Feeding/weight gain				
mAMPK	mPASK	UK	altered activation in hypothalamus	[175]
mTORC1	mPASK	UK	altered activation in hypothalamus	[175]
Glycogen Storage				
mGT1 scGsy2	hPASK, Psk2	DP	inactivation; decreased glycogen storage;	[168]
mRNA and Translational control				
scRok1	Psk2	binds 5'UTR	repression of ROK1 expression	[176]
scPbp1 mAtxn2	hPASK, Psk1/2	DP	activation and stress granule localization of Pbp1; altered Ataxin-2 protein levels	[15, 177]
scCAF20	Psk2	DP	negatively regulates translation by blocking the association of eIF4E and eIF4G	[178]

*The abbreviation "sc" is for *Saccharomyces cerevisiae*, meaning that the data comes from studies with the yeast proteins in yeast, while "m" is for mammalian proteins. DP is for direct phosphorylation, UK is unknown. This table does not include *in vitro* substrates that have been identified but lack *in vivo* evidence.

2.13 The Regulation of PAS Kinase

In yeast, PAS kinase is activated by growth conditions that promote respiration (non-fermentative carbon sources), as well as by cell wall stress [179]. The activation of yeast PAS kinase by respiratory conditions is thought to primarily occur via phosphorylation by the AMPK ortholog in yeast termed SNF1 [15]. Mammalian PAS kinase also appears to be activated by growth conditions that stimulate respiration (high glucose) in pancreatic ß-cell isolates [34]. PASK mRNA expression has also been shown to decrease in response to fasting and is reactivated by feeding in mice. A study performed in Jared Rutter's laboratory demonstrated increased PASK mRNA expression upon re-feeding of either a normal chow or high-fat diet to mice after a fast [169]. The decrease of PAS kinase mRNA upon fasting was recently confirmed

in mice [180], along with increases in PASK mRNA expression upon re-feeding in hypothalamus tissue [175] by the Elvira Alvarez laboratory. Thus, PAS kinase appears to be regulated by nutritional status and cellular stress; it functions in a wide variety of pathways to control metabolism in both yeast and mammalian systems [172] (a summary is found in Table 2-4).

2.14 The Functions of PAS Kinase in Mammals

2.14.1 Pancreatic Function. Guy Rutter's laboratory was the first to demonstrate a role for PAS kinase in pancreatic insulin production. PAS kinase depletion in clonal pancreatic β-cells resulted in abnormal proinsulin gene expression, with a corresponding decrease in *Ins2* and *PDX1* gene expression [167]. Vincent Poitout's laboratory confirmed a role for PAS kinase in insulin production using cultured mouse and rat islets, where overexpression of *PASK* increased insulin and *PDX1* gene expression when islets were incubated with glucose and palmitate [181]. Expectedly, expression of kinase-dead *PASK* in these rat islets decreased insulin and *PDX1* gene expression. Jared Rutter's laboratory reported PAS kinase-deficient mice ($PASK^{-/-}$) displayed impaired plasma insulin secretion upon glucose stimulation in the whole-animal as well as in isolated islets [182]. In addition, they reported increased whole-animal insulin sensitivity. These results were recently supported by a study from the Elvira Alvarez laboratory who confirmed the increased insulin sensitivity in the $PASK^{-/-}$ mice [180]. Alternative but related results for the $PASK^{-/-}$ whole-animal studies were reported by Guy Rutter's laboratory, who observed little effect on *in vivo* insulin secretion upon stimulation, but did observe a significant decrease in total pancreatic insulin content as well as glucagon release. In order to further investigate these effects on insulin, they created the first two tissue-specific knockouts – one in pancreatic α-cells and the other in β-cells [183]. In the α-cell knockout mice, no difference in

weight gain or glucose/insulin tolerance was observed, however the α-cell mass was increased, and glucagon secretion was impaired at basal glucose levels as well as low glucose concentrations (hypoglycemia). In contrast to this increase upon tissue-specific depletion of PAS kinase, the α-cell mass was decreased in the global $PASK^{-/-}$ knockout. In the β-knockout mice, no differences in weight gain were observed, however β-cell mass was significantly decreased upon either tissue-specific β-cell knockout or global knockout in mice on both a normal or high-fat (HF) diet. Furthermore, global knockout mice also displayed defects of *in vivo* insulin secretion which were not observed in the β-cell specific knockout, perhaps due to whole-body development cues or downstream regulation from elsewhere in the body (the brain or secreted hormones/nutrients from other tissues). From the studies in three different research groups (Guy Rutter, Jared Rutter, and Elvira Alvarez laboratories), it was shown that PAS kinase clearly affects insulin production and/or secretion, and is dependent on both nutritional and whole-organism effects. However, there is one study from Roland Wenger's lab where no PASK-dependent insulin induction was observed, along with no changes in GTT or ITT. These results could be due to the mouse construct or to a difference in diet.

Limited data on the effects of PAS kinase on pancreatic function in humans comes from a study by Guy Rutter's group on familial maturity onset diabetes of the young (MODY) [184]. This study identified two unrelated individuals with apparent MODY that harbored mutations in PAS kinase. One mutation resulted in increased basal insulin hypersecretion (a 4.5-fold increase in insulin release at low glucose) when expressed in mouse islets. Combined with the results in mice, these findings support a role for PAS kinase in insulin and glucagon secretion, however, this role may be nutrient-responsive, which explains differences in the observed phenotypes.

The effect of PAS kinase on insulin secretion may be through the regulation of pancreatic duodenal homoeobox-1 (PDX-1), a transcription factor that regulates the insulin promoter. Vincent Poitout and Guy Rutter's laboratory provided evidence for the PAS kinase-dependent regulation of glycogen synthase kinase 3β (GSK3β), which directly phosphorylates Pdx-1 triggering proteosomal degradation [185]. The direct phosphorylation of GSK3ß by PAS kinase at Serine-9 leads to its inactivation *in vitro*. In addition, overexpression of PASK or kinase-dead (KD) GSK3ß stabilizes Pdx-1 protein, while overexpression of knockdown PASK prevents glucose-induction of insulin expression. Therefore, PAS kinase acts to inhibit GSK3ß, thereby stabilizing Pdx-1 protein and promoting insulin expression.

2.14.2 Liver Function. One of the most dramatic phenotypes associated with PAS kinase-deficient mice is the protection from hepatic liver triglyceride accumulation on a high-fat (HF) or high-fat high-sugar (HFHS) diet. Jared Rutter's laboratory reported complete protection from hepatic lipid accumulation for male mice on the HF diet by enzymatic quantification [182], which was recently confirmed in a study by the Elvira Alvarez laboratory [180]. We recently reported a similar effect for male mice on the HFHS diet and further analyzed the triglycerides through mass spectrometry [186]. PAS kinase appeared to reduce the accumulation of 25 of 44 triglycerides studied, and these 25 triglycerides made up a majority (90%) of triglycerides by cellular abundance. Interestingly, the female mice had a decreased accumulation of triglycerides independent of genotype, masking any significant effects from PAS kinase. These effects on liver triglyceride accumulation were verified pharmacologically by Jared Rutter's laboratory, where inhibition of PAS kinase protected rats from liver triglyceride accumulation on a HF diet [182]. Furthermore, they demonstrated that these effects may be due to the impaired SREBP- 1c maturation observed in cultured HepG2 cells treated with shRNA or pharmacological inhibitors

of PAS kinase as well as in mouse and rat liver from pharmacologically-inhibited rodents [169]. SREBP-1c is a transcription factor implicated in the development of metabolic syndrome and diabetes. It requires feeding-induced proteolytic maturation in the endoplasmic reticulum to become an active nuclear transcription factor, therefore the reduction in maturation should reduce fatty acid biosynthesis. Accordingly, the reduction of SREBP-1c maturation was accompanied by decreased expression of SREBP-1c target genes including GPAT1, and Fasn in pharmacologically treated rodents. We have recently provided evidence that Upstream Stimulatory Factor 1 (USF1), a transcription factor intimately linked to SREBP-1c through co-regulation of fatty acid biosynthesis, is also inhibited by PAS kinase in yeast [171]. In this case, the inhibition is through direct phosphorylation. USF1 has been linked to human hypertriglyceridemia in several Genome-Wide Association Studies (GWAS) [187]. Thus, the effects of PAS kinase on both SREBP-1c and USF1 may account for the dramatic effects observed in liver tissue.

