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Characterizing Novel Pathways for Regulation and Function of Ataxin-2

Elise Spencer Melhado
Brigham Young University

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Elise Spencer Melhado

A thesis submitted to the faculty of
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Master of Science

Julianne House Grose, Chair
William Russell McCleary
Kelly Scott Weber

Department of Microbiology and Molecular Biology

Brigham Young University

ABSTRACT

Characterizing Novel Pathways for Regulation and Function of Ataxin-2

Elise Spencer Melhado
Department of Microbiology and Molecular Biology, BYU
Master of Science

Ataxin-2 is an RNA-binding protein that is involved in many crucial cellular processes such as R-loop regulation, mRNA stability, TOR signaling regulation, and stress granule formation. Ataxin-2 is highly conserved, found in organisms ranging from *Saccharomyces cerevisiae* to *Caenorhabditis elegans* and *Homo sapiens*. Recently, ataxin-2 has been linked to the neurodegenerative disease Amyotrophic Lateral Sclerosis (ALS). ALS is a fatal disease that causes loss of motor neurons. In addition to ataxin-2 interacting with known ALS risk factor proteins, research into the relationship between ataxin-2 and ALS shows that polyglutamine expansions in ataxin-2 are gain-of-function mutations that lead to overactivity of ataxin-2 and probable neurodegeneration. In fact, targeting ataxin-2 using gene silencing techniques dramatically slows the progression of ALS in both mice and man.

The Grose laboratory has characterized a serine-threonine kinase, PAS kinase as upstream kinase and putative activator of ataxin-2. We hypothesize that knockdown of PAS kinase could, therefore, have similar effects to directly downregulating ataxin-2 and its cellular functions. Characterization of Ataxin-2 has revealed that its gain or loss of function lead to distinct cellular phenotypes. One study concluded that lowering ataxin-2 levels reduced the size and number of stress granules in mammalian cells, which was observed through microscopy. Another study found that activation and overexpression of ataxin-2 lead to reduced mTOR levels because of its sequestration to stress granules. Lastly, preliminary data obtained by the Grose laboratory suggests that yeast deficient in Pbp1 (the yeast homologue of ataxin-2) have altered cell cycles.

This project describes the cellular readouts used to determine if PAS kinase downregulation confers the same cellular phenotypes as ataxin-2 downregulation. First, we found that PAS kinase does influence ataxin-2 abundance in mammalian cells. Using yeast as a model, we found that Pbp1 influences the cell cycle through its binding partners, causing a reduction in the percentage of cells in the G2 phase compared to the G1 phase. PAS kinase conferred an opposite change, most likely due to the activity of other PAS kinase substrates. Additionally, we found that Pbp1 deficiency is synthetically lethal when in conjunction with deficiency of any one of its cell cycle-related binding partners. The cellular changes cause by Pbp1 deficiency highlight not only the importance of ataxin-2 in the cell, but also the importance of understanding the effects of downregulation of ataxin-2.

Keywords: PAS kinase, Ataxin-2, Pbp1, stress granules, TORC1, Amyotrophic Lateral Sclerosis, cell cycle

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ABBREVIATIONS

ALS: Amyotrophic Lateral Sclerosis

ATXN2: Ataxin-2

FBS: Fetal Bovine Serum

PSK/PASK: PAS Kinase

YPD/YPAD: Yeast extract, Peptone, (Adenine), Glucose

WT: Wildtype

CHAPTER 1: ALS and Ataxin-2

My contributions:

Chapter 1 serves as an introduction to this project and to highlight the foundational preliminary data I produced. The final data are presented in Chapters 2 and 3. This chapter is entirely my own.

1.1 Introduction

1.1.1 Ataxin-2 is an important cellular regulator whose dysfunction causes disease

Ataxin-2 is highly conserved and multifaceted protein that performs many functions within the cell (Figure 1-1) including RNA processing, mRNA stability, stress granule and processing body localization, circadian rhythmicity, R-loop regulation, and physiological functions, including apoptosis, embryonic development, actin development, insulin signaling, metabolic processes, and cellular proliferation (3). Ataxin-2 acts within the nucleus to promote transcript polyadenylation, which stabilizes mRNA (4); additionally, ataxin-2 directly binds to uridine-rich parts of 3' untranslated regions, which has been shown to stabilize mRNAs and increase their protein productivity (5).

The roles of ataxin-2 go beyond interactions with protein-coding mRNAs when ataxin-2 binds to non-coding RNAs to prevent the formation of R-loops, which are potentially deleterious because of the genome instability it causes (6). This interaction led to suggestions that ataxin-2 plays a role in normal progression of the cell cycle, and its disruption could lead to abnormal genome replication and cell proliferation, but further investigation is needed (7, 8). Additionally, ataxin-2 is involved in circadian rhythmicity through activation of pacemaker proteins in *D. melanogaster* and through uncharacterized interactions in *M. musculus* and *H. sapiens* (9-11). The diverse roles Ataxin-2 can be categorized as RNA controlling functions or physiological

functions, although these categories often overlap. When ataxin-2 regulation goes awry, the cell often suffers, and disease ensues. Ataxin-2 deregulation has been linked to numerous diseases including ALS, diabetes, and obesity.

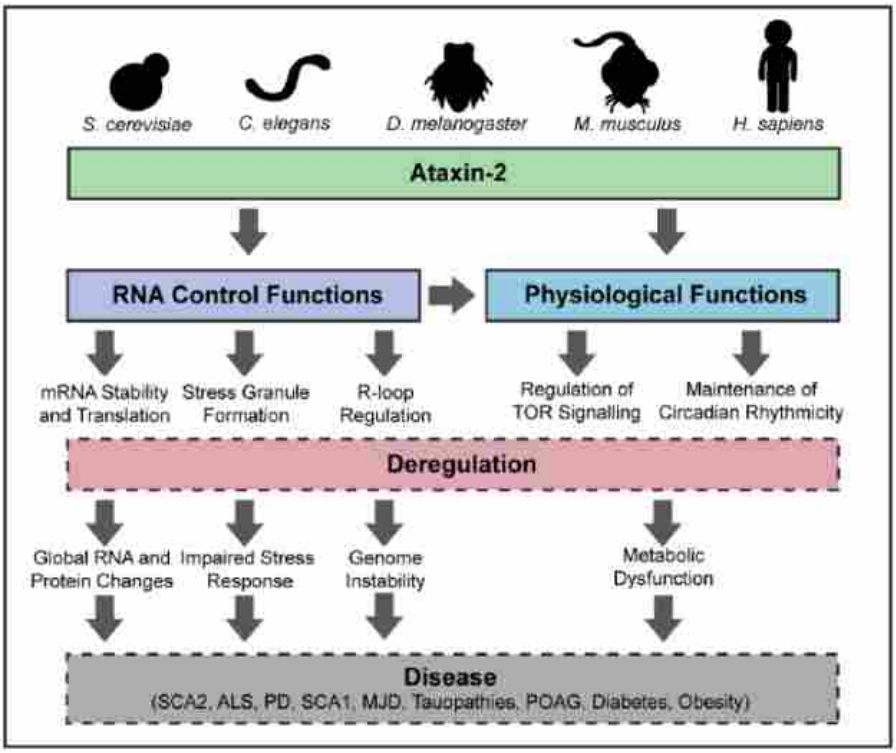

Figure 1-1: Ataxin-2 has diverse cellular functions.
Because of its diverse functions, disfunction of ataxin-2 often leads to disease. (14)

1.1.2 ALS is a fatal neurodegenerative disease with many unknown risk factors

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by the loss of motor neurons in the motor cortex, spinal cord and brain stem; the loss of neurons causes progressive paralysis that eventually leads to respiratory failure and death within five years of onset. ALS usually presents between the ages of 55 and 75 but can affect people of any age. It is estimated to effect more than 20,000 Americans (12). Currently, there is no cure for ALS, and treatments are limited; one of the most effective treatments, riluzole, prolongs patient survival by only two to three months (13).

ALS cases are divided into two groups: sporadic and familial. Only 5 to 10 percent of ALS cases fall into the familial category: most cases are classified as sporadic (12). In 1993, the

first causative mutations were found in the superoxide dismutase (SOD1) gene, accounting for approximately 10-20 percent of familial ALS cases (14).

Other mutations found to be causative in a significant number of cases include TAR DNA-binding protein 43 (TARDBP), fused-in sarcoma/translated in liposarcoma (FUS/TLS), and a hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (CRORF72) (3, 14, 15). The mechanisms by which mutated FUS and TDP-43 confer neurodegeneration are not well-characterized, but both play important roles in mRNA transport, motor neuron development, and axonal maintenance (16). ALS-linked mutations of FUS and TDP-43, which are both normally expressed at higher levels in the nucleus and low levels in the cytoplasm, show gain of cytoplasmic function (17). These mutated genes can cause many disease-related phenotypes, but account for a relatively low number of cases themselves.

Thus far, researchers have uncovered about 15 genes as causative for familial ALS and approximately the same number for sporadic ALS, but the mechanisms through which these mutations contribute to neurodegeneration remain largely uncharacterized (3). Despite the discoveries of these causative genetic mutations, identified genes account for fewer than 20% of sporadic ALS cases and only about 50% of familial cases (18, 19)

1.1.3 Protein-protein interactions may cause differences in disease characteristics

Research into the genetics of ALS is significant, but incomplete. Genetics do not account for variability in age of onset and severity of disease in patients with the same genetic mutation, suggesting that other disease factors exist (3). Ataxin-2, an RNA-binding protein that interacts with the previously discussed FUS and TDP-43 (TARDBP) proteins, has been identified as a risk factor for ALS through interactions with cytosolic aggregates of mutated FUS and TDP-43 (20, 21). The toxicity enhancement of mutated FUS and TDP-43 by ataxin-2 seems to be RNA-

dependent, but the exact mechanism remains uncharacterized (22). Ataxin-2 has been found not only to enhance the cellular toxicity of FUS and TDP-43, but ataxin-2 polyglutamine repeat sequence extensions are themselves a risk factor for ALS (3, 16).

1.1.4 Mutated ataxin-2 is a risk factor for ALS and other neurodegenerative diseases

Polyglutamine repeats are found in ataxin-2 in all cells, and gene with 15-27 repeats is considered normal; however, mutations that cause expansions of this repeat section are linked to ALS when the repeats are between 27 and 34, expansions between 35 and 49 glutamine are linked to parkinsonism, and repeats greater than 33 are linked to Spinocerebellar ataxia type 2 (23). In addition to the length of the expansion being considered a risk factor for various diseases, the purity of CAG repeats in the mRNA can contribute to disease type and age of onset (23). CAA interruptions in the glutamine repeats lessen the toxicity of the mutation, are thought to provide stability to the normally unstable CAG repeats, and may influence the levels of mRNA or translated protein (23). ALS patients usually have 1-3 CAA interruptions in the CAG repeat (23). Although the exact biochemical events that cause these mutations to be pathogenic remain unknown, it is accepted that these repeats form aggregates that cause gain-of-function toxicity. Recently, the polyglutamine expansions have been shown cause abnormal conformation of protein (24). Additionally, the mRNA with CAG/CAA repeats is suggested to be toxic to cells in a repeat length-dependent manner (25).

1.1.5 Ataxin-2 mediates stress granule formation

In response to stressful stimuli cells abort normal processes, like translation. This abrupt end causes mRNA to be stranded in the cytoplasm where it forms into stress granules or processing bodies (P-bodies) (26-28). P-bodies differ from stress granules, but often colocalize with stress granules, implying a connection between the two. Under stressful conditions, ataxin-2

promotes the formation of stress granules, as shown by its localization to stress granules, association with known stress granule proteins and the reduction of stress granules in the absence of ataxin-2 (26). Ataxin-2 also physically interacts with Dhh1, a key P-body component, and colocalizes with its homologue DDX6 implying a direct or indirect connection between ataxin-2 and P-bodies (29, 30). One study modified ataxin-2 expression levels using siRNA; this study found that manipulation of ataxin-2 expression reduced the number and size of stress granules through interference with stress granule assembly but does not prevent the formation of stress granules entirely (31). Ataxin-2 knockdown disrupts normal formation of stress granules and P-bodies, which may further contribute to the diseases caused by the ataxin-2 knockdown itself.

1.1.6 Ataxin-2 is a regulator of TORC1

Ataxin-2 is involved in numerous physiological functions through its mRNA stability and translation influencing functions, however, this study will focus on only one of these regulatory interactions: ataxin-2 as a regulator of TORC1.

TORC1 is a complex that regulates cellular growth in response to nutrient availability and is sensitive to caffeine as well as rapamycin, an immune-suppressant drug. Caffeine is a purine analogue that leads to cell death through uncharacterized mechanisms, but one proposed mechanism is a mechanism in which caffeine directly targets Tor1 kinase (a component of TORC1 complex) in addition to other unknown targets that can interfere/phosphorylate critical proteins. The inhibition of Tor1 kinase leads to the activation of Pkc1p-Mpk1p pathway that controls the expression of cell cycle-related genes at the G1/S

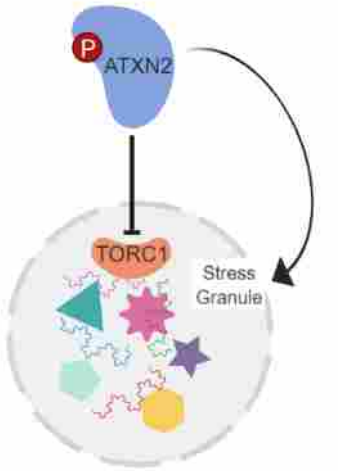

Figure 1-2: Ataxin-2 sequesters TORC1 to stress granules in addition to promoting stress granule formation.

phase transition as well as cell wall biosynthesis and repair genes (32-34). Because Tor1 is unique to the TORC1 complex and not found in TORC2, the caffeine inhibition of Tor1 only affects the TORC1 complex (1). Consistent with TORC1 conferring sensitivity to caffeine, previous studies found that *S. cerevisiae* cells that express overactive TORC1 are resistant to rapamycin and caffeine.

