Characterizing Interaction Between PASK and PBP1/ATXN2 to Regulate Cell Growth and Proliferation

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ABSTRACT

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Pbp1 is a component of glucose deprivation induced stress granules and is involved in P-body-dependent granule assembly. We have recently shown that Pbp1 plays an important role in the interplay between three sensory protein kinases in yeast: AMP-regulated kinase (Snf1 in yeast), PAS kinase 1 (Psk1 in yeast), and the target of rapamycin complex 1 (TORC1), to regulate glucose allocation during nutrient depletion. This signaling cascade occurs through the SNF1-dependent phosphorylation and activation of Psk1, which phosphorylates and activates poly(A)-binding protein binding protein 1 (Pbp1), which then inhibits TORC1 through sequestration at stress granules. In this study we further characterized the regulation of Pbp1 by PAS kinase through the characterization of the role of the Psk1 homolog (Psk2) in Pbp1 regulation, and the identification of functional Pbp1 binding partners. Human ataxin-2 (ATXN2) is the homolog of yeast Pbp1 and has been shown to play an important role in the development of several ataxias. In this study we have also provided the evidence that human ataxin-2 can complement Pbp1 in yeast, and that human PAS kinase can phosphorylate human ataxin-2. Further characterizing this interplay between PAS kinase and Pbp1/ATXN2 aid in understanding pathways required for proper glucose allocation during nutrient depletion, including reducing cell growth and proliferation when energy is low. In addition, it yields valuable insights into the role of ataxin-2 in the development of devastating ataxias.

Keywords: nutrient sensing kinases, AMP regulated kinase (AMPK), PAS kinase (PSK or PASK), Target of Rapamycin (TOR), Pbp1, Ataxin-2 (ATXN2)
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SPECIFIC AIMS

1. To determine whether the pathway between PAS kinase, Pbp1 and TORC1 is conserved in yeast.
   A) To determine whether the human homolog of Pbp1 can complement yeast Pbp1 in the inhibition of TORC1.
   B) To determine whether human PAS kinase can directly phosphorylate ataxin-2 using in vitro kinase assays.

2. To further characterize the regulation of Pbp1 by PAS kinase in yeast.
   A) Yeast has two homologs of PAS kinase, Psk1 and Psk2. The role of Psk2 in Pbp1 regulation will be explored through phenotypic assays.
   B) To determine whether human PAS kinase 2 can directly phosphorylates Pbp1 using in vitro kinase assays.
   C) To characterize the PAS kinase-phosphorylation of Pbp1 through phosphosite mapping and mutagenesis.

3. To define the function of Pbp1, and hence PAS kinase, through identifying the binding partners of Pbp1 using yeast 2-hybrid method.
1 INTRODUCTION

Eukaryotic cell proliferation is controlled by growth factors, hormones, and/or the availability of nutrients. In the absence of a corresponding proliferation signal, cells may enter into a specific, reduced growth and proliferation state, generally associated with increased resistance to various environmental stresses. An essential element of biological adaptation to changing environmental conditions is the use of sensory proteins that transduce various stimuli into changes in the activity of downstream signaling pathways [1].

In the yeast *Saccharomyces cerevisiae*, cell growth and proliferation is regulated by the availability of nutrients through nutrient sensing enzymes that sense and distribute nutrients properly. Nutrient-sensing kinases maintain metabolic homeostasis by allocating cellular resources in response to nutrient status. This allows organisms to adapt to the circumstances of the environment, being able to switch between energy consumption pathways to storage pathways. The ability to control multiple central metabolic pathways has made nutrient sensing kinases the target of many therapeutic approaches [1, 2]. Three of these nutrient sensing kinases are the AMP-regulated kinase (AMPK or Snf1), PAS kinase, and the target of rapamycin (TOR) each playing a unique role in sensing and utilizing nutrients in the cell.

1.1 AMPK or Snf1

The AMP-activated protein kinase (AMPK) is a highly conserved kinase complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits [3]. AMPK is a highly conserved sensor of cellular energy status that is activated under conditions of low intracellular ATP. When energy status is compromised, the system activates catabolic pathways and switches off protein, carbohydrate and lipid biosynthesis, as well as cell growth and proliferation [4].
AMPK was first known for its role in regulating fatty acid and cholesterol synthesis. AMPK was discovered as an inhibitor of Acetyl-CoA carboxylase, the rate limiting enzyme in long chain fatty acid synthesis [6]. It was later found that this inhibition occurred with low ATP availability [7], with AMPK activated when the AMP to ATP ratio is high. AMP directly binds to the AMPK complex and allosterically activates it [44]. AMPK responds to energy stress by suppressing cell growth and biosynthetic processes, in part through its inhibition of the rapamycin-sensitive mTOR (mTORC1) pathway in mammalian cells [43]. Recent studies indicate that the AMPK system is also important in functions that go beyond the regulation of energy homeostasis, such as the maintenance of cell polarity in epithelial cells [5].

Snf1 is a homologue of AMPK in *Saccharomyces cerevisiae* and is essential for responses to glucose starvation as well as for other cellular processes. Snf1 functions similar to AMPK in that it activates respiration, and decreases fatty acid biosynthesis in yeast [9]. The Snf1 complex acts primarily by inducing expression of genes required for catabolic pathways that generate glucose, probably by triggering phosphorylation of transcription factors [8]. Snf1 kinase activity allows for the expression of genes, including those required for respiration, normally repressed when glucose is readily available. Thus, Snf1 mutants in yeast lose their ability to grow on non-fermenting carbon sources such as sucrose [10]. In contrast to mammalian AMPK, Snf1 seems to be activated by a ligand other than AMP.