2.14.3 Cellular Respiration. In the study of PASK$^{-/-}$ male mice on the HF diet performed by Jared Rutter's lab, the PASK$^{-/-}$ mice displayed a hypermetabolic phenotype in that they had a higher whole-animal O_2 uptake and CO_2 output despite no significant difference in food intake or activity (beam breaks) [182]. This hypermetabolic phenotype was supported by increased ATP production from succinate in permeabilized soleus muscle fibers from PASK$^{-/-}$ mice, as well as by shRNA silencing of PASK in L6-derived cells where increased [^{14}C] glucose and [^{14}C] palmitate oxidation was observed. This apparent effect on respiration was recently confirmed by our study of PASK$^{-/-}$ mice, where PASK-deficiency resulted in a 1.5-fold increase in soleus tissue basal respiration independent of diet and sex [186]. In addition, PASK$^{-/-}$ male mice also displayed a two-fold increase in oxidative phosphorylation capacity of soleus tissue when

compared to the wildtype littermates. The effects of PAS kinase on respiration may be through its substrate USF1, which is currently under investigation (see discussion of USF1 below).

2.14.4 Weight Gain. In addition to the resistance to insulin insensitivity and liver triglyceride accumulation, $PASK^{-/-}$ male mice on the HF diet also displayed lower weights than their wildtype littermates [182]. This may be partially due to hormonal effects, since the Elvira Alvarez laboratory has reported effects of PAS kinase on hypothalamus function. In the hypothalamus, PAS kinase is regulated by fasting/feeding and knockout of PAS kinase blunts the exendin-4 feeding-based activation of AMPK and mTOR/e1.

2.14.5 Glycogen Storage. Human PAS kinase (hPASK) has also been shown to directly phosphorylate and inactivate human glycogen synthase at a key regulatory residue, Serine 640 [168]. In addition, a study by Wayne Wilson, Peter Roach, and Jared Rutter, showed direct interaction of hPASK with glycogen synthase in cultured cells, with disruption of this interaction by glycogen itself.

2.14.6 mRNA Processing and Translational Control. The primary evidence for a role for mammalian PAS kinase in regulating translation comes from a study by Roland Wenger's laboratory in which eukaryotic translation elongation factor eEF1A1 was identified as a substrate and *in vivo* binding partner of mammalian PAS kinase [188].

2.14.7 Cell Differentiation. Jared Rutter's lab recently discovered a role for PAS kinase in promoting the differentiation of embryonic stem cells, as well as myogenic and adipogenic progenitor cells [172]. This control of differentiation occurs through direct phosphorylation of Wdr5, a subunit of histone H3 Lysine 4 trimethylase (H3K4me3), which stimulates myogene gene expression via H3K4me3 marks on its promoter.

2.15 The Functions of PAS Kinase in Yeast

Many of the phenotypes observed in the whole animal are mirrored in the single-celled eukaryote *Saccharomyces cerevisiae*. As with TOR, some effects appear to be mediated by both yeast PAS kinase paralogs (Psk1 and Psk2), while others appear to be specific.

2.15.1 Cellular Respiration. The effects of PAS kinase on respiration seen in mammalian cells appear to be well conserved, since PAS kinase-deficiency increases respiration in yeast [15, 171]. This effect on respiration is at least partially due to the phosphorylation and inhibition of Centromere-Binding Factor 1 (Cbf1) at Threonine-212, since CBF1-deficient yeast display decreased respiration, the triple combination yeast (cbf1psk1psk2) show a reduction in respiration, and a phosphosite mutant (T212A) displays increased respiration [171]. However, the reduction of respiration is not complete in the triple knockout, suggesting alternative pathways in the respiratory function of PAS kinase.

The CBF1-dependent regulation of respiration in yeast is at least in part to due to the direct transcriptional regulation of the gamma subunit of ATP synthase (ATP3). ATP3 has a known Cbf1-binding site in its promoter and is regulated by Cbf1 as demonstrated by both ß -galactosidase and mass spectrometry analysis. In addition, we have shown that Cbf1 directly regulates the expression of Psk1, thereby inhibiting its inhibitor.

Cbf1 is the only known substrate for which Psk1 and Psk2 appear to have different roles, however, there is some evidence that Psk2 is the dominant kinase for Ugp1 phosphorylation (see below). Psk2 only very weakly phosphorylates Cbf1 *in vitro* [171]. In addition, Psk2 has been shown to have decreased activity (possibly through decreased expression) under respiratory conditions, suggesting the contributions of PAS kinase under respiratory conditions are primarily through Psk1 [179].

The regulation of respiration by PAS kinase through the phosphorylation of Cbf1 may be conserved in mammalian cells since, first, hPASK can phosphorylate USF1 (the mammalian Cbf1-ortholog) *in vitro*, and second, USF1 can complement the respiration phenotype of Cbf1-deficient yeast [171]. This is the first evidence that mammalian USF1 may have a role in respiratory metabolism, in addition to its role in fatty acid biosynthesis.

2.15.2 mRNA Processing and Translational Control. Several findings have supported a role for yeast PAS kinase in translational control. First, yeast Psk2 directly phosphorylates and activates Cap-Associated Factor (CAF20), a phosphoprotein of the mRNA cap-binding complex involved in translation control of mRNA expression [178]. CAF20 is an eIF4E-binding protein that directly competes with eIF4G for binding to eIF4E, which association nucleates assembly of the translational apparatus with the 5' cap of mRNAs. Mammalian eIF4E-binding proteins (4E-BPs) are a key point in translational control and are often regulated through phosphorylation. Interestingly, in this same study by Steven McKnight's lab, Psk2 was also found to phosphorylate two other proteins *in vitro* which were involved in translational control, TIF11 and SRO9. TIF11 is an essential translation initiation factor that forms a complex with eIF1 and the 40S ribosomal subunit, scanning for the initiating AUG. Sro9 binds RNA and interacts with translating ribosomes. Many proteins involved in translation were also retrieved from a *psk1psk2* suppressor screen, and Psk2 overexpression suppresses the growth and protein synthesis defects of eIF4B-deficient yeast. These effects on translation are consistent with the phosphorylation of eEF1A1 by mammalian PAS kinase reported above. It is unknown whether Psk1 also regulates CAF20 in yeast.

Both Psk1 and Psk2 phosphorylate and activate poly(A)-binding protein 1 (Pbp1) [15]. This phosphorylation leads to increased Pbp1 localization at stress granules. Pbp1, like its

mammalian ortholog Ataxin-2, is known to recruit both mRNA and proteins (including yeast TORC1) to stress granules, thereby inhibiting their expression and/or activity. In fact, deletion of *PSK1* and *PSK2* suppresses the caffeine sensitivity seen in yeast overexpressing Pbp1, suggesting rescue of TORC1 function. This was confirmed through SK1 immunoblot, a key TORC1 substrate. We have recently demonstrated the *in vitro* phosphorylation of mammalian Ataxin-2 by hPASK and have demonstrated the ability of Ataxin-2 to complement Pbp1 function in yeast, including its apparent regulation by PAS kinase. This evidence suggests that this pathway by which PAS kinase regulates TORC1 may be conserved.

A third pathway may tie PAS kinase to mRNA processing and translation through the regulation of Rok1 mRNA [176]. In a three-hybrid screen, Psk2 was identified as a ROK1 5'UTR-interacting partner. In addition, overexpression of Psk2 repressed Rok1 protein expression.

2.15.3 Cytoskeleton Polarization and Cell Wall Integrity. One of the best characterized substrates of yeast PAS kinase is UDP-glucose pyrophosphorylase (UGP1) [178], which catalyzes the reversible formation of UDP-glucose from glucose-1-phosphate and UTP. Ugp1 can be phosphorylated *in vitro* by both Psk1 and Psk2. Although the phosphorylation appears to have no effect on its enzymatic activity, it alters where its product (UTP) is utilized, favoring cell wall biosynthesis over glycogen storage [173]. Although it has not been demonstrated, it is hypothesized that phosphorylation localizes UGP1 to the cell periphery, perhaps by altering protein binding partners, thereby favoring UTP utilization for cell wall biosynthesis. Accordingly, *psk1psk2* yeast are sensitive to cell wall perturbing agents, and have increased glycogen (this may also be due to phosphorylation of Gsy2). It is unknown whether the phosphorylation of UGP1 by PAS kinase is conserved in mammalian cells.

In addition to its role in cell wall biosynthesis through the phosphorylation of Ugp1, overexpression of Psk2 is also capable of suppressing a *tor2(ts)* mutation through the activation of Rho1 [189]. This suppression requires the Ssd1 protein, which interacts with TOR components to play a role in the polarization of the cytoskeleton and cell wall integrity. In addition, suppression requires phosphorylated Ugp1 and Rom2, which appear to form a signaling complex with Ssd1 to activate RhoI. Psk1 overexpression does not appear to suppress the *tor2(ts)* mutant, suggesting this role is primarily a Psk2 function. However, it is unknown whether the mPASK is capable of suppressing defects in mTORC2.

2.15.4 Glycogen Storage. Similar to the regulation of mammalian glycogen synthase by mammalian PAS kinase, yeast Psk2 phosphorylates and inactivates yeast glycogen synthase 2 (Gsy2). Psk2 directly phosphorylates Gsy2 *in vitro* at Serine-654, a key residue known to influence inhibition. In addition, deletion of both Psk1 and Psk2 paralogs results in an increase in glycogen synthase activity as well as a three to four-fold increase in glycogen accumulation [178], although this accumulation seen in the double mutant is greater than either mutant alone; it is unknown whether Psk1 can directly phosphorylate Gsy2.