Previous studies show that if the yeast homologue of ataxin-2 (Pbp1) is overexpressed in conjunction with TORC1 overexpression, TORC1 sensitivity is restored to both inhibitors (35). When overexpressed, Pbp1 can efficiently sequester overactive TORC1 to stress granules, inhibiting its ability to confer caffeine resistance, which makes the cell once again sensitive to caffeine. This indicates that ataxin-2 (Pbp1) is an important regulator of TORC1. As a regulator, Pbp1 does not interfere with the assembly of TORC1, but interferes with its ability to activate other proteins by sequestering TORC1 to stress granules; when sequestered, the complex cannot function as a kinase and phosphorylate its substrates, rendering it non-functional. This can be visualized by analyzing the general abundance of intact TORC1 through immunoblot or visualization in fluorescence microscopy.

1.1.7 Ataxin-2 is a potential target for disease treatment

Therapeutic reduction of ataxin-2 abundance has been shown to be the most promising treatment for ALS; not only does ataxin-2 enhance the toxicity of FUS and TDP-43—and, conversely, reduction reverses the enhanced toxicity, but ataxin-2 also contributes to other cellular disease manifestations, namely stress granules and P-bodies. Some suggested approaches to therapeutically target ataxin-2 include the use of transcription activator-like effector nucleases, CRISPR/CAS, and epigenetic silencing (3, 36).

1.1.8 PAS kinase directly phosphorylates and activates ataxin-2

PAS kinase is a serine-threonine kinase that phosphorylates a threonine residue in ataxin-2, causing its activation (37). When cells experience stress, an activation pathway is initiated that leads to the eventual activation of ataxin-2. Stress activates a series of upstream regulators that, in turn, activate an AMP-regulated kinase called Snf1. PAS kinase is then activated by phosphorylation from Snf1 (38). Activated PAS kinase goes on to activate many proteins through phosphorylation, including ataxin-2 (37). In short, a pathway exists in which Snf1 activates PAS kinase through phosphorylation, which, in turn, activates Pbp1 through phosphorylation, allowing Pbp1 to perform its downstream functions (7). The activation pathway is likely to be conserved throughout eukaryotic organisms. In fact, lowering PAS kinase levels in yeast decreases Pbp1/Ataxin-2 function, including localization to stress granules as well as sequestration of TORC1. Ataxin-2 has also recently been shown to sequester TORC1 to stress granules in mammalian cells.

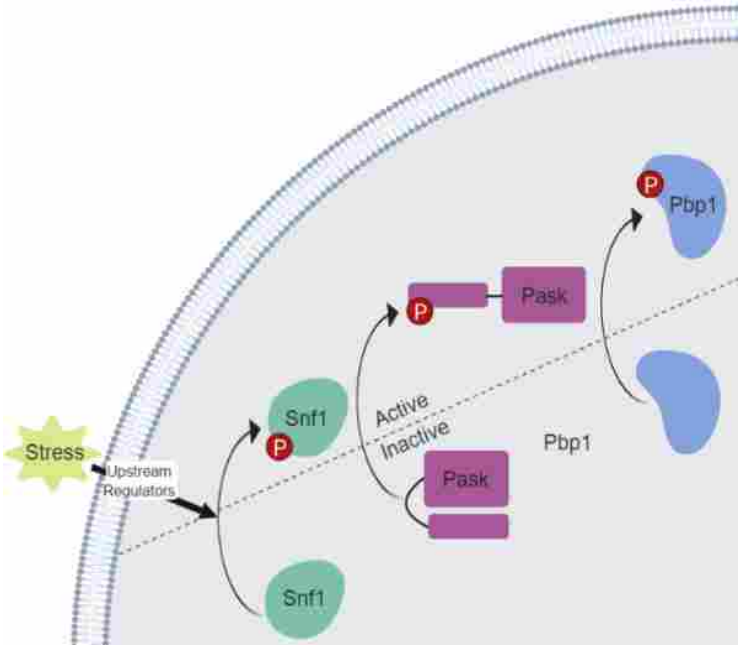

Figure 1-3: Ataxin-2 (Pbp1) is activated by stress in healthy cells.
Stress activates upstream regulators that activate Snf1. Activated Snf1 activates PAS kinase. Activated PAS Kinase activates Pbp1.

1.1.9 Altering PAS kinase abundance could mimic altering ataxin-2 abundance in cellular phenotypes

Both increasing and decreasing general abundance of ataxin-2 cause distinct cellular phenotypes to arise in cells, such as reduction in size and number of stress granules, changes in abundance of TORC1, and possible cell cycle disruption. Inactive ataxin-2 cannot perform these functions, so preventing the activation of ataxin-2 would likely lead to similar losses. As an activator of ataxin-2, PAS kinase is a putative therapeutic target. Inactivation of PAS kinase could lower the cellular levels of active ataxin-2, leading to the same phenotypes as direct downregulation of ataxin-2. Understanding these pathways that surround an important RNA-binding protein like ataxin-2 is vital in the quest to understand the underlying cellular phenotypes that cause devastating disease, such as ALS. This study will investigate the relationship of PAS kinase and ataxin-2 as well as the phenotypic outcomes associated with downregulation of ataxin-2.

1.2 Preliminary data

This project characterized the effects of PAS kinase regulation of ataxin-2 (and Pbp1) and the downstream effects. Specifically, this project focused on identifying the effect of PAS kinase knockdown on ataxin-2 levels and describing the downstream effects of altered Pbp1 levels on the cell cycle.

1.2.1 Identify the effect of PAS kinase knockout or knockdown on general ataxin-2 levels in a mammalian model

PAS kinase is known to phosphorylate many proteins involved in cellular metabolism, including ataxin-2 (37). This phosphorylation activates ataxin-2, allowing it to perform its

functions in the cell. If PAS kinase is removed or downregulated, it is logical to assume that the general abundance of ataxin-2 may be altered.

To test the effect of PAS kinase downregulation on ataxin-2, PAS kinase-deficient ($PASK^{-/-}$) and wild type (WT) mice were utilized (Figure 1-4). To further mimic human habits, the mice were fed two different diets: a normal chow diet, and a high-fat, high-sugar diet reflective of Western diets. Because ALS affects motor neurons, the neural cells from the brain were analyzed for ataxin-2 expression levels via immunoblotting (for materials and methods see Section 2.3.5). Our preliminary results showed that mice on a normal chow diet did not have a significant difference in the amount of ataxin-2 when comparing the two genotypes, but mice on a high-fat, high-sugar diet did display a significant reduction of ataxin-2 levels in PAS kinase-deficient mice compared to wild-type mice. It is likely that the high-fat, high-sugar (HFHS) diet causes stress on cells that activates the stress response pathway activation of ataxin-2; wild-type mice that have functioning PAS kinase have nearly double the ataxin-2 when consuming HFHS

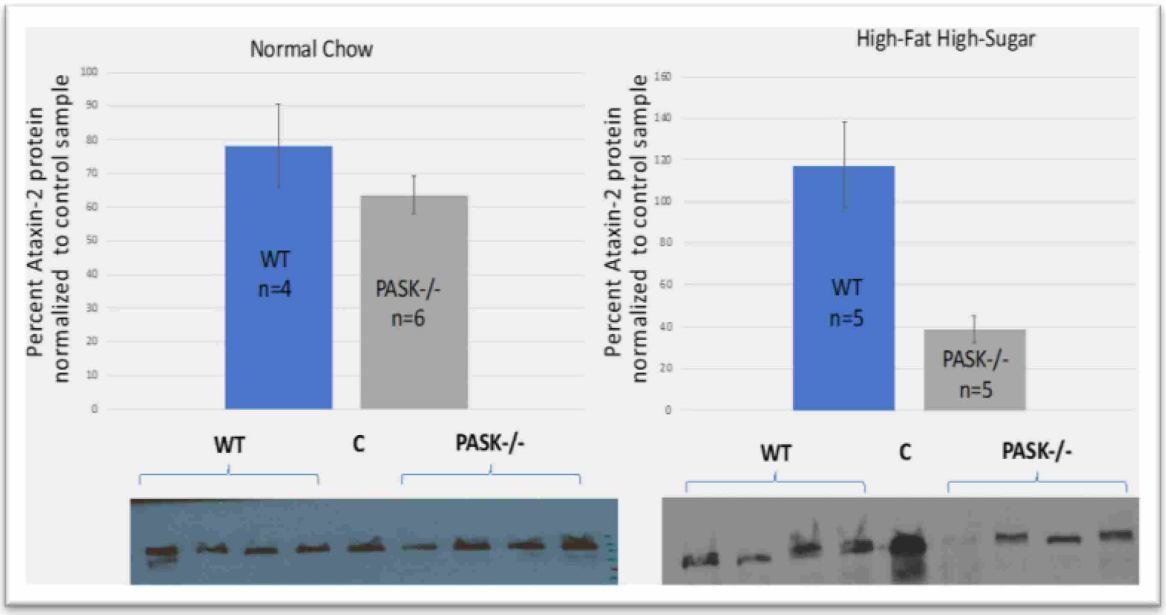

Figure 1-4: ALS model mice who consume a high-fat, high-sugar diet have lower levels of ataxin-2 than wild-type. Four groups of mice consisting of two genetic groups (wild-type and PAS kinase-deficient) and two diet groups (normal chow and high-fat, high-sugar). Tissue from the brain was homogenized and run on a polyacrylamide gel for immunoblotting. Samples were normalized to a control sample.

diet rather than a normal chow diet, but ataxin-2 levels in PAS kinase knockout mice remain constant. These results demonstrate the manipulating PAS kinase can affect ataxin-2 protein levels in conjunction with stress.

1.2.2 Characterize the effects of Pbp1 and PAS kinase on the cell cycle in yeast

A recent screen for Pbp1 interacting partners performed by Nidhi Choksi of the Grose lab, revealed many binding partners associated with cell cycle (Table 1). Ataxin-2 has been posited by many to control the cell cycle, and these binding partners may explain the molecular mechanisms of this control. We have focused on BNR1, APC9, FUN19, and SPR28 (all Pbp1 interacting partners) that were analyzed for roles in cell cycle changes due to their non-essential nature. Since Pbp1 has been shown to sequester proteins (such as TORC1) to stress granules, our hypothesis is that Pbp1 sequesters cell cycle components to the stress granule, inhibiting their function and altering cell cycle progression.

BNR1 is a nonessential protein that nucleates linear actin filaments involved in budding and actin spindle orientation. Although its effects on cell cycle are not known, BNR1 mutations result in altered spindle and actin cable formation as well as abnormally elongated buds, thus we expect an altered cell cycle. In addition, we expect that Pbp1 or PAS kinase overexpression should result a similar phenotype to BNR1 deletion in that BNR1 may be sequestered to stress granules and thus inactivated. SPR28 is also in the family of bed-neck microfilament genes. APC9 is a subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C) that is required for degradation of anaphase inhibitors including mitotic cyclins during the metaphase/anaphase transition. Thus, active BNR1, SPR28 or APC9 (in the absence of activated Pbp1) may increase metaphase/anaphase, perhaps explaining the truncated S phase or extended G_2 of Pbp1 or PAS kinase-deficient yeast as we have observed. FUN19 is included because it seems to interact with

both Pbp1, and it is a cell cycle protein, likely involved in regulation of transcription, and it could also be sequestered by Pbp1.

Table 1-1: Putative Pbp1 binding partners identified by the mass spectrometry
The gene name, human homolog, description, cellular localization (Local.) and the number of times the protein was retrieved from two independent runs are provided. For localization: C, Cytoplasm; M, Mitochondrion; N, Nucleus; ER, Endoplasmic Reticulum; PM, Plasma Membrane; W, Cell Wall; G, golgi; SG, Stress Granule; S, meiotic Spindle; ES, Endosome; CD, Cell Division.