1.2 Target of Rapamycin (TOR)

TOR is a nutrient sensing kinase that is activated under nutrient rich conditions [22]. TOR can form two complexes, TORC1 and TORC2 [23]. TORC1 is sensitive to rapamycin. The core components of TORC1 include tor1, tor2, Lst8 and Kog1 that control protein synthesis in response to growth factors and nutrients [25, 27]. TORC2 is core components include rapamycin
insensitive protein tor2, Sin1, PRR5, PRR5L and lst8. TORC2 regulates cell survival, cell cycle progression, and metabolism by phosphorylating and activating other effector kinases. TORC2 is also involved in actin organization during cell division [24].

TORC1 is activated during times of nutrient abundance, such as high amino acid concentrations, high glucose availability or the presence of growth factors and activates ribosomal translation. TORC1 can phosphorylate two proteins that modulate components of the protein translation machinery. TORC1 phosphorylates S6 kinase (S6K). S6K in turn phosphorylates ribosomal protein S6 and thus increases cell growth [28]. The second link between TORC1 and protein synthesis is TORC1 can phosphorylate eukaryotic initiation factor (eIF) 4E binding proteins (4E-BPs). When 4E-BP is phosphorylated it prevents the mechanism of translation [50]. TORC1 can also upregulates lipid biosynthesis to allow for cell growth [51]. In summary TORC1 is activated in the presence of nutrient rich conditions and promotes cell growth and proliferation via protein, ribosome and lipid biosynthesis.

TORC1 signaling upon heat stress is regulated by stress granules (SGs), which are cytoplasmic foci formed under certain stresses [22]. Ectopic formation of SGs achieved by Pbp1 overexpression in unstressed cells sequesters TORC1 in this compartment, thereby blunting TORC1 signaling [52]. Upon heat stress, a physiological SG-inducing condition, TORC1 is also recruited to SGs, which delays reactivation of TORC1 signaling during recovery from heat stress. Moreover, TORC1 reactivation is directed through SG disassembly, suggesting that SGs act as a key determinant for TORC1 reactivation during recovery from heat stress [22]. Furthermore, this mechanism contributes to reduction of heat-induced mutations. Thus, TORC1 signaling is coupled to heat-induced SGs to protect cells from DNA damage [26].
### 1.3 PAS kinase

PAS kinase is a recently discovered sensory protein kinase. PAS kinase is highly conserved from yeast to man [11]. 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 [12, 13] while the kinase domain regulates other proteins through phosphorylation. The PAS domain binds the kinase domain to inhibit its catalytic activity [45]. PAS kinase has been shown to be involved in the regulation of glucose homeostasis in both yeast and mice [14].

PAS kinase is important in the allocation of glucose in the cell. PAS kinase-deficient mice (PASK-/-) have an increased metabolic rate and are resistant to weight gain, liver triglyceride accumulation and insulin resistance; when placed on a high-fat diet [15]. There is no significant difference between PAS kinase deficient mice and wild type in their food intake or activity levels; instead PAS kinase deficient mice appear to be hyper-metabolic, with more energy being released as heat [15]. Also, PAS kinase deficiency in mice appears to confer a protective effect against the development of type II diabetes, and human mutations in PAS kinase (hPASK) have been found that lead to development of maturity-onset diabetes in the young (MODY) [49].

A *psk1psk2* mutant yeast strain accumulates excess carbohydrate and shows a reduction in glucans necessary for cell wall biosynthesis [46]. In addition, the cells have an impaired ability to grow on galactose. PAS kinase is able to phosphorylate UDP-glucose pyrophosphorylase (*Ugp1*) and glycogen synthase [14]. *Ugp1* is the major producer of UDP-glucose, which can be used for glycogen synthesis and cell wall synthesis [47]. When PAS kinase phosphorylates *Ugp1* it is believed to change its cellular location, from the cytoplasm to
the cell periphery, which results in the donation of glucose to cell wall components at the expense of glycogen formation [46]. Human PAS kinase was also found to phosphorylate mammalian glycogen synthase and inhibit glycogen synthase activity [48].

In addition to its role in glycogen accumulation, PAS kinase has also been shown to regulate respiration in yeast through the phosphorylation and inactivation of Cbf1 [49]. In this large-scale study, 93 binding partners were identified for yeast PAS kinase 1 (Psk1), and four were shown to be in vitro substrates of PAS kinase, namely Cbf1, Pbp1, Zds1 and Utr1. Cbf1 is a transcription factor that regulates respiration as well as lipid and amino acid biosynthesis, and is inhibited by Psk1-dependent phosphorylation. Pbp1, or Pab1p - binding protein, is activated by PAS kinase [52]. It is known to function by regulating mRNA tail length, localizing to stress granules, and inhibiting TORC1 through sequestration at these granules [22]. Thus we see that PAS kinase plays an important role in coordinating a metabolic response in yeast, mice and humans.

TOR and PAS kinase proteins also define key signaling pathways that control cell proliferation in response to growth factors and/or nutrients. In yeast, inactivation of PAS kinase and/or TOR causes cells to arrest growth in early G1 and induces a program that is characteristic of G0 cells [16]. Interestingly, PAS kinase was found to phosphorylates Cap-Associated Factor 20 (Caf20) which is a part of eukaryotic initiation factor 4E (eIF4E) complex and negatively regulates translation by blocking assembly of the translation initiation complex [17, 18]. PAS kinase can also phosphorylate Tif1, which is the yeast eukaryotic translation initiation factor 1A (eIF1A) [19]. eIF1A transfers Met-tRNA to the 40S preinitiation complex [20]. These data indicate that PAS kinase may, like TOR, regulate protein synthesis through phosphorylation of downstream effectors, but the significance of its effect on translation has not been defined [21].
1.4 Crosstalk between nutrient sensing enzymes

PAS kinase is activated by Metabolic and Cell Integrity Stress. Interestingly, Snf1 was found to be required for activation of PAS kinase under stressing conditions [49], PAS kinase and Snf1 actives under glucose deprivation [17]. Another interesting observation was that the yeast orthologs \textit{Psk1} and \textit{Psk2} play a different role in response to the two stimuli, with Psk1 having the major role in response to metabolic stress [17].