2.16 Crosstalk Between PAS Kinase and TOR

PAS Kinase and TOR appear to have co-evolved to work together in the cell to regulate various cellular processes (see Figure 2-3). PAS kinase and TOR have many overlaps in function, including their roles in regulating translation initiation. The two paralogs in yeast - PAS kinase and TOR - appear to have paired functions. One of the primary functions of TORC1 in yeast is to modulate translation initiation. TORC1 consists of either Tor1 or Tor2 together with Kog1, Lst8 and Tco89. TORC1 is inhibited by both Psk1 and Psk2p through the phosphorylation and activation of Pbp1, which in turn sequesters TORC1 to stress granules,

inhibiting growth and proliferation [15]. In contrast, Psk2 regulates cell division (cytoskeleton polarization and cell wall integrity) through the activation of RhoI, suppressing lethality due to TORC2 deficiency [16]. Thus, both TOR and PAS kinase have overlapping functions for their paralogs (Tor1 and Tor2, Psk1 and Psk2) in cell growth and proliferation, and specific functions for Tor2 and Psk2 in cytoskeletal polarization and cell integrity. These roles for PAS kinase in TORC1 regulation and the suppression of TORC2 deficiency by Psk2 remain unstudied in mammalian cells.

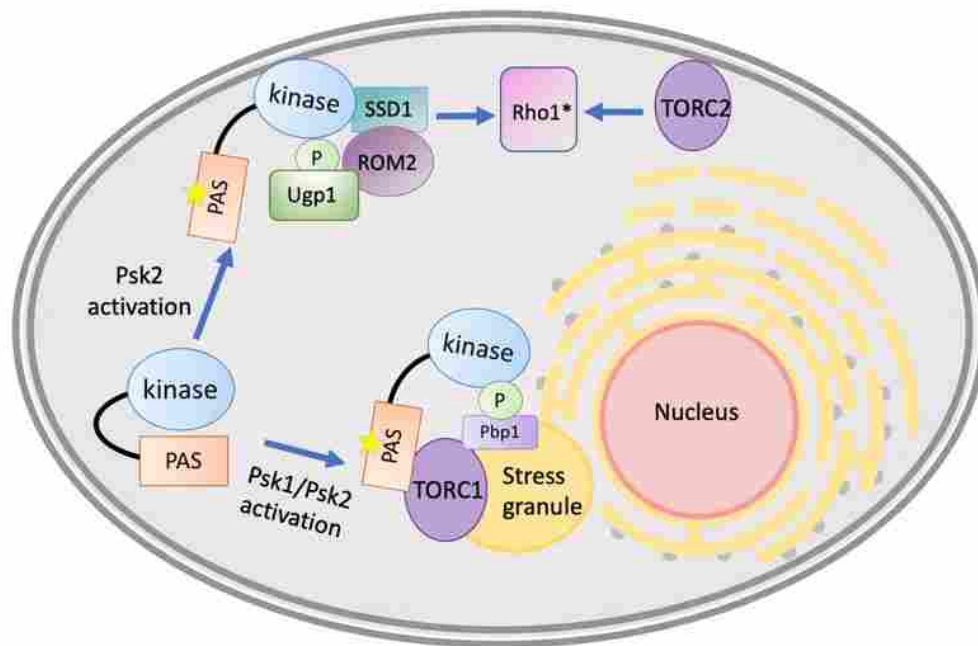

Figure 2-3. PASK and TOR have Overlapping Functions.

2.17 Conclusions

The two paralogs of both PAS kinase and TOR have evolved to have unique functions in the cell, while sharing some overlapping functions. This co-evolution has allowed for cells to coordinate their efforts for a concerted action, such as protein synthesis/translation, mRNA

processing, glucose homeostasis, respiration, cell differentiation, cellular signaling, cellular proliferation, pancreatic function, etc.

Not only have the PASK and TOR paralogs evolved to have different functions, but the entire TOR complex of proteins has co-evolved to control key cellular processes. The core group of proteins is highly conserved, and depending on the organism and whether it is TOR complex 1 or 2, non-conserved interactors may be involved. TORC1 is mainly responsible for protein synthesis/ translation, mRNA processing, cell growth, nitrogen-catabolite repression, and the retrograde response. TORC2 mostly regulates respiration, cellular differentiation, cellular signaling, and cytoskeleton polarization, however, there are lots of overlaps in function between the complexes, even in species as diverse as yeast and humans. TORC1 and TORC2 are both known to contribute to the critical cellular processes of cell survival, cellular lifespan, and various stress responses.

PAS kinase has evolved to work alongside TOR. PAS kinase and TOR have evolved to coordinate and control the pathways of protein synthesis/translation, mRNA processing, glucose homeostasis, respiration, cell differentiation, cellular signaling, and pancreatic function, while PAS kinase also has its own unique functions of cell wall biosynthesis and proliferation, glycogen storage, liver function, and feeding/ weight gain. Yeast Psk1 and Psk2 have also evolved to have distinct, yet overlapping functions, as Psk1 has been found to control respiration in yeast, while Psk2 affects glycogen storage. They are also capable of coordinating their efforts in the regulation of cellular signaling. Even more interesting is that although TOR and PASK have both evolved by gene duplication, Psk1 and Tor2 adapted related secondary functions that Psk2 and Tor1 don't do, another indication of their co-evolution.

As discussed earlier, both TOR and PASK have evolved to regulate respiration (see Figure 2-4). When there is a low ATP:AMP ratio in the cell, AMPK is activated, which activates Psk1. Psk1 then phosphorylates CBF1, which inhibits respiration (yeast preferentially ferment). CBF1 also has the direct effect of upregulating ATP3 (an ATP synthase) in yeast. When there is a high ATP:AMP ratio in the cell, AMPK is inhibited and TSC1/2 are free to inhibit Rheb, the activator of mTOR. Once mTOR is activated, it upregulates the glycolytic enzyme PKM2 and phosphorylates cPKC (important for glycogenolysis and gluconeogenesis) to increase cellular respiration.

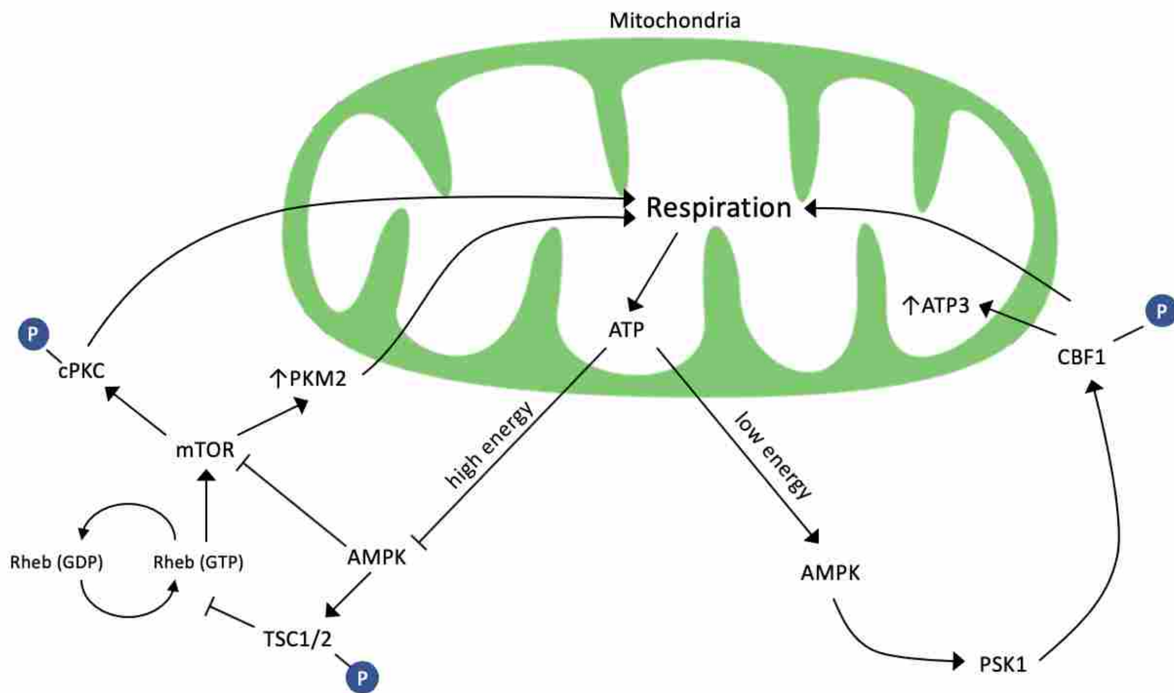

Figure 2-4. PAS Kinase and TOR have Co-evolved to Regulate the Critical Cellular Function of Respiration.