Gene	Human Homolog	Name description		Local.	#
	RNA Processing				
IMP	IMP4	snoRNA-binding rRNA-processing protein		N	2
SSD1	N/A	mRNA-binding translational repressor		N/C	1
	Protein Transport				
TRS31	TRAPPC5	Core component of transport protein particle (TRAPP) complexes I-III		C/G	1
VPS29	VPS29	Vacuolar Protein Sorting		ES/ V	1
SSY1	N/A	Component of the SPS plasma membrane amino acid sensor system		PM	1
SEC24	SEC24	COPII vesicle coat component		ER/G/C	1
TIM9	TIMM9	Mitochondrial inner membrane translocase		M	1
	Cell Cycle				
CDC50	TMEM30	Cell division control protein 50		ES	1
MCM5	MCM5	MiniChromosome maintenance protein 5		N	1
APC9	N/A	Anaphase Promoting Complex subunit 9		CD	1
BNR1	FMNL1	BNI1-related protein 1		CD	1
CYR1	PHLPP2	Adenylate Cyclase & magnesium ion binding		CD	1
CDC3	SEPT7	component of 10 nm filaments of mother-bud neck		CD	1
SPR28	SEPT1	Septin-related protein sporulation induced		W/ S	1
FUN19	TADA2A	SWIRM domain-containing protein		C	1
	Other				
INA22	N/A	Inner membrane assembly complex subunit 22		M	2
RAD33	N/A	Nucleotide excision repair		N	1
APE3	ERMP1	Aminopeptidase Y		V	1
FRE7	CYBB	Ferric reductase PM		PM	1
NIT1	N/A	Nitrilase			1
ZRT2	N/A	low-affinity Zn(2+) transporter		PM	1
MOT3	N/A	Involved in cellular adjustment to osmotic stress		N/C	1
	Protein of Unknown function				
YHR131CP	N/A	Protein of unknown function		C	1
FRT1	N/A	Tail-anchored ER membrane protein of unknown function		ER	1
SYG1	XPR1	Signal Transduction		PM/ER/G	1

Saccharomyces cerevisiae was used to study the effects of PAS kinase knockout and Pbp1 knockout on the cell cycle (for materials and methods see Section 3.3.8). Preliminary data shown in Figure 1-5 that suggests Pbp1, the yeast homologue of ataxin-2, and PAS kinase knockout yeast have altered phases in the cell cycle. For this data, methods from Section 3.3.8 were followed, but Propidium Iodide was used as dye. Because Pbp1 is activated by PAS kinase, the effects of the Pbp1 knockout are expected to be like the knockout of PAS kinase. Further investigation is needed to better evaluate the effect of these knockouts on the cell cycle.

The other interacting partners identified above were analyzed by obtaining yeast strains deficient in one of each of the identified genes and testing these yeast strains along with

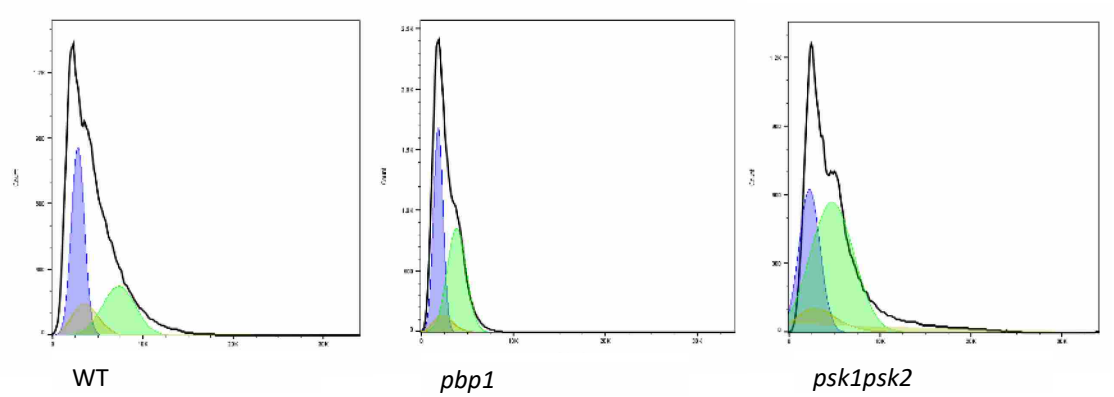

Figure 1-5: Pbp1 and PASK knockout yeast show differences in cell cycle stage lengths.
Flow cytometry was used to detect PI in stained yeast cells. Wildtype yeast (JGY 43), Pbp1 deficient (JGY 1122), and PASK deficient (JGY 1244) are shown.

constructed double-knockout strains. Double-knockout strains were created by designing primers for each flanking region of the knocked-out gene to amplify the flanking regions without the gene but containing a selectable marker. These fragments were transformed into either Pbp1 deficient or PAS kinase deficient yeast so homologous recombination will substitute the gene-lacking portion with the gene-containing portion of the genome, as shown in Figure 1-6. These double-knockouts were tested alongside all the single-knockout strains to check for other

proteins causing cell cycle changes. Growth assays will also be performed to check for viability of the constructed strains.

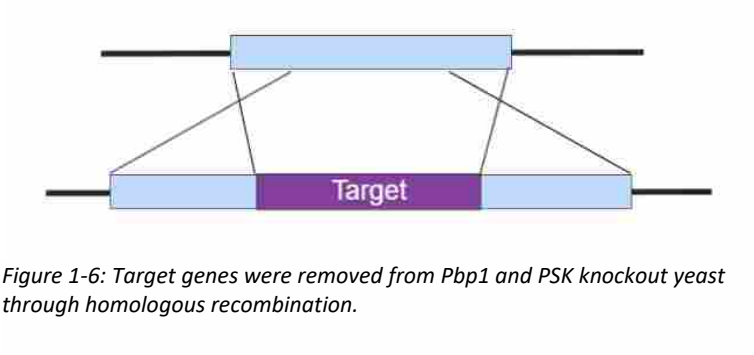

Figure 1-6: Target genes were removed from Pbp1 and PSK knockout yeast through homologous recombination.

1.3 Conclusion

Knockdown of ataxin-2 activity has been shown to be a potential treatment for ALS. As an upstream activator, PAS kinase may be manipulated to reduce ataxin-2 activity. This hypothesis will be explored. Additionally, exploring the multifaceted roles of ataxin-2 plays is crucial to understanding how ataxin-2 contributes to neurodegeneration in ALS and how it may contribute to other disease. We will investigate binding partners of ataxin-2 to better understand how it interacts with other cellular components, especially those components related to the cell cycle.

CHAPTER 2: Identification and Characterization of PAS kinase as a Regulator of Ataxin-2 Function

Nidhi R. Choski, Brittany A. Pielstick, Elise S. Melhado, Aaron D. Gitler, Laura C. Bridgewater, Julianne H. Grose, Ph. D.

My contributions to this paper:

Working with Brittany Pielstick, I processed and analyzed the protein from PAS kinase deficient mice (Figure 2-4). We also optimized the protocols for homogenization, running on polyacrylamide, and immunoblotting samples. Because ataxin-2 is not a highly expressed protein and is prone to degradation, the task of detecting ataxin-2 levels is difficult. We wrote sections 2.3.5 and 2.4.4, as well as the corresponding discussion section.

2.1 Abstract

Mutations in the ATXN2 gene in humans cause neurodegeneration. The ataxin-2 protein contains a tract of repeated glutamine (Q) residues – a polyglutamine (polyQ) tract. Long polyQ expansions in ataxin-2 cause spinocerebellar ataxia type 2 (SCA2), whereas intermediate-length polyQ expansions increase risk for the motor neuron disease amyotrophic lateral sclerosis (ALS). The mechanisms by which polyQ expansions cause neurodegeneration are not fully understood but it is likely caused by a gain of one more toxic properties. Thus, strategies to reduce levels of ataxin-2 could be an effective therapeutic approach. Indeed, in two recent preclinical studies, targeting the ataxin-2 gene in mouse using antisense oligonucleotides was sufficient to alleviate disease symptoms in models of ALS and SCA2. These results illuminate ataxin-2 as a potential therapeutic target for at least two neurodegenerative diseases. We recently identified PAS kinase 1 (Psk1) as an activator of the yeast ataxin-2 homolog poly(A)-binding protein binding protein 1 (Pbp1). Here we characterized the regulation of Pbp1/ataxin-2 by Psk1 and provide evidence that

PAS kinase 2 (Psk2) also phosphorylates and activates Pbp1. Importantly, we show that human ataxin-2 complements Pbp1-deficiency in yeast and that it can also be activated by PAS kinase. We provide evidence that human PAS kinase can phosphorylate ataxin-2 in vitro and knockout of PAS kinase in mouse results in reduced ataxin-2 protein levels, consistent with the reduction of Pbp1/Ataxin-2 activity observed in PAS kinase-deficient yeast. We propose PAS kinase as a regulator of ataxin-2 and thus a novel therapeutic target for ALS and SCA2.

2.2 Introduction

Nutrient-sensing kinases maintain metabolic homeostasis by allocating cellular resources in response to nutrient status through the phosphorylation and subsequent regulation of protein substrates (39). PAS kinase is a nutrient-sensing kinase that contains a catalytic serine/threonine kinase domain and a sensory/regulatory PAS domain (40) and is highly conserved from yeast to man (41). PAS kinase is regulated by glucose levels and in turn regulates glucose homeostasis in both yeast and mice (for reviews see (42-47)). Underscoring PAS kinase's central role in regulating metabolism, when placed on a high fat diet, PAS kinase-deficient mice (Pask-/-) show an increased metabolic rate and are resistant to weight gain, liver triglyceride accumulation and insulin resistance (48). In mammals, this regulation may occur in part through the phosphorylation of PDX1 and glycogen synthase (44, 49). In yeast, PAS kinase has a conserved role in the regulation of glucose metabolism through the phosphorylation of UDP-glucose pyrophosphorylase (Ugp1), which controls the glucose flux between glycogen storage and cell wall growth (50, 51). In addition, PAS kinase phosphorylates centromere binding factor (Cbf1) to control yeast cellular respiration and lipid biosynthesis (52, 53).

We recently identified another substrate of PAS kinase and characterized its role as an intermediate between SNF1 and TORC1, inhibiting cell growth and proliferation when energy is

low. This pathway involves the SNF1-depedent phosphorylation and activation of PAS kinase (37, 53), which then phosphorylates and activates Pbp1, a poly(A)-binding protein (Pab1)-binding protein and a component of stress granules (SGs) (4, 54, 55). Once activated, Pbp1 induces SG formation, where target of rapamycin complex 1 (TORC1) is then sequestered (4, 35, 37, 54, 55). Specifically, deletion of PAS kinase in yeast leads to recovery of TORC1 and reduced Pbp1 localization to stress granules (37).

The human homolog of Pbp1 is ataxin-2, encoded by the ATXN2 gene. Ataxin-2 is an RNA-binding protein that regulates translation and other aspects of RNA metabolism (56-59). Ataxin-2, like yeast Pbp1, is also involved in SG formation (31, 55). Human ataxin-2 harbors a polyQ domain, which is almost always 22Qs long. PolyQ expansions greater than 34 cause SCA2, which is associated with Purkinje neuron degeneration in the cerebellum. Intermediate-length polyQ expansions (60-66) increase risk for the fatal motor neuron disease ALS (2, 19, 22, 23, 60-94). For both SCA2 and ALS, disease is thought to be caused by a toxic gain of function in ataxin-2. Reducing ataxin-2 levels either by genetic knockout or by administering antisense oligonucleotides targeting the ataxin-2 gene to the central nervous system is sufficient to mitigate degenerative phenotypes and improve survival in mouse models of SCA2 and ALS (36, 95). Thus, strategies to reduce levels or activity of ataxin-2 could be an effective therapy for these diseases.

In mouse, knockout of ataxin-2 is not lethal but intriguingly the knockout animals develop obesity, insulin resistance, and dyslipidemia (96). This phenotype is in striking contrast to the marked protection from these phenotypes on a high-fat diet conferred by the PAS kinase knockout. We reasoned that if PAS kinase positively regulates ataxin-2, it could represent an attractive therapeutic target for diseases in which reducing levels or activity of ataxin-2 would be

efficacious (e.g., ALS and SCA2). Here we show that PAS kinase can phosphorylate and regulate the activity of ataxin-2, similar to how it regulates the yeast homolog Pbp1. Importantly, we demonstrate reduced ataxin-2 levels in the brains of PAS kinase knockout mice, highlighting a new therapeutic avenue for targeting ataxin-2.

2.3 Materials and methods

2.3.1 Growth assays

Lists of strains, plasmids, and primers used in this study are provided in Supplementary Table 1. For plasmid construction, standard PCR-based cloning methods were used. All restriction enzymes were purchased from New England BioLabs (Ipswich, MA). 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 days until colonies were apparent.

2.3.2 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 -80ºC. 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 (Roche).

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. Supernatants 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. HIS-tagged proteins were eluted three times with 0.3 ml of Elution Buffer (lysis buffer containing 270 mM imidazole, 10 mM KCl and 50 mM NaCl pH 7.8). Beads containing Myc-tagged proteins were used directly for in vitro kinase assays without eluting.

2.3.3 Pbp1 PAS kinase in vitro kinase assay

Myc-tagged Pbp1 and HIS-tagged Psk1 (pJG858), Psk2 (pJG173), and Psk2-KD (pJG174) were purified from psk1psk2 yeast (JGY4). Purified ΔN419Pbp1-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.

2.3.4 ATXN2 hPASK in vitro kinase assay

ΔN544ATXN2 was purified from psk1psk2 yeast (JGY4) using HIS-tag purification. The truncation was used due to an inability to express the full-length protein in yeast. Purified ATXN2 protein was assayed for human PAS kinase–dependent phosphorylation by incubating purified ΔN544ATXN2 protein in 30 μL of reaction buffer (0.4 M HEPES, 0.1 M KCl, 5 mM MgCl2, pH 7.0), 1 mM ATP, 32P-ATP (5 μCi; MP Biomedicals, Santa Ana, CA) with or without purified human PAS kinase. Samples were incubated for 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 for 6 hours and visualized on film.