In yeast the interplay between three sensory protein kinases, AMP-regulated kinase (AMPK, or Snf1 in yeast), PAS kinase 1 (Psk1 in yeast), and the target of rapamycin complex 1 (TORC1), plays an important role to regulated cell growth and proliferation when energy is low. This signaling cascade occurs through the SNF1-dependent phosphorylation and activation of Psk1, which phosphorylates and activates Pab1p - binding protein 1 (Pbp1), which then inhibits TORC1 through sequestration at stress granules [52].

1.5 Pbp1

An important part of the cellular response to stress or environmental stimuli is the modulation of cytoplasmic mRNA translation and degradation. One aspect of this process in eukaryotic cells is the remodeling of translating mRNAs into non-translating mRNPs that accumulate in cytoplasmic foci known as stress granules and P-bodies to allow for storage and decay of mRNA [29]. Stress granules arise when translation initiation is strongly inhibited, such as during different environmental stresses or drug-induced translational repression, and contain translationally inactive mRNA and translation initiation factors [30]. Understanding the composition and formation of these two granules will provide insight into how mRNA fate is controlled during stress and during normal growth. While the function of these large aggregates is not entirely known, their composition provides some clues as to their roles.
Recent studies in both yeast and mammalian cells implicate a role for Pbp1 (Pab1-binding protein) and Ataxin-2 (mammalian homolog of Ppb1), in the assembly of stress granules. Deletion of Pbp1 in yeast or siRNA knockdown of Ataxin-2 in mammalian cells leads to significant decreases in stress granule formation under glucose deprivation or arsenite treatment, respectively [31, 32]. These results suggest that Pbp1 and its interacting proteins may localize to and play roles in stress granule assembly. Pbp1 interacts with some interesting proteins with possible roles in the control of cytoplasmic mRNA function. Pbp1 was identified by an interaction with the C-terminal domain of Pab1, and was found to exist with both the translating and nontranslating pools of mRNA [33, 34]. Pbp1 also interacts with the Pbp4 and Lsm12 proteins, and these three proteins all associate with the translation machinery [35, 36]. Moreover, a physical interaction between Pbp1 and Dhh has been demonstrated by a protein-fragment complementation assay, as well as a physical interaction between Dhh1 and Lsm12 [37].

It has been shown that overexpression of Pbp1, a poly(A)-binding protein (Pab1)-binding protein I and a component of stress granules (SGs) [38], downregulates TORC1 by inducing SG formation, where TORC1 is sequestered [22]. Since, Pbp1 is activated by PAS kinase, and PAS kinase phosphorylates Pbp1, Pbp1 plays a critical role in the interplay between three sensory protein kinases in yeast to regulate glucose allocation during nutrient depletion. Although Pbp1 signaling is elevated by various stresses in yeast, the underlying mechanisms remain elusive.

### ATXN2

Human ataxin-2 (ATXN2) is a homolog of yeast Pbp1. This gene is a product of the spinocerebellar ataxia type 2 gene (SCA2)[39, 40, 41]. Spinocerebellar ataxia type 2 (SCA2) is a member of the growing group of familial neurodegenerative diseases characterized by unstable CAG repeats which are expanded in patients. The trinucleotide repeat encodes a polyglutamine...
stretch in the N-terminal region of ATXN2. This stretch is expanded in SCA2 patients and confers an increased propensity towards functional defects leading to ataxia and ultimately death [42]. The mechanism, however, by which the expansion triggers neuronal cell death, has remained unclear [53].

The causes of amyotrophic lateral sclerosis (ALS), a devastating human neurodegenerative disease, are poorly understood, although the protein TDP-43 has been suggested to have a critical role in disease pathogenesis. Scientists have shown that ataxin-2 (ATXN2), a polyglutamine (polyQ) protein mutated in spinocerebellar ataxia type 2, is a potent modifier of TDP-43 toxicity in animal and cellular models [54]. ATXN2 and TDP-43 associate in a complex that depends on RNA. In spinal cord neurons of ALS patients, ATXN2 is abnormally localized; likewise, TDP-43 shows mislocalization in spinocerebellar ataxia type 2.

Figure 1-1: Domain architecture of human ATXN2 and its yeast homolog Pbp1.

In summary, mutations in ATXN2 are strongly associated with ataxias, yet little is known about the function of ATXN2. Thus, as ATXN2 functions similar to Pbp1, our studies may help us understand why human mutations in this protein lead to ataxia. In support of conserved roles for Pbp1 and ATXN2, our study shows that ATXN2 can actually complement Pbp1 phenotype in
yeast cells. This provides an incredibly powerful tool to study human mutations using yeast as a model organism.

The proposed study aims to investigate another level of cross talk between PAS kinase and Pbp1/ATXN2. Preliminary data suggests that PAS kinase phosphorylates and activates Pbp1. This study will further our understanding on how PAS kinase interacts with Pbp1. It will also provide an understanding of how Pbp1 and ATXN2 function, emphasizing the effects of PAS kinase on Pbp1 and ATXN2 function.
2 BACKGROUND AND SIGNIFICANCE

In yeast, Psk1 is activated in a SNF1-dependent manner in response to energy/nutrient deprivation [17]. Phosphorylation activates PAS kinase by interfering with inhibitory binding of the PAS domain. Once activated, Psk1 phosphorylates Pbp1 [52], which sequester TORC1 to stress granules, inhibiting cell growth [22].