In the cellular proliferation pathway, PAS kinase and TOR work together to control cell wall biosynthesis (see Figure 2-5). PAS kinase is activated by low energy or another stressor to phosphorylate Pbp1 (ATXN2), which sequesters TORC1 to a stress granule and inactivates it, thus decreasing cellular proliferation. On the other hand, when TORC1 is activated by Rheb in

the lysosome, it activates ribosomal protein S6 kinase -1 (S6K1)1 and inactivates 4E-BP1, thereby increasing translation and cell proliferation/ growth. When PAS kinase phosphorylates Ugp1 in *S. cerevisiae*, it has the effect of increasing cell wall biosynthesis.

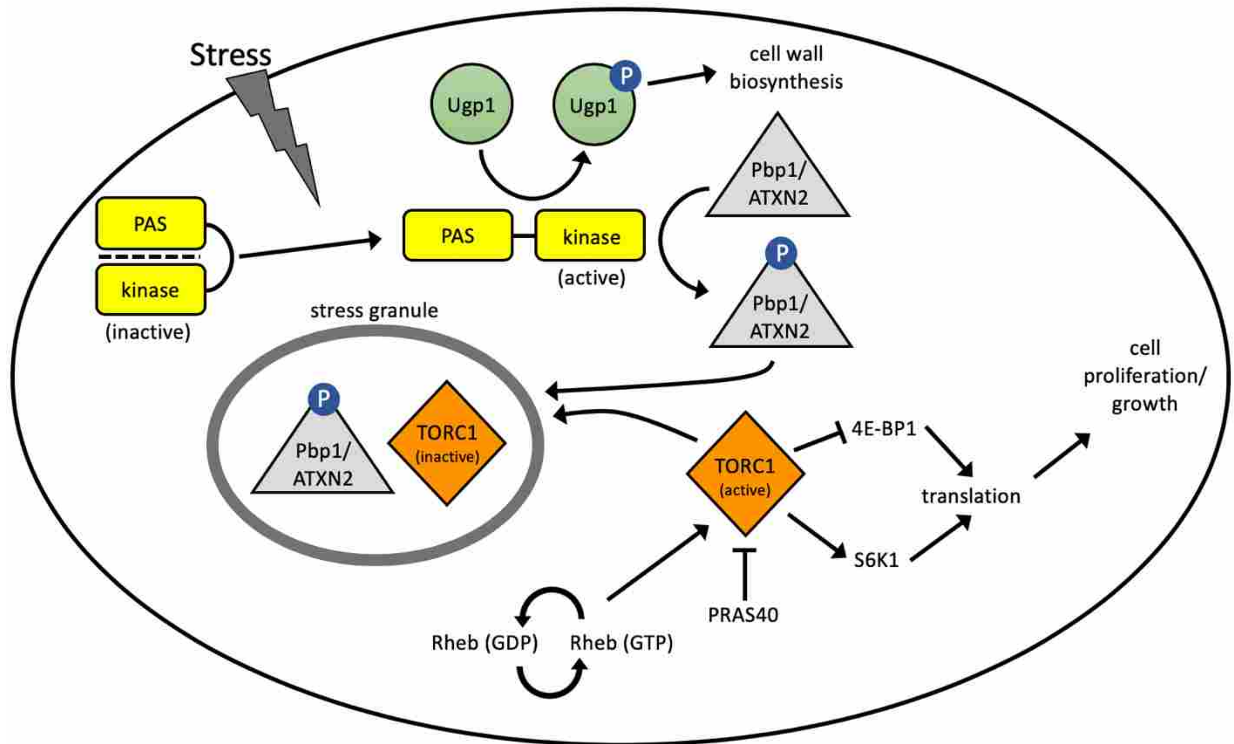

Figure 2-5. PAS Kinase and TOR have Co-evolved to Regulate the Critical Cellular Functions of Cellular Proliferation and Growth.

As we continue to study the interactions and evolutionary history of these proteins, we may begin to uncover new metabolic pathways and potential treatment targets for human diseases that are controlled by these co-evolved proteins (such as cancer and diabetes). It may be possible to identify downstream targets of these pathways, potentially allowing for a drug treatment with fewer side effects than the current options.

CHAPTER 3: Using DCA to Understand Kinase-Substrate Interactions

3.1 Importance of Protein Kinases

Protein kinases make up one of the largest and most important protein families. Because of their evolutionary significance, kinase domains are highly conserved, with great sequence homology existing in species as diverse as yeast and humans [190]. Even though protein kinase genes constitute only about 2% of the genomes in most eukaryotes, they phosphorylate more than 30% of cellular proteins [191]. By catalyzing the transfer of phosphate groups from ATP (or GTP), kinases direct the activity and localization of many proteins, and are critical in the regulation of nearly all cellular processes [192].

Protein kinases phosphorylate hundreds of downstream targets and control processes from cell cycle progression to transcription and nervous system responses [193]. Tight regulation of protein kinases is imperative because of all the critical functions they provide in the cell; when they malfunction, diseases such as cancer [194-196], diabetes [196], or other metabolic diseases may occur. In fact, Alzheimer's Disease is believed to be caused by aberrant protein kinases [197]. The brain has about six different isoforms of the tau microtubule-binding protein, and when they are unphosphorylated, it inhibits their ability to bind microtubules and prevents correct neuronal development and function. In short, when there isn't a functional protein kinase to phosphorylate the tau proteins, Alzheimer's can result [198]. Because kinases control so many cellular processes, protein kinases are often used as drug treatment targets. Type 2 Diabetes, a disease marked by insulin resistance, is often treated with AMP-activated kinase (AMPK) activators. AMPK plays a key role in the coordination of cellular energy homeostasis, so when it malfunctions, diseases such as diabetes and cancer may result [23].

From these examples, we can begin to understand the immense impact that kinases have on our health, and the importance of studying them to provide better treatment options for people afflicted with kinase-triggered diseases. Each year, billions of dollars are spent on laboratory research [199], with many scientists trying to understand the *in vivo* functionality of the hundreds of kinases in our bodies. We have discovered much about protein kinases using the current standard procedure - co-immunoprecipitation and mass spectrometry - however, this method is insufficient.

Notwithstanding the vital importance of protein kinases within the cell, they can be difficult to study. Protein-kinase interactions are generally highly transient, so typical protein-protein interaction discovery methods, such as co-immunoprecipitation and mass spectrometry, rarely give the full "protein-kinase interaction picture." In addition to the transient nature of these interactions, there are often multiple sites which are phosphorylated on a target protein, often by multiple kinases, so understanding the regulation and functions of these proteins can be a difficult, but very important task because of the critical cellular processes involved. The sheer number of proteins phosphorylated in the cell – 30-50%, depending on needs in the body – also complicates the study of protein kinases [17]. The sheer quantity of kinases and their multitude of targets makes them mechanistically difficult to study.

We are particularly interested in studying Per-Arnt-Sim (PAS) kinase because of its significance in cellular metabolism. In a 2007 study [182], it was found that PAS kinase-knockout mice were hypermetabolic and more resistant to liver triglyceride accumulation than the wildtype, despite having similar activity levels. Because of its tie to cellular metabolism, PASK could potentially serve as a therapeutic target for cancer and diabetes. The common

issues that occur in the study of nearly all protein kinases also, unsurprisingly, affect the study of PAS kinase.

Kinases often induce conformational changes when they phosphorylate their substrates, effectively "hiding" the phosphosite on the inside of the protein and potentially changing how the substrate interacts with other proteins in the cell, including its location in the cell [18]. Structure affects function in proteins, so proteins are commonly crystallized to better understand how they could interact with their substrates, however, large proteins such as PAS kinase are difficult to crystallize [19]. In addition, kinases often phosphorylate multiple sites on its substrates, and PAS kinase is no exception, making identifying the critical phosphorylation sites difficult.

Because of the difficulty, we propose a novel method of studying protein kinases and their phosphosites using Rice University's Direct Coupling Analysis (DCA) algorithms [20] with PAS kinase and five of its known substrates as a case study. Using DCA, the co-evolution of PAS kinase with its confirmed substrates, Cbf1, Ugp1, Utr1 (three from yeast species), USF1, and ATXN2 (two from mammalian species) is estimated by Mutual Information, allowing us to predict the most likely interaction points, as well as potential phosphosites for the proteins. We can potentially predict sites that are not captured by current kinase-study techniques, allowing us to be more thorough in our research, and then use site-directed mutagenesis of the sites predicted by DCA to confirm cellular effects caused by PAS kinase.