2.3.5 Protein extraction and Western blotting

PASK-/- mice were generously donated by Jared Rutter (University of Utah) and were previously described (48). Male and female wild type and PASK-/- mice, generated by breeding PASK+/- mice, were placed on a HFHS diet (D12266Bi Condensed Milk Diet from Research Diets - 17% protein, 32% fat, 51% carbs) or standard diet (Teklad Rodent Diet 8604 from Envigo) at 12 weeks old and maintained on the diet for 25 weeks. All procedures were approved by the Brigham Young University Institutional Animal Care and Use Committee (protocol number 13-1003 submitted by L.C.B.). Mouse brain was frozen down immediately after dissection and stored at -80°C. The brain was thawed in triple detergent buffer (100Mm tris pH 7.4 HCl, 1% NP40, 0.5% SDS, 0.5% deoxycholic acid, 1mM EGTA, Halt Protease Inhibitor

Cocktail (Thermo Fisher Scientific) and Phosphatase Inhibitor Cocktail 2 (Sigma Aldrich)) and homogenized using the Bullet Blender from Next Advance and stainless-steel beads. The samples were then spun at 20,817 rcf at 4°C for 10 minutes. The supernatant was extracted and spun at 20,817 rcf at 4°C for an hour. Protein quantification was done using Bradford Assay kit from Thermo Fisher Scientific. Samples were boiled for 3 min at 99°C and stored at -80°C. 50 ug of protein from each sample were run on SDS-page 8% gel at 50-100V for 1-2 hrs. The samples were then transferred to a nitrocellulose membrane at 0.35 Amps for 1.5 hrs. The membrane was washed with TBS (40 mM Tris, 100mM NaCl pH 7.5), blocked in 5% milk, incubated at 4°C overnight with desired primary antibody dilution, and imaged with WesternBright ECL (Advansta). Each sample had 2-4 technical replicates performed and was normalized to a control sample on the blot. Antibodies used were: Anti-Ataxin-2 from BD Transduction Laboratories and anti-GAPDH from Cell Signaling Technology.

Table 2-1: Phosphosite mutants of Pbp1

Plasmid	Mutation in amino acid
pJG1561	S106A, T193A, S436A, S479A, T708A, T718A
pJG1562	S106A, T193A, T355A, S436A, S479A, T708A
pJG1563	S106A, T215A, T355A, S436A, S479A, T708A, T718A

2.4 Results

2.4.1 Complementation of Pbp1 phenotype by ATXN2 in yeast

We previously found that PAS kinase-dependent phosphorylation of Pbp1 leads to the sequestration of TORC1 into stress granules, inhibiting growth and proliferation (DeMille, 2015 #24). Ataxin-2 (ATXN2) is the human homologue of yeast Pbp1, and both ATXN2 and Pbp1 have conserved Lsm, LsmAD and self-interacting domains (Figure 2-1A) (26, 57). To test whether ATXN2 could complement Pbp1 function in its ability to reduce cell growth and

proliferation during caffeine-induced stress, we expressed three truncations of ATXN2 (ΔN378ATXN2, ΔN543ATXN2 and ΔN699ATXN2) in pbp1 yeast and grew transformants on selective media containing caffeine to assay for complementation of the TORC1 phenotype. Expression of ΔN543ATXN2 and ΔN699ATXN2 rendered the cells sensitive to the TORC1 inhibitor caffeine but did not affect growth on standard media, suggesting that ATXN2 can functionally replace yeast Pbp1 to inhibit TORC1 activity (Figure 1B). To our knowledge, this is the first evidence that human ATXN2 can complement its yeast homolog Pbp1. In support of this finding, overexpression of ATXN2, like Pbp1, has also previously been shown to rescue the petite (pet-) phenotype of yeast lacking mitochondrial group II introns (26, 37).

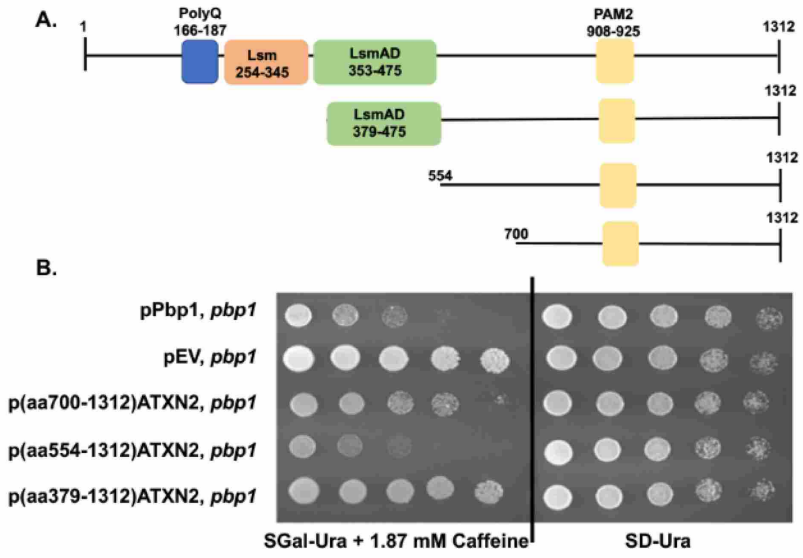

Figure 2-1: Evidence for the conservation of Pbp1/ATXN2 function.
A) A schematic of ATXN2 functional domains as well as ATXN2 truncations used in this study. Numbers indicate amino acids. Boxes are functional domains reported (69) including Lsm, LsmAD and the self-interaction domain. B) Evidence of the inhibition of TORC1 by human ΔN553ATXN2 in yeast. ATXN2, like Pbp1, causes sensitivity to caffeine when overexpressed, most likely due to the inhibition of TORC1 (17, 21). 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 days until colonies were apparent.

2.4.2 Evidence for direct phosphorylation of human ATXN2 by human PAS kinase

We previously provided evidence for the phosphorylation of Pbp1 by PAS kinase 1 (Psk1) in yeast (37). Psk1 and Psk2 are two partially redundant PAS kinase homologs that likely arose via whole genome duplication in yeast (97). 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 (41, 50, 51). However, gene duplication often leads to differentiated function allowing selection for both copies to occur (98, 99). We therefore analyzed the impact of Psk2 on Pbp1. We recombinantly expressed and purified His-HA tagged Psk1, Psk2 and kinase-dead Psk2 (K870R) (41) and used them for in

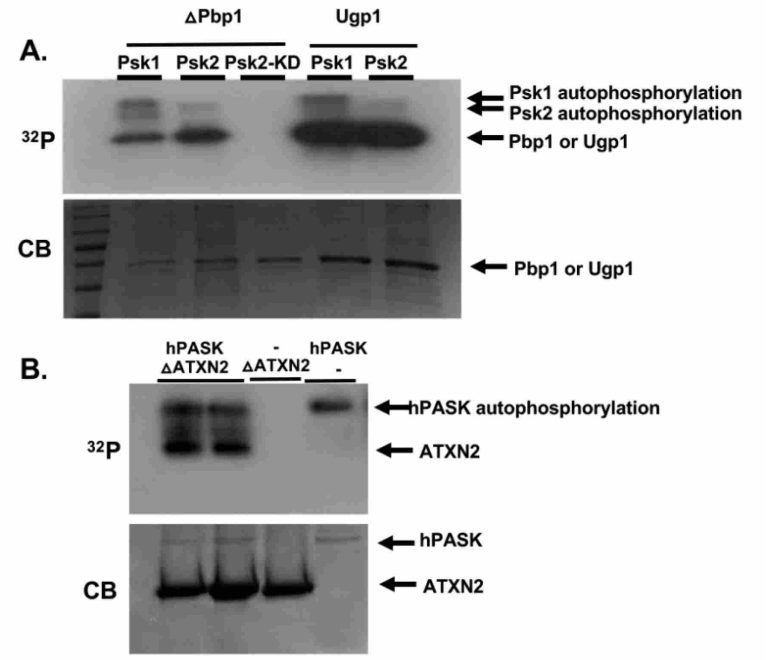

Figure 2-2: Investigation into the regulation and function of yeast Pbp1. A) Evidence for in vitro phosphorylation of Pbp1 by Psk2. ΔN419Pbp1 was expressed and purified from psk1psk2 yeast cells and then assayed for in vitro phosphorylation by Psk2 in the presence of 32P ATP. Pbp1 was verified as Psk2 substrate using a kinase-dead mutant (Psk2- K870R) as a control. B) Evidence for in vitro phosphorylation of ΔN553ATXN2 by hPASK. ΔN553ATXN2 was expressed and purified from psk1psk2 yeast cells and then assayed for in vitro phosphorylation by hPASK in the presence of 32P-ATP. Phosphorylation of ΔN553ATXN2 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.

vitro kinase assays with purified Pbp1 (53). Note that we used a truncation (ΔN419Pbp1) because the full-length protein was not functional in vitro (37). We observed Psk2-dependent Pbp1 phosphorylation (Figure 2-2A), providing evidence for the direct phosphorylation of ΔN419Pbp1 by Psk2.

Given the ability of ATXN2 to complement Pbp1 function, we next asked if the regulation by PAS kinase was also conserved. We assessed the ability of human PAS kinase to directly phosphorylate human ATXN2. We HIS incubated purified ΔN553ATXN2 with human PAS kinase (hPASK) in the presence of radioactive ATP and assayed the phosphorylation of ATXN2 by autoradiography. We detected PAS kinase-dependent phosphorylation of ATXN2 in the in vitro kinase assay (Figure 2-2B), providing evidence that hPASK can phosphorylate human ATXN2 and that the phosphorylation site is somewhere between aa554-1312 of ATXN2.

2.4.3 Evidence for the regulation of Pbp1/ATXN2 by PAS kinase in yeast

To determine whether the phosphorylation of ATXN2 by PAS kinase can regulate the inhibition of TORC1, we transformed wild type and psk1psk2 yeast with ΔN553ATXN2 or full length Pbp1 and checked the effect of growth on media containing caffeine. Upregulation of ΔN553ATXN2 driven by the Gal promoter caused significant growth inhibition in wild-type cells compared to psk1psk2 cells (Figure 2-3A). 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 like the effects of Pbp1. We assessed TORC1-dependent caffeine sensitivity in psk1 (JGY2) and psk2 (JGY3) yeast cells. In the presence of PSK2 (in PSK1-deficient yeast), upregulation of Pbp1 or ATXN2 in yeast cells induced caffeine sensitivity (35) when compared with the psk1psk2 yeast (Figure 2-3B). These results are

consistent with Psk2 acting in a similar manner as Psk1 to inhibit TORC1 activity by phosphorylating either Pbp1 or ATXN2.

2.4.4 Evidence for regulation of mammalian ATXN2 by PAS kinase in mice

Having established that PAS kinase can phosphorylate ATXN2 in vitro and that ATXN2 can complement PAS kinase-dependent functions in yeast, we finally sought to assess the effects of PAS kinase on ATXN2 in mammalian cells. We analyzed brain homogenates from wild type and PAS kinase deficient (PASK-/-) male mice for ATXN2 levels by immunoblot. The PASK-/- showed a marked decrease in ATXN2 protein compared to their wild type littermates when

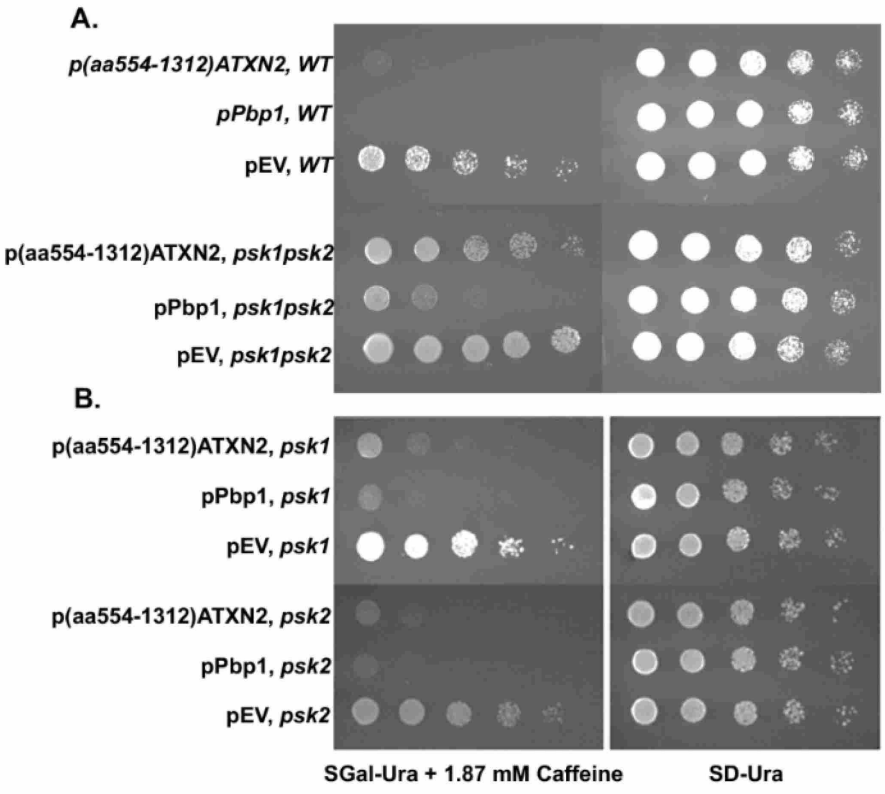

Figure 2-3: Evidence for the regulation of ATXN2 by PAS kinase.
A) Evidence that PAS kinase-deficiency (psk1psk2) ameliorates caffeine toxicity due to Pbp1 or ΔN553ATXN2 overexpression. B) Evidence that PSK1 and PSK2 can function to activate Pbp1 or ΔN553ATXN2 and thus inhibit TORC1 as determined by the caffeine sensitivity phenotype. Wild type (JGY1), psk1 yeast (JGY2), psk2 yeast (JGY3), or the double mutant (JGY4) were transformed with an empty vector (EV, pJG859), a plasmid overexpressing Pbp1 (pJG925) or a plasmid overexpressing ΔN553ATXN2 (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 days until colonies were apparent.

placed on a High-Fat High-Sugar (HFHS) diet, whereas we did not observe significant effects when analyzing tissue from the mice on a normal chow diet (Figure 2-4). In addition, no significant difference was seen when we immunoblotted for GAPDH levels. Thus, PAS kinase can regulate ATXN2 levels in mouse brain.