Figure 2-1: A model for the cross-talk between the nutrient-sensing kinases SNF1, TORC1, and Psk1.

The fact that overexpression of Pbp1 leads to apparent growth inhibition by inhibition of TORC1, makes it an efficient target of anti-cancer therapies. Therefore identifying the molecular mechanism behind Pbp1 activation and its function will increase our understanding of glucose regulation, and cell growth and proliferation.
Human ATXN2 is a homolog of yeast Pbp1. The molecular functions of ATXN2 have not been understood to date. However, some evidence provided by studying ATXN2 homologs from yeast, *C. elegans*, and *D. melanogaster*, indicates that they may be involved in RNA-processing pathways [53, 54, 55]. In this study, we will also further characterize the role of ATXN2. In addition, we will investigate whether PAS kinase also phosphorylates ATXN2. This study will increase our understanding of the nutrient sensing kinase PAS kinase, and its role in regulating basic cellular metabolism. Therefore, discovering the molecular mechanisms behind PAS kinase function is essential in that it will not only increase our understanding for the treatment of metabolic disease, but contribute to our basic understanding of metabolic regulation in cells. In addition, the proposed research project could help us understood the molecular mechanisms behind neuronal death reported in ATXN2-associated ataxia. If PAS kinase regulates ATXN2, it may be a therapeutic target in the treatment of ataxias, a disease with few successful treatment options.
3 MATERIALS AND METHODS

3.1 Growth assays

Lists of strains, plasmids, and primers used in this study are provided in APPENDIX A. For plasmid construction, standard PCR-based cloning methods were used. All restriction enzymes were purchased from New England BioLabs (Ipswich, MA).

Yeast two-hybrid bait plasmid was made by PCR amplification of Pbp1 and subsequent cloning into the EcoRI/SalI sites of pGBD-C1 yeast two-hybrid Gal4 bait vector (pJG424)[57] (pJG1386 [JG2916/3163]). Yeast two-hybrid libraries are described in an earlier work [49]. Yeast two-hybrid Gold cells (Clontech, Mountain View, CA) were used to transform in bait and prey plasmids for interaction studies.

For serial dilution growth assays, spot-dilutions were performed by growing yeast in overnight liquid culture, serially diluted 1:10 in water and spotted on selective plates as well as control plates. Plates were incubated at 30°C for 7–10 d until colonies were apparent.

3.2 Histidine- and Myc-tagged protein purification

Yeast harboring plasmids for 6X histidine (HIS) or Myc-tagged protein expression were grown in either SD-Ura media overnight, diluted 1:100-fold into 100 or 250 ml of SD-Ura, and grown for 10–12 h, pelleted, and resuspended in 500 ml of SGal-Ura for 36 h to induce expression under the GAL1-10 promoter. Yeast were pelleted and flash frozen at -80°C. Yeast were then resuspended in lysis buffer for HIS-tag purification (50 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid [HEPES], 300 mM NaCl, 20 mM imidazole, 10 mM KCl, 1 mM β-mercaptoethanol, and complete Protease Inhibitor Cocktail Tablet [Roche], pH 7.8, with phosphatase inhibitors, 50 mMNaF, and glycerophosphate when necessary) or for Myc purification (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, 10%
glycerol, 1 mM β-mercaptoethanol, complete Protease Inhibitor Cocktail Tablet, pH 7.4, with phosphatase inhibitors when necessary). Resuspended cultures were lysed using the Microfluidics M-110P homogenizer (Microfluidics, Westwood, MA) for 500-ml cultures or bead blasted for 1 min, followed by 1 min on ice repeated three times using 0.2-mm glass beads for small cultures (100 to 250 ml). Cell debris were then pelleted at 12,000 rpm for 20–30 min. Supernates were transferred to new tubes and incubated with either 200 μl of nickel-nitrilotriacetic acid (Ni-NTA, Qiagen, Valencia, CA) agarose beads for 500-ml HIS purification, or 5–10 μl of Myc-conjugated magnetic beads (Cell Signaling, Danvers, MA) for Myc purification, for 2–3 h at 4°C. For HIS-epitope purification, beads were washed twice with 15 ml of lysis buffer and then transferred to a polypropylene column and washed with 30–50 ml of lysis buffer. For Myc-protein purification, beads were separated using magnetic force and washed four times with 500 μl of lysis buffer. HIS-tagged proteins were eluted three times with 0.3 ml of Elution Buffer (lysis buffer containing 270 mM imidazole, 10 M KCl and 50 mM NaCl pH 7.8). Beads containing Myc-tagged proteins were used directly for in vitro kinase assays without eluting.

3.3 **Pbp1 PAS kinase in vitro kinase assay**

Psk1 (pJG858), Psk2 (pJG173), and Psk2-KD (pJG174) was HIS purified from psk1 psk2 yeast (JGY4). Myc-tagged Pbp1 was purified from psk1 psk2 (JGY4) yeast. Purified ∆N419Pbp1-Myc–tagged proteins were assayed for PAS kinase–dependent phosphorylation by incubating purified protein in 30 μl of reaction buffer containing 1× PAS kinase buffer (0.4 M HEPES, 0.1 M KCl, 5 mM MgCl2, pH 7.0), 0.2 mM ATP, 32P-ATP [5 μCi; MP Biomedicals, Santa Ana, CA]) in the presence or absence of HIS purified full-length Psk2 or Psk1, or kinase-dead Psk2-K870R. Kinase assays were started with the addition of PAS kinase and stopped with
SDS–PAGE sample buffer. Reactions were incubated for 12 min at 30°C, separated on SDS-PAGE gels, stained with Coomassie Blue and dried. The protein gels were then incubated on film overnight, and visualized the next day. By developing film using kinase dead PAS kinase 2 to check for auto-phosphorylation of Pbp1.