3.2 Methods

3.2.1 Direct Coupling Analysis. To collect sequences for the Direct Coupling Analysis, we found organisms that had orthologs of both proteins of interest (for this study, yeast and mammalian PAS kinase, yeast Cbf1, Ugp1, Utr1, and mammalian USF1 or ATXN2). For the *S.*

cerevisiae DCA, we included other fungal species, and the *H. sapiens* analysis was limited to other mammals. We used the longest isoform (see Table 3-1 for protein sequences) of Psk1 (for fungal species) or hPASK (for mammalian species) and performed a regular protein BLAST to find orthologs of the proteins in other species. To confirm that the listed protein was truly an ortholog, we performed a non-organism-specific protein BLAST and checked that PASK was at the top of the results. After verifying it was our protein of interest, we completed an organism-specific tBLASTn using organisms from the PASK BLAST search and the longest isoform of the other protein of interest (Cbf1, Ugp1, Utr1, USF1, or ATXN2). We then downloaded the protein alignments (starting with the smallest e-value) and re-BLASTed the proteins to confirm that it was truly our protein of interest.

Table 3-1. Protein Sequences Used in Direct Coupling Analysis. The longest isoform of each protein from *H. sapiens* (h) or *S. cerevisiae* (sc) was used.

Protein	Sequence
hPASK	MEDGGLTAFEEDQRCLSQSLPLPVSAEGPAAQTTAEPSRSFSSAHR HLSRRNGLSRLCQSRTALSEDRWSSYCLSSLAAQNICTSKLHCPAA PEHTDPSEPRGSVSCCSLLRGLSSGWSSPLLPAPVCNPNKAIFTVD AKTTEILVANDKACGLLGYSSQDLIGQKLTQFFLRSDSDVVEALSE EHMEADGHAAVVFGTVVDIISRSGEKIPVSVWMKRMRQERRLCC VVVLEPVERVSTWVAFQSDGTVTSCDSLFAHLHGYVSGEDVAGQ HITDLIPSVQLPPSGQHIPKNLKIQRSVGRARDGTTFPLSLKLKSQPS SEEATTGEAAPVSGYRASVWVFCTISGLITLLPDGTIHGINHSFALT LFGYGKTELLGKNITFLIPGFYSYMDLAYNSSLQLPDLASCLDVGN ESGCGERTLDPWQGQDPAEGGQDPRINVVLAGGHVVPRDEIRKL MESQDIFTGTQTELIAGGQLLSCLSPQPAPGVDNVPEGSLPVHGEQ ALPKDQQITALGREEPVAIESPGQDLLGESRSEPVDVKPFASCEDS EAPVPAEDGGSDAGMCGLCQKAQLERMGVSGPSGSDLWAGAAV AKPQAKGQLAGGSLLMHCPCYGSEWGLWWRSQDLAPSPSGMAG LSFGTPTLDEPWLGVENDREELQTCLIKEQLSQLSLAGALDVPHAE LVPTECQAVTAPVSSCDLGGRDLCGGCTGSSSACYALATDLPGGL EAVEAQEVDVNSFSWNLKELFFSDQTDQTSSNCSCATSELRETPSS LAVGSDPDVGSLQEQGSCVLDDRELLLLTGTCVDLGQGRRFESC

	VGHDPTEPLEVCLVSSEHYAASDRESPGHVPSTLDAGPEDTCPSAE EPRLNVQVTSTPVIVMRGAAGLQREIQEGAYSGSCYHRDGLRLSI QFEVRRVELQGPTPLFCCWLVKDLLHSQRDSAARTRLFLASLPGS THSTAAELTGPSLVEVLRARPWFEEPPKAVELEGLAACEGEYSQK YSTMSPLGSGAFGFVWTAVDKEKNKEVVVKFIKKEKVLEDCWIE DPKLGKVTLEIAILSRVEHANIIKVLDIFENQGFFQLVMEKHGSGL DLFAFIDRHPRLDEPLASYIFRQVRAGQSRLVSAVGYLRLKDIIHR DIKDENIVIAEDFTIKLIDFGSAAYLERGKLFYTFCGTIEYCAPEVL MGNPYRGPELEMWSLGVTLYTLVFEENPFCELEETVEAAIHPPYL VSKELMSLVSGLLQPVPERRTTLEKLVTDPWVTQPVNLADYTWE EVFRVNKPESGVLSAASLEMGNRSLSDVAQAQELCGGPVPGEAP NGQGCLHPGDPRLLTS
scPsk1	MPYIGASNLSEHSFVNLKEKHAITHKGTSSSVASLQTPPSPDQENHI DNELENYDTSLSDVSTPNKKEGDEFEQSLRDTFASFRKTKPPPSLD FEQPRLPSTASSSVDSTVSSPLTDEDIKELEFLPNESTHSYSYNPLSP NSLAVRLRILKRSLEIIIQNPSMLLEPTPDDLPPLKEFAGRRSSLPRT SASANHLMNRNKSQIWNTTSATLNAFVNNTSSSSAASSALSNKKP GTPVFPNLDPTHSQTFHRANSLAYLPSILPEQDPLLKHNNSLFRGD YGNNISPERPSFRQPFKDQTSNLRNSSLLNERAYQEDETFLPHHGP SMDLLNEQRANLKSLLNLLNETLEKNTSERASDLHMISLFNLNKL MLGDPKKNNSERDKRTEKLKKILLDSLAEPFFEHYNFIEDNPIADT DELKEEIDEFTGSGDTTAITDIRPQQDYGRILRTFTSTKNSAPQAIFT CSQEDPWQFRAANDLACLVFGISQNAIRALTLMDLIHTDSRNFVL HKLLSTEGQEMVFTGEIIGIVQPETLSSSKVVWASFWAKRKNGLL VCVFEKVPCDYVDVLLNLDEFGVENIVDKCELLSDGPTLSSSSTLS LPKMASSPTGSKLEYSLERKILEKSYTKPTSTENRNGDENQLDGDS HSEPSLSSSPVRTKKSVKFANDIKDVKSISQSLAKLMDDVRNGVVF DPDDDLLPMPIKVCNHINETRYFTLNHLSYNIPCAVSSTVLEDELK LKIHSLPYQAGLFIVDSHTLDIVSSNKSILKNMFGYHFAELVGKSIT EIIPSFPKFLQFINDKYPALDITLHKNKGLVLTEHFFRKIQAEIMGDR KSFYTSVGIDGLHRDGCEIKIDFQLRVMNSKVILLWVTHSRDVVFE EYNTNPSQLKMLKESELSLMSSASSSASSSKKSSSRISTGTLKDMS NLSTYEDLAHRTNKLKYEIGDDSRAHSQSTLSEQEQVPLENDKDS GEMMLADPEMKHKLELARIYSRDKSQFVKEGNFKVDENLIISKISL SPSTESLADSKSSGKGLSPLEEEKLIDENATENGLAGSPKDEDGIIM TNKRGNQPVSTFLRTPEKNIGAQKHVKKFSDFVSLQKMGEGAYG KVNLCIHKKNRYIVVIKMIFKERILVDTWVRDRKLGTIPSEIQIMAT LNKKPHENILRLLDFFEDDDYYYIETPVHGETGCIDLFDLIEFKTN MTEFEAKLIFKQVVAGIKHLHDQGIVHRDIKDENVIVDSKGFVKII DFGSAAYVKSGPFDVFVGTIDYAAPEVLGGNPYEGQPQDIWAIGI LLYTVVFKENPFYNIDEILEGDLKFNNAEEVSEDCIELIKSILNRCV PKRPTIDDINNDKWLVI
hUSF1	MKGQQKTAETEEGTVQIQEGAVATGEDPTSVAIASIQSAATFPDP NVKYVFRTENGGQVMYRVIQVSEGQLDGQTEGTGAISGYPATQS MTQAVIQGAFTSDDAVDTEGTAAETHYTYFPSTAVGDGAGGTTS GSTAAVVTTQGSEALLGQATPPGTGQFFVMMSPQEVLQGGSQRSI