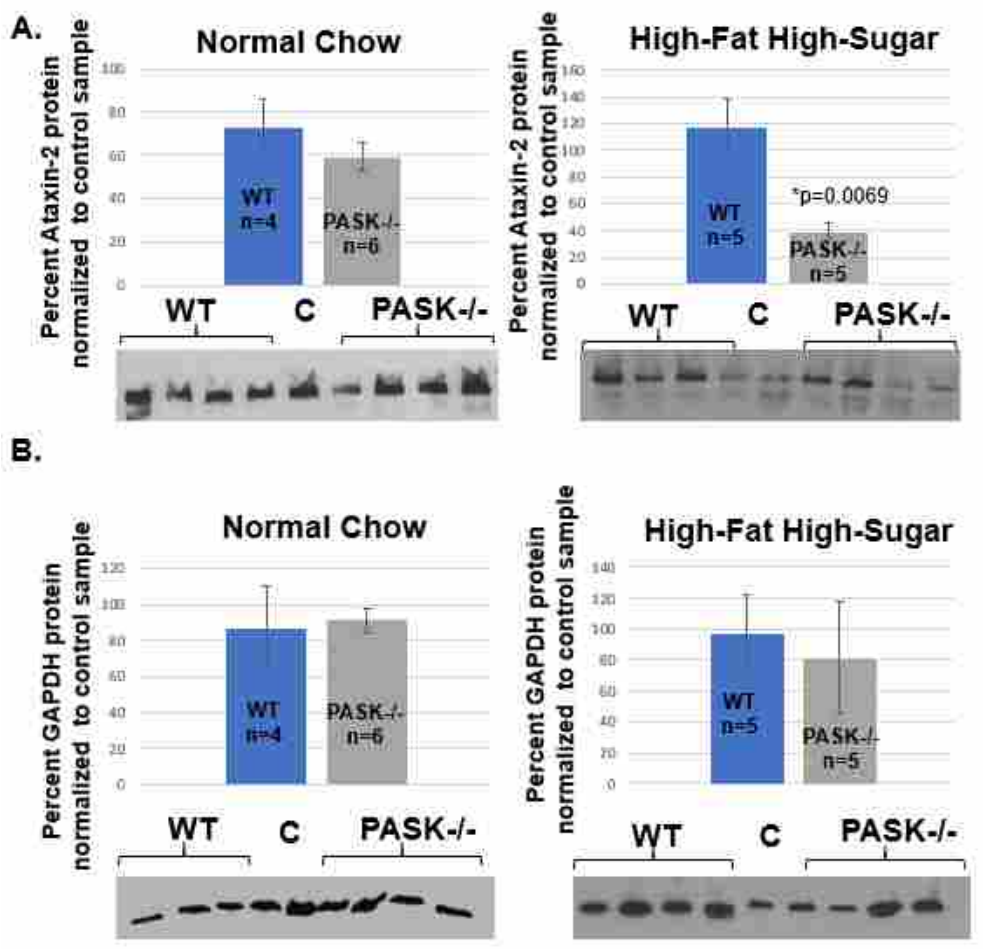

*Figure 2-4: PASK deficiency decreases Ataxin-2 abundance in brain tissue from mice on a HFHS diet. Brain was homogenized from WT and PASK-/- male mice on a normal chow diet or placed on a high-fat high sugar (HFHS) diet for 25 weeks. Proteins was normalized by Bradford assay using bovine serum albumin as a standard, run on SDS-PAGE and transferred to nitrocellulose paper for imaging with anti-ataxin-2 antibody (BD transduction laboratories) and anti-GAPDH antibody (Cell Signaling Technologies). All samples were run three times to check technical replicates, normalized to a loading control sample, and quantified using ImageJ (73). Error bars represent SEM. Representative western blots are shown. Data was analyzed using one-way analysis of variance (2) with Tukey's HSD post hoc test. *p-value <= 0.05.*

2.5 Discussion

Herein we have provided evidence for regulation of Pbp1/ATXN2 by the nutrient response serine-threonine protein kinase PAS kinase (see Figure 2-5 for a summary model).

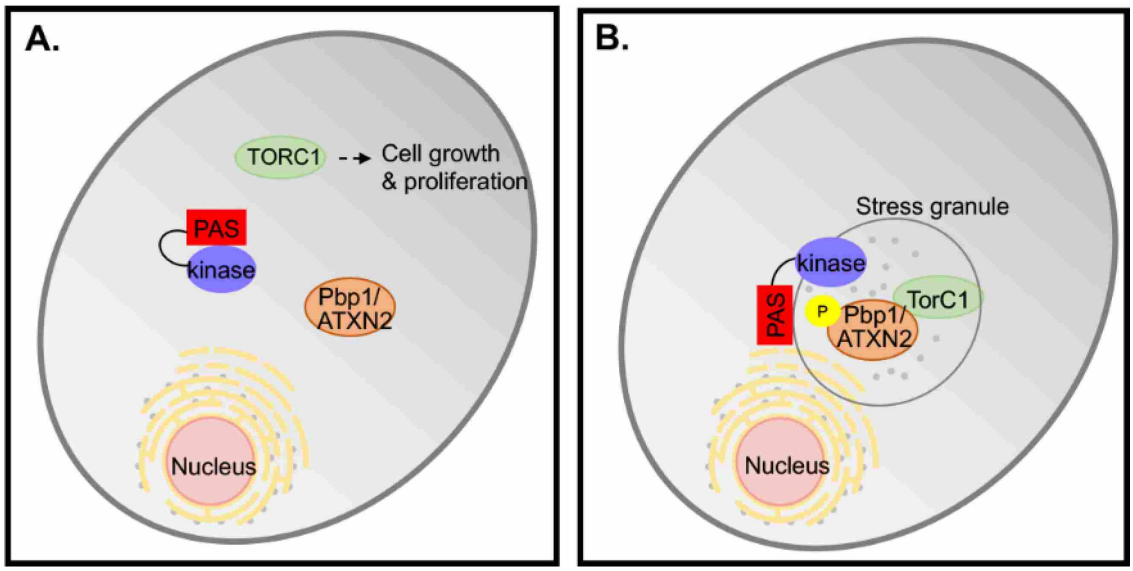

Figure 2-5: A model for the regulation of Pbp1/ATXN2 by PAS kinase.
A) When PAS kinase is inhibited, Pbp1 is inhibited and TORC1 functions to increase cell growth and proliferation. B) When PAS kinase is activated, it phosphorylates Pbp1/ATXN2 which then inhibits TORC1 function through sequestration at stress granules.

We have previously demonstrated the direct phosphorylation of Pbp1 by Psk1 as well as the reduced localization of Pbp1 at stress granule and recovery of TORC1 activity in PAS kinase-deficient yeast (37). There are two homologs of PAS kinase in yeast, Psk1 and Psk2. This is of interest because proteins that arise from gene duplication often evolve related but differential function (99). 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. TORC1 contains the protein encoded by TOR1 or TOR2 as well as Kog1(Raptor), Tco89 and Lst8, and regulates protein synthesis and cell size in response to

nutrients (100). In contrast, TORC2 contains only Tor2 along with Avo1, Avo2, Avo3, Bit61 and Lst8 and is responsible for polarization of the actin cytoskeleton as well as cell division (101). We, therefore, hypothesized that Psk1 and Psk2 may have differential roles and explored the role of Psk2 in Pbp1 regulation. In addition to its phosphorylation by Psk1, we provide evidence for in vitro phosphorylation of Pbp1 by Psk2 (Figure 2A). In addition to in vitro kinase assays, our phenotypic assays also suggest that Psk2 ameliorates caffeine toxicity due to Pbp1 upregulation similar to its homolog Psk1 (Figure 3).

Crosstalk between PAS kinase and TORC1 has been previously reported in mammalian cells. However, the molecular mechanisms of this crosstalk are unknown. Our findings suggest it could be through the phosphorylation and regulation of ATXN2, the mammalian homolog of Pbp1. First, we provide evidence for the complementation of PBP1-deficient yeast by ATXN2 (Figure 1). Second, PAS kinase is able to phosphorylate ATXN2 in vitro (Figure 2A) and to regulate its function in vivo (Figure 3B). Specifically, under caffeine induced stress, upregulation of ATXN2 inhibits TORC1 and thus inhibits cell growth and proliferation. Caffeine sensitivity can be rescued in psk1psk2 cells, suggesting that phosphorylation of ATXN2 by PAS kinase activates it which then inhibits TORC1 (Figure 3B). Thus, this pathway, the suppression of ATXN2 activity by PAS kinase, may be conserved in mammalian cells.

Our discovery of a kinase regulator of ATXN2 could have important therapeutic implications for neurodegenerative diseases. Mutations in ATXN2 can cause SCA2 or ALS. These mutations appear to confer a gain of function. Indeed, reducing ATXN2 levels can mitigate phenotypes in mouse models of ALS and SCA2 (36, 95). Studies of Atxn2 knockout mice have implicated ATXN2 in the development of obesity, insulin resistance, and dyslipidemia. Our study provides evidence that ATXN2 protein levels are significantly decreased

in brain tissue from PASK-/- mice that are on a western (HFHS) diet (Figure 4). However, no significant difference was seen when mice are on the standard diet, suggesting diet may play a role and/or that the effects of PAS kinase are smaller and require a larger sample number. The available data on the effects of a western diet on ALS progression are relatively unknown, and diet impacts in general appear to be complex (for recent reviews see (102, 103)). The published studies suggest high calorie diets (high fat, high protein or high carbohydrate) may aid in weight stabilization (104, 105), which is a common marker for poor prognosis in ALS patients (106, 107), but further studies are needed to understand the effect of diet on disease progression. Although further study is needed, diet may be part of a combinatorial therapeutic approach for improving outcomes in ALS.

The decrease in ATXN2 protein in PAS kinase-deficient mice on a HFHS diet is consistent with the in vitro phosphorylation of ATXN2 by hPASK and the suppression of ATXN2 function by PAS kinase deletion in yeast. Thus, the characterization of ATXN2 and its potential regulation by PAS kinase may be key in understanding the development of this devastating disease and nominate PAS kinase as a potential therapeutic target.

2.6 Acknowledgements

We thank Jared Rutter (University of Utah) for the PASK-deficient mice as well as Haley Burrell, Jeralyn Franson and Jenny Pattison for care of the mice (8). Funding for this work was supported by National Institutes of Health Grant R15 GM100376-01 (J.H.G), a grant from the Robert Packard Center for ALS Research At Johns Hopkins (J.H.G. and A.D.G), The Arnold and Mabel Beckman Foundation for the funding of 2018 Beckman Scholar (B.A.P. and J.H.G., Brigham Young University), and the Brigham Young University Department of Microbiology and Molecular Biology and College of Life Sciences.

Table 2-2: Putative Pbp1 binding partners identified by the yeast two-hybrid
The gene name, human homolog, description, cellular localization (Local.), number of times it was retrieved (#), yeast library screened (cDNA or Genomic library), construct description (amino acids fused to the prey domain) and the growth strength of each putative partner (Weak or Strong) is provided. Putative binding partners were placed into functional categories based on their reported function. 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

Gene	Human homolog	Gene description	Local.	#	Pray library	Construct (aa)	Growth strength
RNA Processing							
MUM2	ERC2	mRNA methylation	N/C	3	cDNA	aa170 to aa298 (aa366)	W
PAT1	SLC36A1	Deadenylation-dependent mRNA-decapping factor	N/C	1	Gen	aa2 to aa192 (aa796)	S
PKH1	Pdpk1	Cytoplasmic mRNA processing body assembly	C/V	1	cDNA	aa649 to aa741 (aa766)	M
SGDID:S000003482	-	Hypothetical Protein. Putative RNA binding protein	C/SG	2	Gen	aa509 to aa718 (aa718)	W
Other Functions							
YEN1	GEN1	Response to DNA damage stimulus	N/C	2	Gen	aa504 to aa759 (aa759)	W
PTC6	PPM1K	mitochondrion degradation, macroautophagy	M	1	Gen	aa1 to aa120 (aa442)	S
SEC61	SEC61A2	misfolded protein transport	ER/MEM	1	Gen	aa407 to aa480 (aa480)	M

Table 2-3: Putative Pbp1 binding partners identified by the mass spectrometry
The gene name, human homolog, description, cellular localization (Local.) and the number of times the protein was retrieved from two independent runs are provided. For localization: C, Cytoplasm; M, Mitochondrion; N, Nucleus; ER, Endoplasmic Reticulum; PM, Plasma Membrane; W, Cell Wall; G, golgi; SG, Stress Granule; S, meiotic Spindle; ES, Endosome; CD, Cell Division.