### 3.4 ATXN2 hPAS kinase in vitro kinase assay

ΔN544ATXN2 was purified from psk1psk2 yeast (JGY4) using HIS-tag purification. Purified ATXN2 protein was assayed for human PAS kinase–dependent phosphorylation by incubating purified ΔN544ATXN2 protein in 30μl of reaction buffer (0.4 M HEPES, 0.1 M KCl, 5 mM MgCl2, pH 7.0), 1mM ATP, 32P-ATP [5 μCi; MP Biomedicals, Santa Ana, CA] with or without purified human PAS kinase. Samples were inculcated at 30 min at 30°C. Kinase assays were started with the addition of human PAS kinase and stopped with SDS–PAGE sample buffer. Reactions were separated on 10% SDS polyacrylamide gels and proteins were stained with Coomassie Blue and dried. The protein gels were then exposed to film for 6 hours and visualized on film. GAPDH was used to check non-specific phosphorylation of human PAS kinase, and substrate or kinase alone were used to check for auto-phosphorylation.

### 3.5 Pbp1 phosphosite mutants

Three sets of multiple site-directed mutations were incorporated in Pbp1 using the StrategeneQuikChange kit and cloned into a HIS expression vector under the GAL1-10 promoter by PCR amplification of Pbp1 with primers JG2916/2917 and cloning into the EcoRI/XhoI sites of pJG859. Plasmids and mutations (designated by amino acid change) are shown in Table 3-1. All mutations were confirmed by sequencing and the encoded proteins were subjected to in vivo kinase assays.
Table 3-1: Phosphosite mutants of Pbp1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation in amino acid</th>
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3.6 In vivo kinase assay

HIS-tagged Pbp1 (pJG1560) was expressed in WT yeast (JGY1) overexpressing full-length Psk1 on a plasmid (pJG9) or in psk1psk2 yeast (JGY4) containing an empty vector (pJG124). Phospho-site mutants of Pbp1 (pJG1561, pJG1562 and pJG1563) were expressed in JGY1 (WT) overexpressing full-length Psk1 on a plasmid (pJG9). Proteins were grown in duplicate in SD-Ura-Trp overnight, induced with SGal-Ura-Trp for 36 h, and purified on Ni-NTA as described above. Eluates were run on 10% SDS–PAGE, transferred to nitrocellulose membrane, and incubated overnight with nonspecific PhosphoThreonine antibody (Cell Signaling Technology). Blots were imaged, stripped by incubating in striping reagent (6.75mM Tris, 2% SDS, and 10mM β-mercaptoethanol) for 30 minutes at 50ºC followed by 10x wash in TBST buffer for 5 minutes, and then incubated overnight with PhosphoSerine (Q5; Qiagen) antibody. Total Pbp1 was assessed by stripping the membrane once more, incubating overnight with anti-HIS antibody (Cell Signaling Technology), and imaging.

3.7 Yeast 2-Hybrid Screening

Yeast 2-hybrid system were used to look for a protein-protein interaction. The yeast 2-hybrid modifies the transcription factor for the Gal4 promoter. The transcription factor has a DNA binding domain and an activation domain that recruits RNA polymerase. The yeast 2-
hybrid system cleaves the activation and binding domain and fuses them with a protein that will either serve as the bait (bound to binding domain) or prey (bound to activation domain). When the two proteins interact in the cell it now allows for the Gal4 transcription factor binding domain and activation domain to become close enough to each other to recruit RNA polymerase to the promoter region and allow for expression of the reporter genes [63]. Pbp1 was cloned into binding domain (pJG1386) and the library was previously cloned into activation domain [49] (pJG428, pJG429, JGY1079 and JGY1098). Yeast containing both plasmids were selectively grown on SD-Leu-Trp. Colonies were then streaked onto selective media SD-Leu-Trp-His-Ade, where growth indicates a protein-protein interaction due to expression of reporter genes.

**Yeast 2-Hybrid screening by mating**

For cDNA library screens, α haploid yeast harboring a cDNA Y2H prey library (JGY1074 and JGY1098) [49] was mated to a haploid yeast harboring ΔN419Pbp1 Bait (JGY1383) in 2X YPAD for 24 hours at @ 30°C. Yeast cells where then pelleted and diluted using 10mL of SD-Leu-Trp media followed by plating 100uL on 100 SD-Leu-Trp-His-Ade plates.

**Yeast 2-Hybrid screening by transformation**

For genomic library screens, the Y2HGold (Clontech) strain bearing ΔN419Pbp1 Bait (JGY1383) was transformed with genomic libraries (pJG428, or pJG429) obtained from David Stillman, University of Utah [57]. Yeast cells where then pelleted and diluted using 10mL of SD-Leu-Trp media followed by plating 100uL on 100 SD-Leu-Trp-His-Ade plates.