	APRTHPYSPKSEAPRTTRDEKRRAQHNEVERRRRDKINNWIVQLS KIIPDCSMESTKSGQSKGGILSKACDYIQELRQSNHRLSEELQGLD QLQLDNDVLRQQVEDLKNKNLLLRAQLRHHGLEVVIKNDSN
hATXN2	MRSAAAAPRSPAVATESRRFAAARWPGWRSLQRPARRSGRGGG GAAPGPYPSAAPPPPGPGPPPSRQSSPPSASDCFGSNGNGGGAFRP GSRRLLGLGGPPRPFVVLLLPLASPGAPPAAPTRASPLGARASPPRS GVSLARPAPGCPRPACEPVYGPLTMSLKPQQQQQQQQQQQQQQQ QQQQQQQQPPPAAANVRKPGGSGLLASPAAAPSPSSSSVSSSSAT APSSVVAATSGGGRPGLGRGRNSNKGLPQSTISFDGIYANMRMVH ILTSVVGSKCEVQVKNGGIYEGVFKTYSPKCDLVLDAAHEKSTES SSGPKREEIMESILFKCSDFVVVQFKDMDSSYAKRDAFTDSAISAK VNGEHKEKDLEPWDAGELTANEELEALENDVSNGWDPNDMFRY NEENYGVVSTYDSSLSSYTVPLERDNSEEFLKREARANQLAEEIES SAQYKARVALENDDRSEEEKYTAVQRNSSEREGHSINTRENKYIP PGQRNREVISWGSGRQNSPRMGQPGSGSMPSRSTSHTSDFNPNSG SDQRVVNGGVPWPSPCPSPSSRPPSRYQSGPNSLPPRAATPTRPPSR PPSRPSRPPSHPSAHGSPAPVSTMPKRMSSEGPPRMSPKAQRHPRN HRVSAGRGSISSGLEFVSHNPPSEAATPPVARTSPSGGTWSSVVSG VPRLSPKTHRPRSPRQNSIGNTPSGPVLASPQAGIIPTEAVAMPIPA ASPTPASPASNRAVTPSSEAKDSRLQDQRQNSPAGNKENIKPNETS PSFSKAENKGISPVVSEHRKQIDDLKKFKNDFRLQPSSTSESMDQL LNKNREGEKSRDLIKDKIEPSAKDSFIENSSSNCTSGSSKPNSPSISP SILSNTEHKRGPEVTSQGVQTSSPACKQEKDDKEEKKDAAEQVRK STLNPNAKEFNPRSFSQPKPSTTPTSPRPQAQPSPSMVGHQQPTPV YTQPVCFAPNMMYPVPVSPGVQPLYPIPMTPMPVNQAKTYRAVP NMPQQRQDQHHQSAMMHPASAAGPPIAATPPAYSTQYVAYSPQQ FPNQPLVQHVPHYQSQHPHVYSPVIQGNARMMAPPTHAQPGLVS SSATQYGAHEQTHAMYACPKLPYNKETSPSFYFAISTGSLAQQYA HPNATLHPHTPHPQPSATPTGQQQSQHGGSHPAPSPVQHHQHQAA QALHLASPQQQSAIYHAGLAPTPPSMTPASNTQSPQNSFPAAQQT VFTIHPSHVQPAYTNPPHMAHVPQAHVQSGMVPSHPTAHAPMML MTTQPPGGPQAALAQSALQPIPVSTTAHFPYMTHPSVQAHHQQQL
scCbf1	MNSLANNNKLSTEDEEIHSARKRGYNEEQNYSEARKKQRDQGLL SQESNDGNIDSALLSEGATLKGTQSQYESGLTSNKDEKGSDDEDA SVAEAAVAATVNYTDLIQGQEDSSDAHTSNQTNANGEHKDSLNG ERAITPSNEGVKPNTSLEGMTSSPMESTQQSKNDMLIPLAEHDRGP EHQQDDEDNDDADIDLKKDISMQPGRRGRKPTTLATTDEWKKQR KDSHKEVERRRRENINTAINVLSDLLPVRESSKAAILACAAEYIQK LKETDEANIEKWTLQKLLSEQNASQLASANEKLQEELGNAYKEIE YMKRVLRKEGIEYEDMHTHKKQENERKSTRSDNPHEA
scUtr1	MKENDMNNGVDKWVNEEDGRNDHHNNNNNLMKKAMMNNEQI DRTQDIDNAKEMLRKISSESSSRRSSLLNKDSSLVNGNANSGGGTS INGTRGSSKSSNTHFQYASTAYGVRMLSKDISNTKVELDVENLMI VTKLNDVSLYFLTRELVEWVLVHFPRVTVYVDSELKNSKKFAAG ELCEDSKCRESRIKYWTKDFIREHDVFFDLVVTLGGDGTVLFVSSI FQRHVPPVMSFSLGSLGFLTNFKFEHFREDLPRIMNHKIKTNLRLR

	LECTIYRRHRPEVDPNTGKKICVVEKLSTHHILNEVTIDRGPSPFLS MLELYGDGSLMTVAQADGLIAATPTGSTAYSLSAGGSLVCPTVN AIALTPICPHALSFRPIILPESINLKVKVSMKSRAPAWAAFDGKDRI ELQKGDFITICASPYAFPTVEASPDEFINSISRQLNWNVREQQKSFT HILSQKNQEKYAHEANKVRNQAEPLEVIRDKYSLEADATKENNN GSDDESDDESVNCEACKLKPSSVPKPSQARFSV
scUgp1	MSTKKHTKTHSTYAFESNTNSVAASQMRNALNKLADSSKLDDAA RAKFENELDSFFTLFRRYLVEKSSRTTLEWDKIKSPNPDEVVKYEII SQQPENVSNLSKLAVLKLNGGLGTSMGCVGPKSVIEVREGNTFLD LSVRQIEYLNRQYDSDVPLLLMNSFNTDKDTEHLIKKYSANRIRIR SFNQSRFPRVYKDSLLPVPTEYDSPLDAWYPPGHGDLFESLHVSG ELDALIAQGREILFVSNGDNLGATVDLKILNHMIETGAEYIMELTD KTRADVKGGTLISYDGQVRLLEVAQVPKEHIDEFKNIRKFTNFNT NNLWINLKAVKRLIESSNLEMEIIPNQKTITRDGHEINVLQLETACG AAIRHFDGAHGVVVPRSRFLPVKTCSDLLLVKSDLFRLEHGSLKL DPSRFGPNPLIKLGSHFKKVSGFNARIPHIPKIVELDHLTITGNVFLG KDVTLRGTVIIVCSDGHKIDIPNGSILENVVVTGNLQILEH

After collecting sequences for separate fungal and mammalian DCAs, we performed a multiple sequence alignment for each of the pairwise comparisons (PAS kinase vs. Cbf1, Ugp1, Utr1, USF1, or ATXN2). Using the multiple sequence alignments, we performed DCA (see Figure 3-1 for summary of process). We then extracted the residue numbers from the multiple sequence alignment that correlated to the original residues in *S. cerevisiae* or *H. sapiens*. We made a heatmap of DCA mutual information interactions. The brightest yellow color on the heat map (with mutual information coefficients above 1.5) are indicative of the strongest interactions, and greatest likelihood of co-evolution between proteins (see Figures 3-2 and 3-3).

3.2.2 Site-Directed Mutagenesis. To decide which residues to mutate via site-directed mutagenesis, we sorted the raw data and examined the "pairings" with mutual information coefficients above 1.4. The amino acid residues that appeared most frequently with the "above 1.4" cutoff were chosen for mutagenesis. In addition, potential phosphorylation sites were identified as serines or threonines that had high mutual information coefficients, since we know that PAS kinase is a serine/threonine kinase [200]). Site-directed mutagenesis was performed

according to the QuickChange Mutagenesis protocol (StrateGene) using the primers listed in Table 3-2. The resulting plasmid DNA was sequenced to confirm mutations.

Table 3-2. Primers Used for Site-Directed Mutagenesis of PAS Kinase Substrates.

Protein	Mutation	Forward Primer
Psk1	H1161A	CGT TGA ACA AAA AAC CAG CTG AGA ATA TTT TAC GG
	I1121A	AAG AAG AAT AGG TAT GCT GTG GTG ATT AAG ATG
	E880A	CGA GAG ACG TGG TAT TTG CAG AAT ATA ATA CAA ATC C
	V841A	GGC CAT CAA TAC CCA CCG CGC ACG TAT AAA AGC
	E829A	GGA AAA TTC AGG CAG CGA TTA TGG GTG ATC G
Cbf1	P203A	CTT CCA CGA CGA CCC GCC TGC ATG CTT ATA TCC
	R205A	GCA TGC AGC CGG GTG CTC GTG GAA GAA AAC CTA C
	R252A	GAC CTC CTG CCC GTG GCA GAA TCA AGT AAG GCA G
	Q304A	GTG CAA ATG AGA AAC TGG CGG AAG AAC TGG GAA ATG C
	L320A	GTA CAT GAA ACG CGT TGC AAG GAA GGA GGG AAT AG
	I325A	TTA AGG AAG GAG GGA GCA GAA TAC GAG GAT ATG C
Utr1	T419A	ATT TTA TAA CCA TAT GCG CCG GCC CAT ATG CTT TTC CAA C
	T425A	CGG GCG AGG CTT CCA CGG CTG GAA AAG CAT ATG G
	S451A	GAG GGA ACA ACA AAA GGC CTT TAC GCA TAT TTT GTC CC
USF1	S186A	CCT AGG ACT CAC CCT TAT GCC CCG AAG TCA GAA GCT CCC

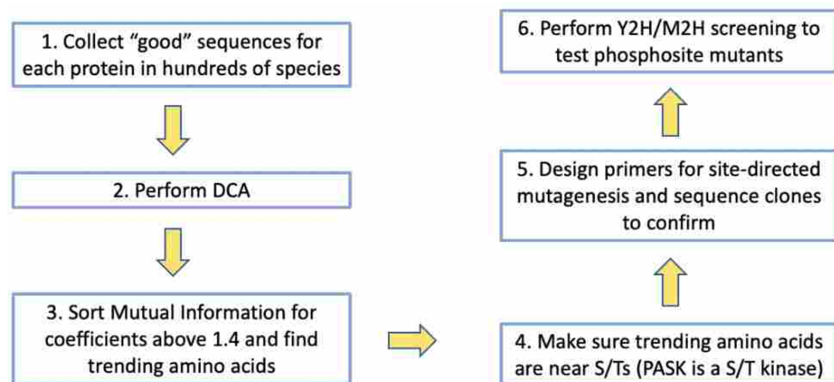

Figure 3-1. Method for Performing Direct Coupling Analysis.