Gene	Human Homolog	Name description	Local.	#
RNA Processing				
IMP	IMP4	snoRNA-binding rRNA-processing protein	N	2
SSD1	N/A	mRNA-binding translational repressor	N/C	1
Protein Transporter				
TRS31	TRAPPC5	Core component of transport protein particle (TRAPP) complexes I-III	C/G	1
VPS29	VPS29	Vacuolar Protein Sorting	ES/ V	1
SSY1	N/A	Component of the SPS plasma membrane amino acid sensor system	PM	1
SEC24	SEC24	COPII vesicle coat component	ER/G/C	1
TIM9	TIMM9	Mitochondrial inner membrane translocase	M	1
Cell Cycle				

CDC50	TMEM30	Cell division control protein 50	ES	1
MCM5	MCM5	MiniChromosome maintenance protein 5	N	1
APC9	N/A	Anaphase Promoting Complex subunit 9	CD	1
BNR1	FMNL1	BNI1-related protein 1	CD	1
CYR1	PHLPP2	Adenylate Cyclase & magnesium ion binding	CD	1
CDC3	SEPT7	component of 10 nm filaments of mother-bud neck	CD	1
SPR28	SEPT1	Septin-related protein expressed during sporulation	W/ S	1
FUN19	TADA2A	SWIRM domain-containing protein	C	1
Other				
INA22	N/A	Inner membrane assembly complex subunit 22	M	2
RAD33	N/A	Nucleotide excision repair	N	1
APE3	ERMP1	Aminopeptidase Y	V	1
FRE7	CYBB	Ferric reductase PM	PM	1
NIT1	N/A	Nitrilase		1
ZRT2	N/A	low-affinity Zn(2+) transporter	PM	1
MOT3	N/A	Involved in cellular adjustment to osmotic stress	N/C	1
Protein of Unknown function				
YHR131CP	N/A	Protein of Unknow function	C	1
FRT1	N/A	Tail-anchored ER membrane protein of unknown function	ER	1
SYG1	XPR1	Signal Transduction	PM/ER/G	1

CHAPTER 3: A Pbp1 Interactome Reveals Novel Roles in Mitophagy and Cell Cycle Regulation Through Protein Sequestration at Stress Granules

Pape JA, Melhado ES, Newey CR, Choksi N, Grose JH

My contributions to this paper:

I produced the data for Figures 3-2 and 3-3 and constructed the related strains. I optimized the flow cytometry protocol for the strains, including unhealthy strains that exhibit higher levels of RNA to interfere with DNA staining. This includes running some of the samples on the flow cytometer and analyzing the data. I wrote all sections pertaining to this data (3.3.1, 3.3.7-3.3.9, 3.4.2, 3.4.3), and the corresponding discussion. Additionally, I merged and edited the sections written by Jenny Pape that pertain to her data (Ptc6) with my own, and in doing so added sections throughout to create a single narrative, rather than two separate projects.

3.1 Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal disease with few non-cure treatment options. Investigations into the molecular mechanisms of ALS have revealed ataxin-2 to be associated with ALS-related neurodegeneration through multiple pathways, including the sequestration of mRNA and proteins at stress granules. Understanding the regulation and function of ataxin-2 may therefore provide key insights into neurodegeneration. Herein, we expand the known function of ataxin-2 through a protein-protein interactome study of the yeast homolog, Pbp1, revealing 32 novel binding partners. Several proteins associated with the known function of Pbp1 in mRNA processing were identified, as well as proteins not previously associated with Pbp1, including proteins in cell cycle and mitophagy pathways. The novel binding partner Ptc6 was investigated further due to its relationship to TORC1 and mitophagy. Pbp1 was found to inhibit Ptc6 via sequestration to stress granules. Additionally, Pbp1 deficient

strains displayed increased mitophagy while Pbp1/Ptc6 deficient strains displayed normal mitophagy. Analysis of the cell cycle revealed Pbp1 affects the cell cycle through increased G2/G1 ratio, and sequestration of APC9 at stress granules. The identification of Pbp1 binding partners, especially those that affect general cell health and viability, contributes to the understanding of ataxin-2/Pbp1 and how it is involved in disease.

3.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease. With no known cure and only a handful of known genetic components, treatment options for patients typically revolve around symptomatic management, such as physical therapy, pain relievers, and dietary supplementation (108, 109). Therefore, there is a desperate need for an effective drug to be developed that will ameliorate disease symptoms and extend patient lifespan. Recently, intermediate-length ataxin-2 polyQ repeat expansions were identified to be significantly associated with susceptibility of ALS in humans (110). While little is known about ataxin-2, initial studies have revealed that therapeutic knockdown of ataxin-2 in TDP-43 transgenic ALS model mice extends lifespan when compared controls (36). This suggests that higher levels of ataxin-2 are associated with shorter lifespan, while lower levels of ataxin-2 are associated with a longer lifespan. Using ataxin-2 as a therapeutic target is promising, though not without side-effects, especially when considered complete inhibition of ataxin-2. Deletion of ataxin-2 in mice has been shown to have adverse side-effects, including obesity, insulin resistance, and hyperlipidemia (111, 112), suggesting that the deletion significantly impairs healthy cellular metabolism. It is unclear what other pathways are being altered in response to ataxin-2 deficiency, suggesting that reducing ataxin-2 levels, rather than depleting them, may restore healthy function of the gene instead of ameliorating its function.

A newly identified regulator of ataxin-2 is PAS kinase. PAS kinase is a nutrient sensing protein kinase that is conserved between yeast, mice, and humans (113). PAS kinase is a promising regulator because PAS kinase deficiency protects against obesity, insulin resistance, and hyperlipidemia in mice on a high-fat diet, many of the phenotypes associated with ataxin-2 deficiency in mice (114, 115). Furthermore, PAS kinase is influenced by both diet and sex, two traits also seen to differ ALS pathology in both human and mouse studies (114). This suggests that PAS kinase is working in conjunction with ataxin-2 to regulate many cellular functions, especially those related to metabolic and mitochondrial health.

In yeast, PAS kinase has been shown directly phosphorylate the homolog of ataxin-2, Poly A binding protein binding protein 1, Pbp1. Under cellular stress, PAS kinase phosphorylates and activates Pbp1, sequestering it to stress granules. This function is not surprising, as Pbp1 is reported to be involved with sequestration of mRNA and proteins to stress granules (55, 116). This model has been verified by monitoring the known interactions of Pbp1 and TORC1 (Figure 1). Pbp1 overexpression has been shown to cause caffeine sensitivity and resulting inhibition of cellular growth through the sequestration of TORC1 to stress granules (116). In PAS kinase deficient yeast, Pbp1 is no longer phosphorylated and activated by PAS kinase, thus preventing the sequestration of TORC1 to stress granules by Pbp1 and promoting cellular growth on caffeine (117).

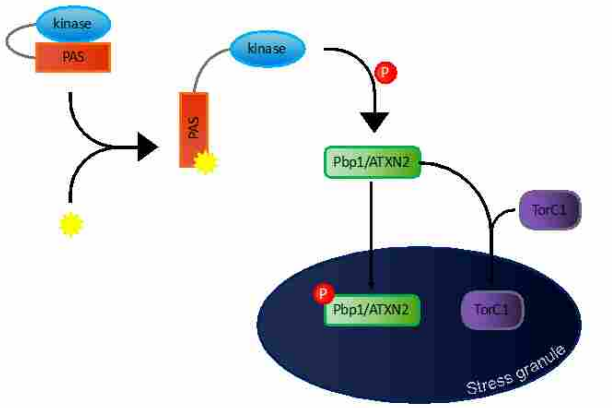

Figure 3-7: PAS kinase phosphorylates and activates Pbp1/ataxin-2, causing sequestration of Pbp1/ataxin-2. Pbp1/ataxin-2 also influences the sequestration of other proteins to the stress granules.

Understanding the PAS kinase-ataxin-2 pathway is key to understanding the molecular pathways involved in stress granule formation as well as neurodegenerative disease. While some functions of ataxin-2 have been outlined, little is known about how it interacts with other proteins to alter conditions in the cell. Currently, only four binding partners of Pbp1 have been characterized—Pab1, Lsm12, Pbp4, and Dhh1, all proteins involved in stress granule formation (55). Herein we report a yeast Pbp1 interactome obtained from both yeast two-hybrid as well as copurification techniques. In addition, two key binding partners, Ptc6 and Apc9, are further characterized and the effects of Pbp1 on mitophagy and cell cycle are analyzed. Identifying additional binding partners and understanding their effects on normal cellular functions will shed light onto how Pbp1 is affecting neurodegeneration and help identify disease-associate genes.

3.3 Materials and methods

3.3.1 Growth assays

Lists of strains, plasmids, and primers used in this study are provided in Table 1-3, respectively. For plasmid construction, standard PCR-based cloning methods were used. Restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Yeast knockouts were from the Saccharomyces Genome Collection (118). Double knockouts were obtained using primers designed 300 bp upstream and downstream of the respective gene and transforming the PCR products PCR products into JGY1122 harboring a Pbp1 overexpression plasmid (pJG925) to recover lost function that was suspected to be preventing the successful creation of double knockouts. Strain knockouts were verified by PCR and plated on 5'FOA agar to remove the plasmid. To transform plasmids or PCR products into strains, 200 microliters of an overnight culture of yeast were centrifuged at 10,000 rpm for 2 minutes prior to resuspension in the transformation mixture, consisting of DTT, PEG, and Tris. PCR amplifications of regions of

interest or plasmids were added into the transformation mixture and this was incubated at 45° C for 45 minutes. After an appropriate recovery time in YPAD, the mixture was plated on the appropriate selection media and allowed to grow for 48-72 hours. 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) (119) (pJG1386 (JG2916/3163)). Yeast two-hybrid libraries are described in an earlier work (120). Yeast two-hybrid Gold cells (Clontech, Mountain View, CA) were used to transform in bait and prey plasmids for interaction studies.

For single mutant phenotype serial dilution growth assays, spot-dilutions were performed by growing yeast in overnight liquid culture for 48 hours, serially diluting 1:10 in water and spotting on selective plates as well as control plates. For synthetic lethal spot-dilutions the first dilution was 1:10 and all subsequent were 1:5. Plates were incubated at 30°C for 1–10 days until colonies were apparent.

Table 3-1: Yeast strains used in this study

Strain	**Bkgd**	**Genotype**	**Abbreviation**	**a/ α**	**Reference or source**
JGY1	W303	ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1	WT	a	David Stillman (University of Utah,)
JGY2	W303	Psk1::his3 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1	PSK2psk1	a	Grose *et al.* (2007)
JGY3	W303	psk2::kan-MX4 ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1	PSK1psk2	a	Grose *et al.* (2007)
JGY4	W303	psk1::his3 psk2::kan-MX4 ade21, can1-100 his3-11,15 leu2-3112, trp1-1 ura3-1	psk1psk2	a	Grose *et al.* (2007)
JGY299	S288C	PSK2-TAPtag::kanMX ura3-0 trp1-0 SUC2 mal mel gal2 CUP1 flo1 flo8-1	WT	α	Jared Rutter (University of Utah,)
Y2H Gold (JGY1031)		LYS2::GAL1UAS-GAL1TATA-His3, GAL2UAS-Gal2TATA-Ade2 URA3::MEL1UAS-MEL1TATA, AUR1-CMEL1, ura3-52 his3-200 ade2-101 trp1-901 leu2-3, 112 gal4del gal80del met-	Y2H Gold	a	Clontech
Y187 (JGY1073)		URA3::GAL1-GAL1-LacZ, MEL1, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4del, gal80del, met-	Y187	α	Clontech