**3.8 Colony check and Dependency assay**

Colonies that arose on Y2H selection plates (SD-Leu-Trp-His-Ade) were again patched on SD-Leu-Trp-His-Ade plates for validation. The library plasmid inserts were then identified by colony PCR with subsequent sequencing (Brigham Young University DNA Sequencing Center)
and National Center for Biotechnology Information (NCBI) BLAST [64] analysis (an unambiguous hit with e-value of $\geq 10^{-45}$). For verification and elimination of false positives, library plasmids were purified from yeast [58], amplified in *Escherichia coli* (GenElute Plasmid Mini-prep Kit, Sigma-Aldrich, St. Louis, MO), and transformed into Clontech Matchmaker Gold yeast with either the Pbp1 bait plasmid (pJG1386) or the empty bait plasmid (pJG424). Colonies arising on the SD-Leu-Trp transformation plates were then streaked onto SD-Leu-Trp-His-Ade in duplicate and allowed to grow for 3–5 d to test for Pbp1 dependence. The strength of growth was determined by comparing growth of yeast on SD-Leu-Trp-His-Ade plates, which is an indication of the interaction strength (S, strong; M, medium; W, weak).
4 RESULTS

4.1 Complementation of Pbp1 phenotype by ATXN2 in yeast

We previously provided evidence for the PAS kinase-dependent phosphorylation of Pbp1 [52], which then sequesters TORC1 into stress granules, inhibiting growth and proliferation [22]. ATXN2 is the human homologue of yeast Pbp1 [26] and both ATXN2 and Pbp1 have conserved Lsm, LsmAD and self-interacting domains [60] (See Figure 1-1). The self interacting region of Pbp1 gets phosphorylated by PAS kinase [52]. To test whether ATXN2 could complement Pbp1 function in its ability to reduce cell growth and proliferation under stress by sequestering TORC1 in to stress granules, three truncation of ATXN2 (∆N379ATXN2, ∆N544ATXN2 and ∆N700ATXN2) (Figure 4-1) were cloned into a HIS-tagged yeast expression vector, transformed into *pbp1* yeast and grown on selective media containing caffeine to check for a TORC1 phenotype. ∆N544ATXN2 overexpression in cells rendered the cells sensitive TORC1 inhibitor, caffeine [27], suggesting that the ATXN2 overexpression inhibits TORC1 activity (Figure 4-2).
Figure 4-1: ATXN2 truncations used to screen for interaction with PAS kinase.

Figure 4-2: Evidence of the inhibition of TORC1 by human ΔN554ATXN2 in yeast. ATXN2, like Pbp1, causes sensitivity to caffeine when overexpressed, most likely due to the inhibition of TORC1 [52]. Pbp1 deficient yeast (JYG1122) were transformed with ΔN700ATXN2 (pJG1361), ΔN554ATXN2 (pJG1360), ΔN379ATXN2 (pJG1359), pPbp1 (pJG925), or empty vector (pJG859) grown in SD-Ura, serially diluted 1:10, and spotted on SGal-Ura + 1.87 mM caffeine plates, and on a control SD-Ura plate. Plates were incubated at 30°C for 7–10 d until colonies were apparent.
To determine whether the phosphorylation of ATXN2 by PAS kinase can regulate the inhibition of TORC1, we transformed wild type and psk1psk2 yeast with ΔN554ATXN2 or Pbp1 and checked the effect of growth on media containing caffeine [22, 52]. Overexpression of ΔN554ATXN2 driven by the Gal promoter used in this study did induce significant growth inhibition in wild-type cells when compared to psk1psk2 cells. These results suggest that PAS kinase may activate ATXN2 under stress conditions, which may then sequester TORC1 in stress granules and reduce cell growth and proliferation similar to Pbp1.

Figure 4-3: Evidence for the activation of Pbp1 and ATXN2 by PAS kinase. Psk1Psk2 deficiency decreased caffeine toxicity due to ATXN2 and Pbp1 overexpression. Wild-type (JGY299) or psk1psk2 yeast (JGY1161) was transformed with an empty vector (EV, pJG859), a plasmid overexpressing Pbp1 (pJG925), or plasmid overexpressing ΔN554ATXN2 grown in SD-Ura, serially diluted 1:10, and spotted on SGal-Ura + 1.87 mM caffeine plates, and on a control SD-Ura plate. Plates were incubated at 30°C for 7–10 d until colonies were apparent.
4.2 Evidence for direct phosphorylation of human ATXN2 by human PAS kinase

The mutations in ATXN2 are found strongly associated with human ataxias [39] and we have recently shown that overexpression of ATXN2 in the presence of PAS kinase can inhibit growth and proliferation in yeast (Figure 4-3), in this aim we checked whether human PAS kinase can directly phosphorylate human ATXN2. HIS purified ΔN554ATXN2 was incubated with human PAS kinase in the presence of radioactive ATP and the phosphorylation of ATXN2 was assayed by autoradiography. Human PAS kinase dependent phosphorylation of ATXN2 was seen on the in vitro kinase assay (Figure 4-4), which suggest that hPASK can phosphorylates human ATXN2 and the phosphorylation site is somewhere between aa554 to aa1312ATXN2. That is consistent with yeast PAS kinase-dependent effects on ATXN2 activity observed in the phenotypic assay (see Figure 4-3). However, the downstream effect of human PAS kinase on ATXN2 is not known in mammalian cells, but it may be involved in neural degeneration in ATXN2 associated ataxias through the inhibition of TORC1.
Figure 4-4: Evidence for in vitro phosphorylation of ∆N554ATXN2 by hPASK. ∆N554ATXN2 was expressed and purified from psk1psk2 yeast cells and then assayed for in vitro phosphorylation by hPASK in the presence of $^{32}$P ATP. Phosphorylation of ∆N554ATXN2 was verified by assessing hPASK auto-phosphorylation in the absence of ATXN2, and in the presence of non hPASK substrate GAPDH. Kinase reactions were visualized on 10% SDS–PAGE gels, stained with Coomassie brilliant blue (CB), and exposed on x-ray film.
4.3 Evidence of in vivo interaction between Pbp1/ATXN2 and Psk2