3.3 Results

Direct coupling analysis was performed on yeast or mammalian PAS kinase compared with yeast Cbf1, Ugp1, Utr1, or mammalian USF1, or ATXN2, respectively. The results are provided in Figures 3-2 and 3-3. In most eukaryotic cells, only one PAS kinase can be identified. However, there are two paralogs in yeast, Psk1 and Psk2, that appear to have arisen via whole-genome duplication. A discussion of each of the substrates and the corresponding DCA results follows.

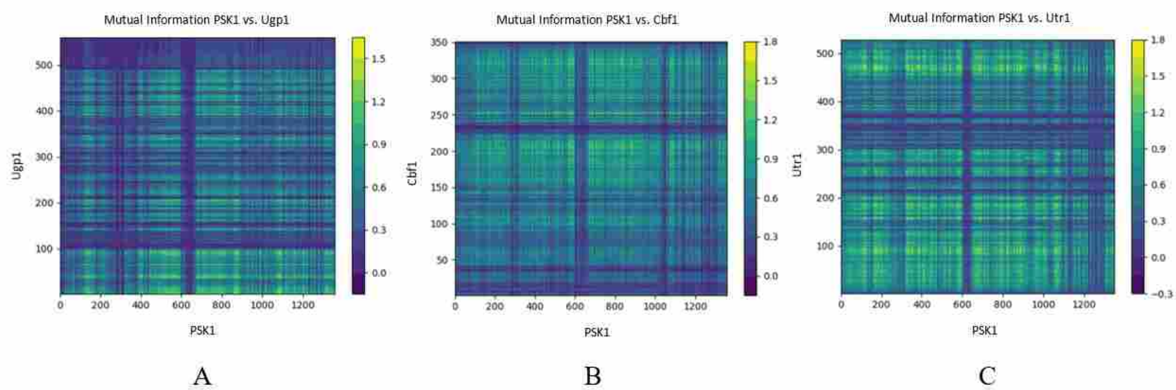

Figure 3-2. Heat Maps Showing DCA Mutual Information of S. cerevisiae Psk1 vs. Ugp1, Cbf1, or Utr1. The brightest yellow indicates a closer evolutionary relationship between the amino acid residues of the two proteins.

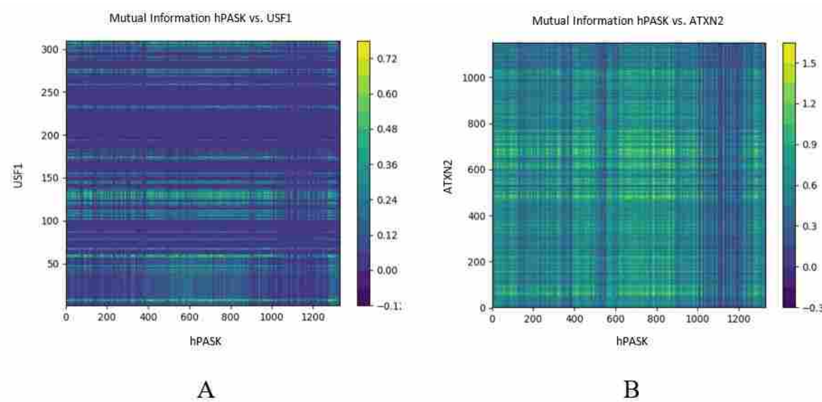

Figure 3-3. Heat Maps Showing DCA Mutual Information of H. sapiens PASK vs. USF1 or ATXN2. The brightest yellow indicates a closer evolutionary relationship between the amino acid residues of the two proteins.

3.3.1 Ugp1 and PSK. UDP-glucose pyrophosphorylase 1 (Ugp1) is the best characterized substrate of yeast PAS kinase (PSK), so it was used as a control for these studies. Ugp1 was first discovered as a substrate by Rutter, et al. in 2002 through *in vitro* kinase screens of yeast proteins [174]. It has since been shown be phosphorylated *in vivo* at Serine-11 [173]. Ugp1 phosphorylates the reversible conversion of glucose 1-phosphate and UDP-glucose to uridine triphosphate (UTP), a cellular glucose storage molecule [201]. Phosphorylation by either yeast paralog, Psk1 or Psk2, does not result in a change in enzymatic activity, but rather a change in the destination of its substrate UTP. Phosphorylation of Ugp1 results in increased cell wall components and decreased glycogen storage [179]. In contrast, PAS kinase-deficient yeast (*psk1psk2*) or yeast harboring the phosphosite mutant of Ugp1 (S11A) display increased glycogen [174] and are sensitive to cell wall perturbing agents [173]. Interestingly, the DCA results show the highest mutual association probabilities at the N-terminus of Ugp1, particularly amino acids 1-80 (Figure 3-2A, Table 3-2). These results are consistent with the known phosphorylation site at Serine-11, providing proof-of-principle. No further studies can be easily conducted with this protein to confirm important kinase-substrate interaction residues since we are unable to tag Ugp1 in any way without disrupting the PSK-Ugp1 interaction [173].

Table 3-3. Trending Amino Acids for Each Protein. The trending amino acids with the best Mutual Information (i.e. a coefficient above 1.4, or above 0.5 for USF1) were identified. The residues with the greatest Mutual Information coefficients are listed in descending order for each protein. Potential phosphosites or interaction sites were noted.

Protein	**Trending Residue #**	**Amino Acid**	***Potential* Phosphosite / Interaction Site**
Ugp1	38	S	phosphosite
	89	near S	phosphosite
	3	T	phosphosite
	206	S	phosphosite

	1	near S	phosphosite
	2	S	phosphosite
	88	Y	interaction site
	392	near S	phosphosite
	5	near S	phosphosite
	94	near S	phosphosite
Cbf1	252	near S	phosphosite
	205	near S and T	phosphosite
	321	R	interaction site
	203	near S	phosphosite
	251	near S	phosphosite
	304	E	interaction site
	320	L	interaction site
Utr1	467	E	interaction site
	90	near S and T	phosphosite
	470	K	interaction site
	475	A	interaction site
	85	near S	phosphosite
	91	T	phosphosite
	474	Q	interaction site
	86	T	phosphosite
	472	R	interaction site
	159	near T	phosphosite
USF1	7	T	phosphosite
	174	S	phosphosite
	132	T	phosphosite
	58	Q	interaction site
	129	near T	phosphosite
	276	D	interaction site
	307	near S	phosphosite
	47	V	interaction site
	59	V	interaction site
	121	S	phosphosite
ATXN2	481	near S	phosphosite
	614	near S and T	phosphosite
	684	S	phosphosite
	489	near T	phosphosite
	678	S	phosphosite
	63	near S	phosphosite
	472	T	phosphosite
	731	near S and T	phosphosite
	687	T	phosphosite
	706	V	interaction site

3.3.2 Cbf1 and PSK. Centromere binding factor 1 (Cbf1) was identified as a yeast two-hybrid partner and an *in vitro* substrate of Psk1 in 2014 by DeMille, et al. [177], and was recently characterized. Cbf1 and Psk1 strongly interacted in the yeast two-hybrid screen, which allowed us to test residues that were expected to be important for the Psk1-Cbf1 interaction. We found that Cbf1 is preferentially phosphorylated by Psk1 (only weakly by Psk2) at Threonine-212, and controls cellular respiration and lipid biosynthesis in yeast [186]. The Cbf1 residues with the highest mutual information occur around the region of phosphorylation (T212, Figure 3-2B, Table 3-3), once again confirming the validity of DCA as a method of determining potential interaction sites between proteins.

3.3.3 Utr1 and PSK. NAD kinase (Utr1) was identified as a yeast two-hybrid partner and an *in vitro* substrate of Psk1 in 2014 by DeMille et al. [177], and is the least characterized substrate used in this analysis. The DCA results suggest the region of phosphorylation is near amino acid 480, or amino acid 90 (both are regions with high mutual association coefficients). We performed mass spectrometry analysis of Utr1 incubated with PAS kinase, which identified two putative phosphorylation sites: Threonine-419 and Threonine-425 (unpublished data). Although protein kinase substrates do not always obey a consensus, there is also one putative phosphorylation site matching the PAS kinase phosphorylation consensus (Strong preference for R/H/K at -3 and/or -5 position, some preference for S/T/C/P/N/Q at the -2 position [19]) in this region – Serine 451 (REQQKS*). These constructs have been made and will be tested via *in vitro* kinase assay for their validity.