JGY1074		pGADT7 Mate and Plate stationary phase YPAD library in JGY1073	YPAD library	α	DeMille *et al.* (2014)
JGY1098		pGADT7 Mate and Plate mid-log YPAGal library in JGY1073	YPAGal library	α	DeMille *et al.* (2014)
JGY1122	BY4743	*Pbp1::KanMx4* His3-1, leu2-0, lys2-0,ura3-0	pbp1	α	Janet Shaw (University of Utah, Salt Lake City, UT)
JGY1161	S288C	psk1::hygro psk2::NAT ura3-0 trp1-0 SUC2 mal mel gal2 CUP1 flo1 flo8-1	psk1psk2	α	DeMille *et al.* (2015)
JGY1383		*pJG1386 Pbp1 in Y2H bait* ura3-52, his3-200, ade2-101, trp1-901,leu2-3,112, gal4del	PBP1-Y2H Gold	a	This study
JGY43	BY4741	His3d1, leu2DO, met15DO, ura3DO	WT		Jared Rutter
JGY1244	BY4741	Psk2::NAT, psk1::hygro, his3D1, leu2DO, met15DO, ura3DO.	psk1psk2		
JGY1598	BY4741	Ptc6::kanMX, his3D1, leu2Do, met15DO, ura3DO	ptc6		
JGY1599	BY4741	Spr28::kanMX, His3d1, leu2DO, met15DO, ura3DO	spr28		
JGY1600	BY4741	Apc9::kanMX, His3d1, leu2DO, met15DO, ura3DO	apc9		
JGY1601	BY4741	Fun19::kanMX, His3d1, leu2DO, met15DO, ura3DO	fun19		
JGY1602	BY4741	Bnr1::kanMX, His3d1, leu2DO, met15DO, ura3DO	bnr1		
JGY1659	BY4741	Spr28::kanMX Pbp1::his3, His3d1, leu2DO, met15DO, ura3DO	spr28pbp1		
JGY1660	BY4741	Apc9::kanMX Pbp1::his3, His3d1, leu2DO, met15DO, ura3DO	apc9pbp1		
JGY1661	BY4741	Fun19::kanMX Pbp1::his3, His3d1, leu2DO, met15DO, ura3DO	fun19pbp1		
JGY1662	BY4741	Bnr1::kanMX Pbp1::his3, His3d1, leu2DO, met15DO, ura3DO	bnr1pbp1		
JGY1603	BY4741	PTC6-GFP::HIS3MX6, his3-1, leu2-0, met15-0, ura3-0	ptc6-GFP		O'shea collection (Thermo-Fisher)
JGY1591	BY4741	OM45-GFP::His, leu2DO, met15DO, ura3DO	WT		
JGY1593	BY4741	OM45-GFP::His, leu2DO, met15DO, ura3DO	pbp1		
JGY1624	BY4741	OM45-GFP::His, leu2DO, met15DO, ura3DO	ptc6		
JGY1594	BY4741	OM45-GFP::His, psk1::hygro, psk2::nat, leu2DO, met15DO, ura3DO	psk1psk2		
JGY1608	BY4741	OM45-GFP::His, psk1::hygro, psk2::nat, pbp1::kanMX, leu2DO, met15DO, ura3DO	psk1psk2pbp1		
JGY1623	BY4741	OM45-GFP::His, psk1::hygro, psk2::nat, ptc6::kanMX, leu2DO, met15DO, ura3DO	psk1psk2ptc6		
JGY1625	BY4741	OM45-GFP::His, atg32::kanmx, leu2DO, met15DO, ura3DO	atg32		

Table 3-2: Plasmids used in this study

Plasmid	Gene	Description	Backbone	Yeast origin	Selection	Reference or source
pJG9	PSK1	Psk1 in pRS424	pRS424	2u	Trp	Jared Rutter
pJG173	PSK2	Psk2 in pJR1765B	pJR1765B	2u	Ura	Jared Rutter
pJG174	PSK2	Psk2 K870R kinase dead mutant	pJR1765B	2u	Ura	Jared Rutter
pJG421	EV	pGAD-C1 empty Y2H prey vector	YEp-GAD	2u	Leu	James *et al.* (1996)
pJG424	EV	pGBD-C1 empty Y2H bait vector	YEp-GBD	2u	Trp	James *et al.* (1996)
pJG428	Library	pGAD-C2 genomic library	YEp-GAD	2u	LEU	James *et al*. (1996)
pJG429	Library	pGAD-C3 genomic library	YEp-GAD	2u	LEU	James *et al*. (1996)
pJG598	PSK1	ΔN692Psk1 in pJG425	YEp-GBD	2u	Trp	DeMille *et al.* (2014)
pJG725	EV	pADH-Myc	pRS416	CEN	Ura	David Stillman
pJG734	PBP1	ADH ΔN420Pbp1-Myc	pRS416	CEN	Ura	David Stillman
pJG858	PSK1	pGAL1-10, Psk1-HIS/HA	pRS426	2u	Ura	DeMille et al. (2014)
pJG859	EV	pGAL1-10, HIS/HA	pRS426	2u	Ura	DeMille et al. (2014)
pJG925	PBP1	Full-length Pbp1 in pJG859	pRS426	2u	Ura	DeMille et al. (2015)
pJG960	PSK1	pGAL1-10, ΔN693Psk1-HIS/HA	pRS426	2u	Ura	DeMille et al. (2015)
pJG1250	PBP1	pGAL1-10, ΔN419Pbp1-Myc	pRS426	2u	Ura	DeMille et al. (2015)
pJG1251	PBP1	pGAL1-10, Full length Pbp1-Myc	pRS426	2u	Ura	DeMille et al. (2015)
pJG1271	PSK1	Psk1-D1230A kinase dead mutant	pRS426	2u	Ura	DeMille et al. (2014)
pJG1331	ATXN2	Full length ATXN2	pGEM-T	2u	Ura	Sino Biological Inc.
pJG1359	ATXN2	ΔN379 ATXN2 in pJG859	pRS426	2u	Ura	This Study
pJG1360	ATXN2	ΔN554 ATXN2 in pJG859	pRS426	2u	Ura	This Study
pJG1361	ATXN2	ΔN700 ATXN2 in pJG859	pRS426	2u	Ura	This Study
pJG1386	PBP1	ΔN419Pbp1 in pJG424	pRS416	CEN	Ura	This Study
pJG1409	PBP1	Full length Pbp1 in pJG725	pRS416	CEN	Ura	This Study
pJG1542	PBP1	Pbp1 with mutations: S106A, T193A, S436A, S479A, T708A, T718A made with pJG1251	pRS416	CEN	Ura	This Study
pJG1523	PBP1	Pbp1 with mutations: S106A, T193A, T355A, S436A, S479A, T708A made with pJG1251	pRS416	CEN	Ura	This Study
pJG1544	PBP1	Pbp1 with mutations: S106A, T215A, T355A, S436A, S479A, T708A, T718A made with pJG1251	pRS416	CEN	Ura	This Study

pJG1546	PAT1	PTA1 in Y2H bait			Leu	DeMille et al. (2014)
pJG1548	Hypothetical gene	Hypothetical gene in Y2H bait			Leu	DeMille et al. (2014)
pJG1549	YEN1	YEN1 in Y2H bait			Leu	DeMille et al. (2014)
pJG1560	PBP1	Full length Pbp1 in pJG859	pRS426	2u	Ura	This Study
pJG1561	PBP1	Pbp1 with mutations: S106A, T193A, S436A, S479A, T708A, T718A made with pJG12560	pRS426	2u	Ura	This Study
pJG1562	PBP1	Pbp1 with mutations: S106A, T193A, T355A, S436A, S479A, T708A made with pJG1560	pRS426	2u	Ura	This Study
pJG1563	PBP1	Pbp1 with mutations: S106A, T215A, T355A, S436A, S479A, T708A, T718A made with pJG1560	pRS426	2u	Ura	This Study
pJG1572	SEC61	SEC61 in Y2H bait			Leu	DeMille et al. (2014)
pJG1573	PTC6	PTC6 in Y2H bait			Leu	DeMille et al. (2014)
pJG1574	MUM2	Mum2 in Y2H bait			Leu	DeMille et al. (2014)
pJG1575	PKH1	PKH1 in Y2H bait			Leu	DeMille et al. (2014)
pJG1669	PBP1	PBP1-RFP	pRS426	2u	Ura	This Study
pJG1690	SEC63	Sec63-RFP			Ura	Katrina Cooper

Table 3-3: Primers used in this study

Primer	**Sequence**
JG2335	TTCGATGATGAAGATACC
JG2761	CTATTCGATGATGAAGATACCCCACC
JG2762	AGATGGTGCACGATGCACAG
JG2916	GCCTCGAGGTTTATGGCCACTGGTACTACTATTATGG
JG2917	GGCGAATTCATGAAGGGAAACTTTAGGAAAAGAG
JG2953	GAAGGAAGGAAAGCTCAATTGGGAG
JG2954	GATTGAAATACTGATTACTTAAAATTTGC
JG2993	CGCCAGGGTTTTCCCAGTC
JG3037	CAACCATAGGATGATAATGCGATTAG
JG3136	GGCGAATTCTCGTTGCCTCCAAAACCGATCAGC
JG3384	GGCGAATTCATGTCGTTGCCTCCAAAACCGATCAGC
JG3704	GGCGTCGACTTACAACTGCTGTTGGTGGTGGGC
JG3758	GGCCAATTGATGCGCTCAGCGGCCGC
JG3759	GGCCAATTGATGACCCCCAGTGGGCCAGTTCTTG
JG3760	GGCCAATTGATGTCGCCTTGCCCATCTCCTTCC
JG3761	GGCCAATTGATGCTCACAGCCAATGAGGAACTTGAG
JG3854	GGCGTCGACGCAACTGCTGTTGGTGGTGGGC
JG4531	GATCGATTCAAAGTTACGACAGTCA
JG4536	ACCCTATTTATATCTCTTCTTTCTAG

JG4537	CAACTGCCGTTCCCACTGC
JG4538	TGAAAGTTCCATAATTGAAATACGTCG
JG4539	CTTTGAGAAACAAGACGTAAAATACG
JG4540	TCGATATAAGGACGAATAATGTCC
JG4542	TTAAAGAGCGCCGCTACCATTTG
JG4543	AGCCCTGATGTTGAAGATTTTCTC

3.3.2 Yeast 2-Hybrid screening

A yeast 2-hybrid system was 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 (121). Pbp1 was cloned into binding domain (pJG1386) and the library was previously cloned into activation domain (120) (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.

3.3.3 Yeast 2-Hybrid screening by mating

For cDNA library screens, α haploid yeast harboring a cDNA Y2H prey library (JGY1074 and JGY1098) (120) 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.

3.3.4 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 (119). 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.

3.3.5 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 (122) analysis (an unambiguous hit with e-value of ≥10e−45). For verification and elimination of false positives, library plasmids were purified from yeast (123), 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).

3.3.6 Mass spectrometry

Full length Pbp1 (pJG1560) into HIS expression under the GAL1-10 promoter was made by PCR amplifying Pbp1 with primers JG2916/2917 and cloning into the EcoRI/XhoI sites of pJG859. The protein was purified, run on 10% SDS PAGE, stained with Coomassie blue and 78 kDa protein purified with it was cut from protein gel using a sterile razorblade. The gel slice was

flash frozen at -80°C and sent to the University of San Diego Biomolecular and Proteomics Mass Spectrometry Facility for analysis.

3.3.7 Cell cycle analysis sample fixing

Strains were grown in plasmid-selective SD-Ura media for 24 hours, then the cultures were centrifuged for 5 minutes at 500 rpm and the supernatant discarded. The cells were resuspended in inducive S-Gal-Ura media and allowed to grow for 24 additional hours. Cultures were then fixed in 95% EtOH at a ratio of 3 mL EtOH to 1 mL culture and placed in -20° C for overnight to one week.

3.3.8 DNA staining

Depending on the OD as read from a spectrophotometer, 50-200 ul of the culture were prepared according to the Rosebrock protocol (124). Samples were centrifuged for 20 minutes at 15,000 rpm and the supernatant discard. Then, samples were washed with Sodium Citrate Buffer twice and allowed to rehydrate. After complete rehydration, samples were treated with RNase and SYTOX-green and incubated in the dark. Initially, samples were treated with RNase for an one-hour digestion time. Later repetitions were digested for up to four hours because of clouding RNA presence. Proteinase K was subsequently added to halt RNase and cells were cooled to 4° C. Prior to running, the samples were sonicated for 30 seconds at 3V using a needle tip sonicator.

3.3.9 Flow cytometry

Samples were run on a Cytoflex cytometer. Samples were run at 400-800 events/second and 80,000 events were collected for each sample. Data gating was kept minimal, only to remove dead cells from the dataset and to reduce dye noise by plotting FITC-H against FITC-A, as shown in the Rosebrock protocol (124). The stages were discerned using Flow Jo's Cell Cycle

automatic mathematic model Watson Pragmatic algorithm. At minimum, three replications for each constructed strain were collected and used in calculations.

3.3.10 PTC6 and APC9 localization studies

Apc9-GFP (JGY 1656) and Ptc6-GFP fusion yeast (JGY1603) were obtained from the O'Shea collection (ThermoFisher). For colocalization with Pbp1 at stress granules, Pbp1-RFP was transformed into JGY1656 or JGY1603 to monitor colocalization of Pbp1, Apc9 and Ptc6 to stress granules. Overnight samples were grown according to Demille *et al.* Briefly, samples were grown in in SD-Ura medium overnight, pelleted and re-suspended in SGal-Ura to induce expression of Pab1-RFP. Cultures were then grown 4 days, pelleted, washed, and re-suspended in synthetic complete medium. Cultures were grown for an additional hour at 30°C. Confocal fluorescent imaging stacks were acquired using an Olympus FV1000 confocal microscope. A 60x lens was used with 10x zoom, 0.4 μm step size and resolution of 640x640 pixels/frame for image acquisition. Deconvolution of images was performed using cellSens (Olympus).

3.3.11 Mitophagy assay

Mitophagy assays were performed via the method outlined by Kanki *et al.* Briefly, Om54-GFP DNA fragment was amplified from JGY1586 using JG4522 and JG4523. PCR product was transformed into JGY43, JGY1122, JGY 1598, and JGY1244 using standard yeast transformation methods. Correct integration was verified through fluorescence microscopy and PCR analysis of the genomic DNA (JG4524/JG4525).