Above we provided evidence for the Psk1-dependent phosphorylation of Pbp1 and ATXN2 in yeast. Psk1 and Psk2 are two partially redundant PASK homologs in yeast [17], and the effects of Psk2 on Pbp1/ATXN2 have not been assessed. Both Psk1 and Psk2 act as nutrient sensing kinases and share similar functions in the regulation of glucose homeostasis in the cell, including the ability to phosphorylate the well-characterized substrate Ugp1 [11]. To determine whether Psk2 can also phosphorylate Pbp1/ATXN2, the TORC1-dependent caffeine sensitivity was assessed in psk1 (JGY2) and psk2 (JGY3) yeast cells. In the presence of Psk2, overexpression of Pbp1 or ATXN2 in yeast cells induced caffeine sensitivity (Figure 4-5) when compared with the psk1psk2 yeast (Figure 4-3). These results are consistent with Psk2 acting in a similar manner as Psk1 to inhibit TORC1 activity by phosphorylating either Pbp1 or ATXN2 [22].
Figure 4-5: Evidence of PSK2 deficiency ameliorates caffeine toxicity due to Pbp1 or ΔN554ATXN2 overexpression. psk1 yeast (JGY2) or psk2 yeast (JGY3) was transformed with an empty vector (EV, pJG859), a plasmid overexpressing Pbp1 (pJG925) or a plasmid overexpressing truncated ATXN2 (pJG1360) grown in SD-Ura, serially diluted 1:10, and spotted on SGal-Ura + 1.87 mM caffeine plates, as well as on a control SD-Ura plate. Plates were incubated at 30°C for 7–10 d until colonies were apparent.
4.4 Evidence for direct phosphorylation of Pbp1 by Psk2

The in vivo effects of Psk2 on Pbp1 activity prompted us to investigate whether ∆N419Pbp1 could be phosphorylated by Psk2. His-HA tagged Psk1, Psk2 and kinase-dead Psk2 (K870R) [49] were purified and subjected to in vitro kinase assays with purified Pbp1. PAS kinase is known to autophosphorylate in vitro [11], making the kinase-dead mutant vital to ensure Psk2 dependent phosphorylation of Pbp1. Psk2-dependent Pbp1 phosphorylation was observed in these in vitro assays (Figure 4-6), providing evidence for the direct phosphorylation of ∆N419Pbp1 by Psk2.

Figure 4-6: Evidence for in vitro phosphorylation of Pbp1 by Psk2. ∆N419Pbp1 was expressed and purified from psk1psk2 yeast cells and then assayed for in vitro phosphorylation by Psk2 in the presence of $^{32}$P ATP. Pbp1 was verified as Psk2 substrate using a kinase-dead mutant (Psk2-K870R). Kinase reactions were visualized on 10% SDS–PAGE gels, stained with Coomassie brilliant blue (CB), and exposed on X-ray film.
4.5 **Characterization of PAS kinase-dependent phosphorylation of Pbp1 through phosphosite mapping and mutagenesis.**

From the previous data we know that PAS kinase phosphorylates Pbp1 at a threonine residue and the phosphorylation site is most likely between aa420 to aa722 [52]. Also, PAS kinase recognizes the consensus phosphorylation motif ([R/K/H]X[R/K/H][S/T/C/P/N/Q]X[T]) [56]. Based on mass spectrometry and in vivo kinase assay [52] data with Pbp1 phosphosite mutants (single amino acid change) (data not shown) we suspect that phosphorylation occurs at multiple sites. On the basis of these knowledge we introduced three sets of site-directed mutation in Pbp1 (Table 3-1) using the StrategeneQuikChange kit. All three sets of Pbp1 mutants were introduced to in vivo kinase assay (3.6 In vivo kinase assay) by western blot. Unfortunately, Psk1 is able to phosphorylates all the mutants, making is difficult to identify the critical phosphorylation site of Pbp1. Thus, more than one site may be the phosphorylated (as has been shown for other kinase mutants), or we have not identified the correct site. Future work will be aimed at testing a triple mutant of Pbp1 (to test all three sites at once), and testing truncations of Pbp1 in the in vitro kinase assay and yeast two-hybrid, to try to narrow in on the critical region.

4.6 **Defining the function of Pbp1 through identification of the binding partners**

Human ATXN2 plays a pivotal role in the development of human ataxias [39], yet little is known about its function. As mentioned above, both Pbp1 and its human homologue contain conserved functional domains (Figure 1-1), are components of glucose stress granules, and are involved in the regulation of TORC1 (Figure 2-1). In an effort to further elucidate the function of these proteins, the yeast two-hybrid (Y2H) approach was employed. Pbp1 was cloned into Bait (pJG1386) and was used to perform large-scale Y2H screens to identify Pbp1 binding partners. Y2H screens were conducted with two yeast genomic libraries (pJG428, and pJG429)
[57] and two cDNA libraries (JGY1098 and JGY1074) [49]. From ~10 million transformants or mated yeast screened, seven Pbp1 binding partners were identified (Table 4-1). Advantages of the Y2H approach include the identification of direct protein–protein interactions and sensitivity due to transcriptional amplification of an interaction [49]. However, the yeast two-hybrid can also yield false positives that allow growth independent of the bait. To minimize false positives, the Y2HGold strain was used, which harbors four different reporters control (Clontech Matchmaker Gold Yeast Two-hybrid System) [57], and each of these binding partners was verified by purifying the prey plasmid from yeast and retransforming into naive Y2HGold along with the ΔN419Pbp1 bait or an empty bait plasmid. The strength of growth is an indication of the interaction strength (Figure 4-7).
Table 4-1: Pbp1 binding partners identified by the yeast two-hybrid