3.3.4 USF1 and PASK. Upstream Stimulatory Factor 1 (USF1) is the human ortholog of yeast Cbf1, which has been shown to be a substrate of yeast PAS kinase. USF1 has been associated with hyperlipidemia in several Genome-Wide Association Studies (GWAS) [202-

204]. Thus, the regulation of USF1 by PAS kinase may explain the dramatic decrease in triglyceride accumulation observed in PAS kinase-deficient mice [182]. In support of the conservation of regulation and function, human USF1 expression can complement the respiration defect observed in CBF1-deficient yeast and appears to be regulated by PAS kinase in yeast [186]. In addition, human PAS kinase can phosphorylate USF1 *in vitro*. To test this association, as well as aid in phosphosite mapping, the evolutionary association of USF1 and PAS kinase was explored through DCA (Figure 3-3A). Although the overall association plot is much darker than other substrates, there is a clear association in the region around amino acid 130. Interestingly, when searching for PAS kinase consensus sequences, there is an excellent match at amino acid 186 (RTHPYS*), a smaller, but also bright, nearby region on the DCA heat map. This amino acid has been mutated to an alanine (S11A) and will be tested as a putative phosphosite in the *in vitro* kinase assays.

3.3.5 ATXN2 and PASK. There is also preliminary data to support Ataxin-2 (ATXN2) as a substrate of mammalian PAS kinase. Human mutations in Ataxin-2 are associated with Amyotrophic Lateral Sclerosis (ALS), a devastating neurodegenerative disease that affects nerves of the brain and spinal cord. The yeast ortholog of ATXN2 (Poly(A)- Binding Protein, or Pbp1) was first identified as a yeast two-hybrid partner and *in vitro* substrate of Psk1 in 2014 by DeMille et al. [177], and was subsequently shown to be activated by PAS kinase *in vivo* [15]. We have recently demonstrated the ability of human ATXN2 to complement yeast Pbp1 and have provided evidence for the regulation of ATXN2 by PAS kinase in yeast. In addition, human PAS kinase can phosphorylate ATXN2 *in vitro*. Despite the clear importance of this substrate, we have been unable to map the critical phosphorylation site by mass spectrometry; over 10 sites were identified, but none appeared to be the critical site for PAS kinase

phosphorylation (unpublished data). This may be due to the presence of other kinases that phosphorylate Pbp1, as we have previously provided evidence for these unknown kinases [205]. We therefore attempted to perform DCA on both the yeast proteins (Psk1 and Pbp1) as well as their human orthologs (PASK and ATXN2). The abundance of Pbp1 orthologs (which lacked a matching organism for PSK1) made the analysis of the fungi difficult, however, we were able to get data on the mammalian orthologs. As seen in Figure 3-3B, there are multiple regions of association between these proteins with two key regions around amino acids 480 and 615 of ATXN2 (Figure 3-4). There are PAS kinase consensus sequences near each of these two regions. There is a consensus sequence at Serine 479 (VQRNSS*) and a consensus at 617 (PKRMSS*), both of which are currently being mutated to check for their requirement through *in vitro* kinase assays. A double mutant with both mutations is also being constructed, since this dual phosphorylation may be the reason for the abundance of phosphorylation events seen by mass spectrometry.

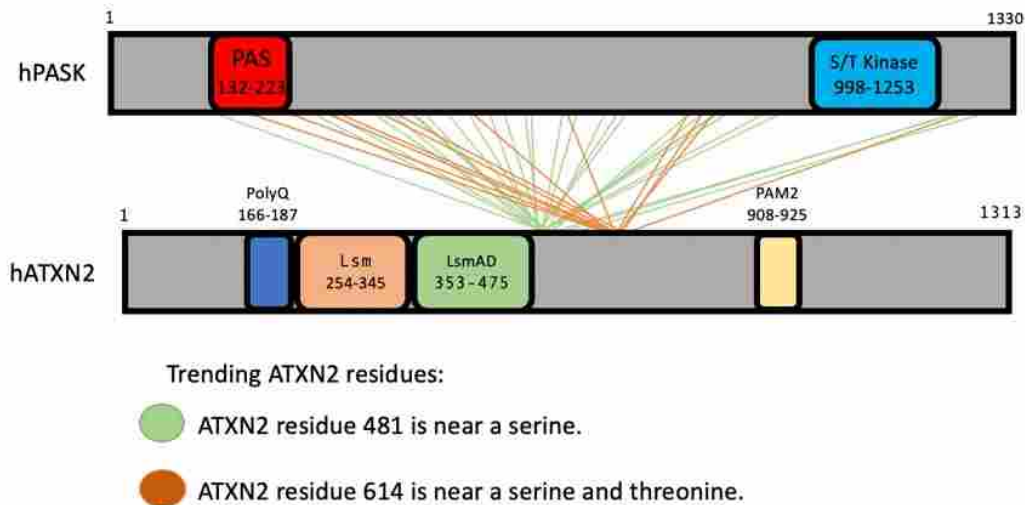

Figure 3-4. A Diagram Depicting the hATXN2 and hPASK Trending Amino Acids.

3.3.6 PASK and its substrates. In addition to identifying critical substrate residues for PAS kinase-substrate interactions, our DCA results also suggest regions of PAS kinase that may be important for substrate recognition. PAS kinase contains a regulatory PAS domain, which is thought to bind the kinase domain and inhibit its activity (Figure 3-5). When a small molecule binds the PAS domain or PAS kinase is phosphorylated, it disrupts the interaction between the PAS and kinase domains, thus activating PAS kinase and allowing it to phosphorylate its substrates. Most protein kinases that function in larger complexes are composed of regulatory subunits and/or substrate-recognition subunits, in addition to the catalytic kinase subunit. We hypothesize that the large middle region of PAS kinase may function as the substrate recognition module of PAS kinase. Our DCA results support this hypothesis, since the residues with the greatest mutual information values are located between the PAS and kinase domains (for example, see Figure 3-4). To confirm this hypothesis, we have mutated several of these residues in Psk1 and will test the ability of PAS kinase to recognize and bind its substrates via the yeast two-hybrid.

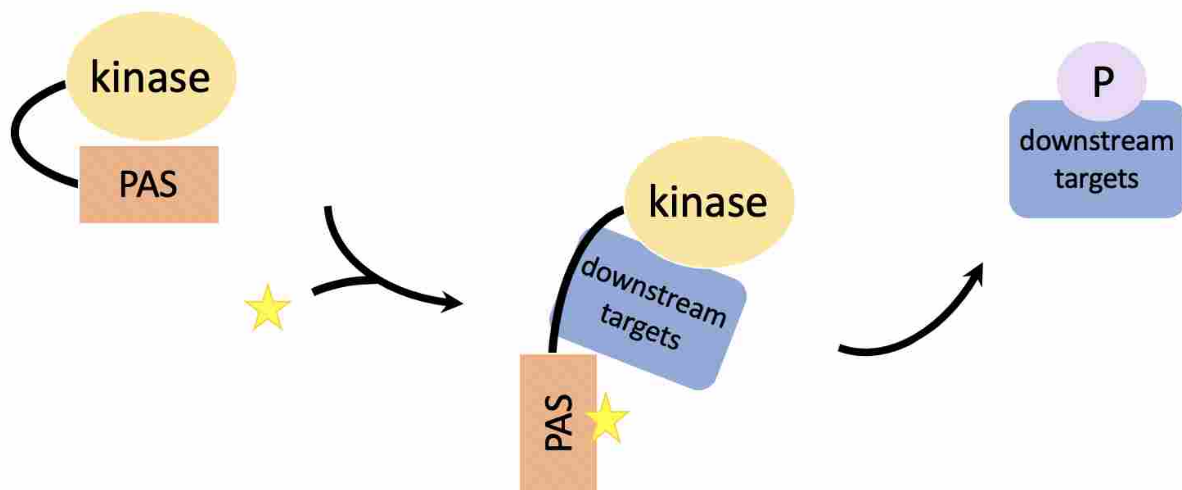

Figure 3-5. Activation of PAS Kinase. A small metabolite binds the PAS domain or PAS kinase is phosphorylated, disrupting the interaction between the PAS and kinase domains, thus activating PAS kinase and allowing it to phosphorylate its substrates.

3.4 Conclusions

By exploring the interactions of Per-Arnt-Sim (PAS) kinase with several of its substrates via Direct Coupling Analysis (DCA) and subsequent kinase assays, it will allow us to determine important phosphosites of the substrates, as well as critical substrate recognition sites on PAS kinase. We have already identified several sites of interest in our data, generated several constructs, and we will be testing the interactions between PAS kinase and yeast Cbf1, Utr1, Ugp1, and mammalian USF1 or ATXN2. As we understand more about PAS kinase and the proteins and pathways that it regulates, we could potentially find new therapeutic treatment targets for metabolic diseases, such as: ALS, diabetes, hyperlipidemia, hypercholesterolemia, or cancer.

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