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<th>Construct (Total number of aa)</th>
<th>Growth strength</th>
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<td>3</td>
<td>cDNA</td>
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<td>SLC36A1</td>
<td>DEADENYULATION-DEPENDENT mRNA-DECAPPING FACTOR</td>
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<td>Pdpk1</td>
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<td>aa649 to aa741 (aa766)</td>
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<tr>
<td>Hypothetical Protein</td>
<td>-</td>
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RNA Processing

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<td>MISFOLDED PROTEIN TRANSPORT</td>
<td>ER/M EM</td>
<td>1</td>
<td>Gen</td>
<td>aa407 to aa480 (aa480)</td>
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</table>

C corresponds to cytoplasm, MEM to membrane, M to mitochondrion, N to nucleus, ER to endoplasmic reticulum, V to vacuole and SG to stress granule. S, strong; M, medium; W, weak.
Figure 4-7: Dependency assay and Growth comparison. For verification and elimination of false positives, library plasmids were purified and transformed into Clontech Matchmaker Gold yeast with either the bait plasmid (pJG1386) or the empty bait plasmid (pJG424). Colonies arising on the SD-Leu-Trp transformation plates were then streaked to SD-Leu-Trp-His-Ade in duplicate and allow growing for 3–5 d to test for Pbp1 dependence. The strength of growth was determined by comparing growth of yeast on SD-Leu-Trp-His-Ade plates, which is an indication of the interaction strength. (a) SEC61: Medium growth; Hypothetical Protein: Weak growth. (b) PAT1 and PTC6: Strong Growth.
5 DISCUSSION

The results of these experiments contribute to our understanding of basic cellular processes and the regulation of an important metabolic enzyme PAS kinase. Our studies here demonstrate a crucial regulation of TORC1, a key protein in the regulation of cell growth and proliferation, by PAS kinase via ATXN2 and Pbp1. The interaction between Pbp1 and PAS kinase 1 (Psk1) in yeast was demonstrated by DeMille et.al. [57]. This study further characterized the phosphorylation of Pbp1 by PAS kinase, explored the conservation of the Pbp1/PAS kinase/TORC1 pathway, and further explored the function of Pbp1 through the identification of novel binding partners.

There are two homologs of PAS kinase in yeast, Psk1 and Psk2. This is of interest since proteins that arise from gene duplication often evolve related but differential function. For example, there are two TOR complexes in eukaryotic cells, TORC1, which primarily regulates cell growth and proliferation in yeast and mammalian cells, and TOR complex 2 (TORC2), which primarily regulates the actin cytoskeleton. Although a single protein works in both complexes in most eukaryotic cells, yeast has two TOR proteins, TOR1 and TOR2, which function in TORC1 and TORC2 respectively. We, therefore, hypothesized that Psk1 and Psk2 may have differential roles and explored the role of Psk2 in Pbp1 regulation. Phenotypic assays suggest that Psk2 ameliorates caffeine toxicity due to Pbp1 or ATXN2 overexpression similar to its homolog Psk1 (Figure 4-5). This study also provided evidence of in vitro phosphorylation of Pbp1 by Psk2 (Figure 4-6). So far we have not been able to identify the critical phosphorylation site of Pbp1 activation. The difficulty in mapping critical phosphorylation sites can be explained by the large size of the protein. However, the ability of Psk1 to phosphorylate the phosphosite mutants of Pbp1 would suggest that there are multiple phosphorylation sites. It is expected that
the phosphosite mapping of shorter fragments of Pbp1, as well as combinatorial mutations, by in vivo kinase assays will allow for a more specific Psk1 phosphorylation site.

In addition to further characterizing the role of yeast PAS kinase in the regulation of Pbp1, this study provided evidence for the conservation of the PAS kinase/Pbp1/TORC1 pathway in yeast. Evidence was provided that the human homolog of Pbp1, ATXN2 can complement its function in yeast cells (Figure 4-2). Under caffeine induced stress, over expression of ATXN2 inhibits TORC1 and thus inhibits cell growth and proliferation. Caffeine sensitivity can be rescued in pslk1psl2 cells, suggesting that phosphorylation of ATXN2 by PAS kinase activates it which then inhibits TORC1 (Figure 4-3).

In addition to identifying an interaction between PAS kinase and Pbp1, we have also identified a possible novel substrate of PAS kinase, ATXN2. Evidence suggests that human mutations in SCA2, the gene encoding ATXN2, is linked to ataxias [60]. In addition, ATXN2 knockout mice studies have also shown the involvement of ATXN2 in the development of obesity, insulin resistance, and dyslipidemia [65]. However, the molecular function of ATXN2 has not been understood to date [31]. Our study provides evidence that human PAS kinase can phosphorylate ATXN2 in vitro (Figure 4-4). This can contribute to understand the molecular mechanisms of neuronal death reported in Ataxin-2-associated ataxia. In addition, we provided evidence that PAS kinase phosphorylates and activates ATXN2 in yeast, suggesting PAS kinase may be a therapeutic target in the treatment of ataxias, a disease with few successful treatment options.

Despite Pbp1’s clear important in the regulation of cell growth and proliferation, only four binding proteins have been identified Pab1, Lsm12, Pbp4, and Dhh1 [29]. This study describes the interactome for Pbp1, identifying 7 novel putative binding partners and expanding
the role of Pbp1 in yeast, which includes 4 proteins that are involved in RNA processing and 3 proteins with other function. As Pbp1 is known component of stress granule it would be interesting to study interaction between Pbp1 and Hypothetical Protein (SGDID: S000003482), a putative RNA binding protein found in stress granules induced by glucose deprivation [66]. Thus, this study not only solidified and further characterized a known role for Pbp1 (localization to P-body), but also provides new pathways for further exploration.
6 REFERENCES


### APPENDIX A

Table 6-1: Yeast strain used

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Table 6-2: Plasmids used in this study

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Table 6-3: Primers used in this study