Single colonies were picked and used to inncoluate 5mL YPD and grown to mid-log phase. Aliquots equal to OD~0.1 were collected for t=0, and the remaining cultures were centrifuged and re-suspended in a flask containing YPL (OD ~0.2). Cultures were grown shaking for 48 hours at 30°C, then aliquots equal to OD~0.1 were collected by centrifugation, re-

suspended in 50 µL of 10% TCA, incubated on ice for 10 minutes, and frozen until ready to be broken open for SDS-Page. To break open, samples were thawed on ice and pelleted by centrifugation at 21,000xg for 10 minutes. Supernatant was removed, pellets were washed twice with 500 uL of cold acetone, and air dried. Samples were then re-suspended in 50 µL sample buffer (150 mM Tris-HCl, pH 8.8, 6% SDS, 25% glycerol, 6 mM EDTA, 0.5% 2-mercaptoethanol, and 0.05% bromophenol blue), disrupted by vortex with an equal amount of glass beads for 3 minutes, and incubated at 100°C for 3 minutes.

5 µL of sample was loaded on a 12% polyacrylamide gel and resolved. Transfer was completed using the TurboBlot program, 2 midi-gel mixed MW program, transferring onto a nitrocellulose membrane. Membranes were blocked with 5% milk, then probed with anti-GFP antibody (1:5000 TBST) at 4°C overnight, washed, then probed with HRP-conjugated anti-mouse (1:10,000 TBS) for 1 hour at room temperature. Membranes were developed using the WesternBright ECL HRP substrate (Advansta Inc., San Jose, CA, USA, catalog number K-12045-D50) according to the manufacturer's protocol. Membranes were then striped and probed with anti-UGP1 to ensure equal loading between samples. Bands were quantified using the ImageJ software version 1.50i (National Institute of Health, Bethesda, MD, USA).

3.4 Results

3.4.1 Uncovering a Pbp1 interactome from yeast two-hybrid and copurification

Human ataxin-2 plays a pivotal role in the development of human ataxias, yet little is known about its function (36, 110, 125). To elucidate the function of ataxin-2, a large-scale interactome screen was performed using the yeast two-hybrid (Y2H) approach and copurification. Y2H screens were conducted with ΔN419Pbp1 as bait and two previously published yeast genomic libraries (pJG428, and pJG429) (119) as well as two cDNA libraries

(JGY1098 and JGY1074) as prey (120). The ΔN419Pbp1 was utilized as bait because only false positive hits were retrieved from several screens from the full-length Pbp1 construct was nonfunctional in our hands in that. From ~10 million transformants or mated yeast screened, seven Pbp1 binding partners were identified (Table 4). Four partners are RNA binding proteins or involved in mRNA processing which is consistent with the known role of Pbp1, while the other three have roles in stress response, mitophagy and misfolded protein transport. The strength of growth is an indication of the interaction strength. Advantages of the Y2H approach include the identification of direct protein–protein interactions and sensitivity due to transcriptional amplification of an interaction (120). 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), 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 (119).

An alternative sensitive and reliable approach for identifying protein binding partners is copurification followed by quantitative mass spectrometry (MS). Full-length Pbp1 fused with histidine (HIS), epitope was affinity purified in triplicate, and samples were subjected to quantitative liquid chromatography (LC)–tandem MS. Twenty-five putative binding partners were identified for Pbp1 (Table 5). The Y2H screen retrieved all novel binding partners, whereas the copurification screens retrieved both expected and novel binding partners. The copurification screen retrieved two proteins involved in RNA processing five associated with protein transport and eight involved in cell cycle.

Table 3-4: Pbp1 binding partners identified by the yeast two-hybrid
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

Gene	Human Homolog	Gene name description		Localization	Number of hits	Pray library used	Construct (Total number of aa)	Growth strength
	RNA Processing							
MUM2	ERC2	mRNA methylation		N/C	3	cDNA	aa170 to aa298 (aa366)	W
PAT1	SLC36A1	Deadenylation-dependent mRNA-decapping factor		N/C	1	Gen	aa2 to aa192 (aa796)	S
PKH1	Pdpk1	Cytoplasmic mRNA processing body assembly		C/V	1	cDNA	aa649 to aa741 (aa766)	M
Hypothetical Protein (SGDID:S000003482)	-	Putative RNA binding protein,localizes to stress granules induced by glucose deprivation		C/SG	2	Gen	aa509 to aa718 (aa718)	W
	Other Functions							
YEN1	GEN1	Response to DNA damage stimulus		N/C	2	Gen	aa504 to aa759 (aa759)	W
PTC6	PPM1K	mitochondrion degradation, macroautophagy		M	1	Gen	aa1 to aa120 (aa442)	S
SEC61	SEC61A2	misfolded protein transport		ER/MEM	1	Gen	aa407 to aa480 (aa480)	M

Table 3-5: Pbp1 binding partners identified by the mass spectrometry
For localization: C, cytoplasm; M, mitochondrion; N, nucleus; ER, endoplasmic reticulum; PM, plasma membrane; W, cell wall; G, golgi; SG, stress granule; S, meiotic spindle; ES, Endosome; CD, cell division sites.

Gene	Human Homolog	Name description	Local-ization	Number of times retrieved
RNA Processing				
IMP	IMP4	snoRNA-binding rRNA-processing protein	N	2
SSD1	N/A	mRNA-binding translational repressor	N/C	1
Protein Transporter				
TRS31	TRAPPC5	Core component of transport protein particle (TRAPP) complexes I-III	C/G	1
VPS29	VPS29	Vacuolar Protein Sorting	ES/ V	1
SSY1	N/A	Component of the SPS plasma membrane amino acid sensor system	PM	1
SEC24	SEC24	COPII vesicle coat component	ER/G/C	1
TIM9	TIMM9	Mitochondrial inner membrane translocase	M	1
Cell Cycle				
CDC50	TMEM30	Cell division control protein 50	ES	1
MCM5	MCM5	MiniChromosome maintenance protein 5	N	1
APC9	N/A	Anaphase Promoting Complex subunit 9	CD	1
BNR1	FMNL1	BNI1-related protein 1	CD	1
CYR1	PHLPP2	Adenylate Cyclase & magnesium ion binding	CD	1
CDC3	SEPT7	component of 10 nm filaments of mother-bud neck	CD	1
SPR28	SEPT1	Septin-related protein expressed during sporulation	W/ S	1
FUN19	TADA2A	SWIRM domain-containing protein	C	1
Other				
INA22	N/A	Inner membrane assembly complex subunit 22	M	2
RAD33	N/A	Nucleotide excision repair	N	1
APE3	ERMP1	Aminopeptidase Y	V	1
FRE7	CYBB	Ferric reductase PM	PM	1
NIT1	N/A	Nitrilase		1
ZRT2	N/A	low-affinity Zn(2+) transporter	PM	1
MOT3	N/A	Involved in cellular adjustment to osmotic stress	N/C	1
Protein of Unknown function				
YHR131CP	N/A	Protein of Unknown function	C	1
FRT1	N/A	Tail-anchored ER membrane protein of unknown function	ER	1
SYG1	XPR1	Signal Transduction	PM/ER/G	1

3.4.2 Pbp1 deficiency alters the cell cycle

Due to the abundance of cell cycle proteins retrieved from the copurification interactome we investigated the role of Pbp1 in cell cycle regulation. The ratio of cells in G1 phase compared to G2 phase is of interest because these phases involve the synthesis of protein and therefore likely involve RNA-binding proteins more than S phase. Pbp1-deficient cells displayed significantly lower ratios of G1 to G2 cells compared wildtype cells when analyzed utilizing Dunnett's Method for t-test in JMP Pro14 (version 14.0) software (SAS Institute, Cary, NC, USA), with a mean G1/G2 ratio of 8.17 compared to the wild type ratio of 11.98 because of an increase in G2 and a decrease in G1. All statistical comparisons were made using Dunnett's Method for t-testing to maintain the wildtype strain as the primary comparison for all other samples with a p-value cutoff of 0.05. Conversely, yeast deficient in PAS kinase (*psk1psk2)* demonstrated higher G1/G2 ratios than wildtype because of a decrease in G2, with a mean G1/G2 ratio of 18.15.

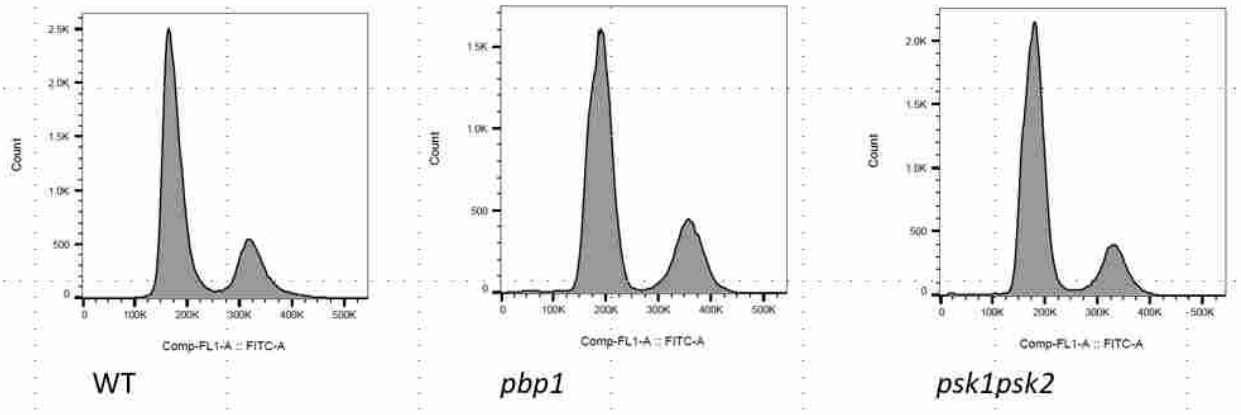

Figure 3-8: Representative histograms of the cell cycle as seen in wildtype, pbp1, and psk1psk2 cells.
Cells were fixed, stained with SYTOX-Green, and run through a Cytoflex Flow Cytometer. Stages of the cell cycle were distinguished by Flow Jo software's integrated cell cycle function. n≥2

3.4.3 Pbp1 deficiency causes synthetic lethality with cell cycle binding partners

Cell cycle alterations were observed in Pbp1-deficient yeast, but the ways by which these proteins affect the cell cycle are unknown. Four cell cycle proteins were chosen from the results listed in Table 5 based on the ability to create single knockout strains. Growth assays were performed to assess the viability of single knockout samples and double knockout samples; serial dilutions were utilized to visualize suspected growth deficiencies and overall growth of strains.

The *pbp1, spr28,* and *apc9* strains show growth comparable to that of the wildtype, but the *spr28pbp1* and *apc9pbp1* strains demonstrate severely stunted growth. Spr28 is a septin-encoding protein, which are conserved family that are required for cytokinesis and the localization of other proteins during cytokinesis and other cell cycle stages (126, 127). Spr28 is highly expressed during meiotic division when it localizes to the prospore membrane (128). Null mutants notably exhibit abnormal vacuolar morphology and are haploinsufficient, however, these mutants remain viable (127-129). Apc9 is a subunit of the anaphase-promoting complex that functions as a ubiquitin transferase. The complex aids the cell in transition from metaphase to anaphase in the cell cycle by degrading anaphase inhibitory proteins and mitotic cyclins, which causes sister

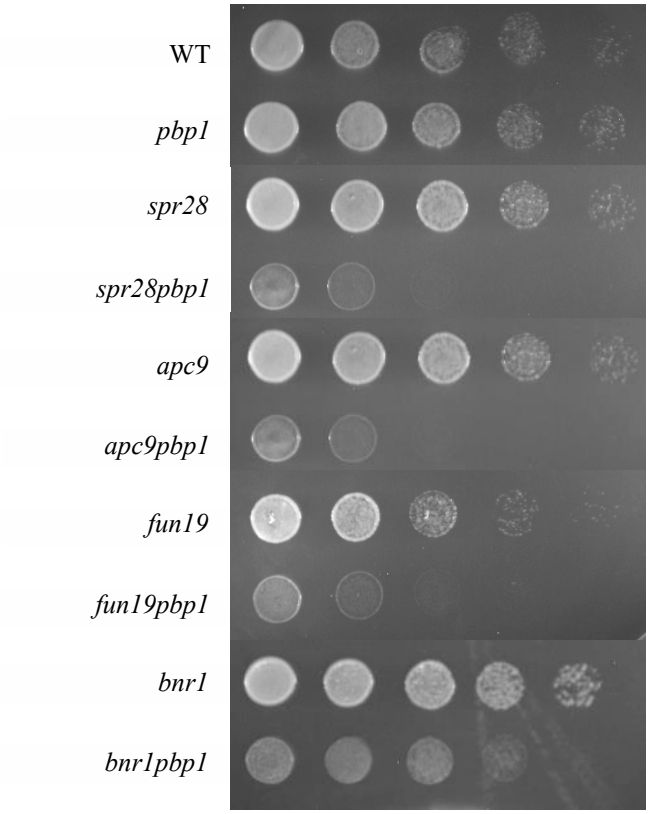

Figure 3-9: Serial dilutions reveal growth defects. No growth defects were observed in single-knockout strains, but double-knockout strains show large defects. All strains were constructed from the same background, despite their various modifications.

chromatids to separate, and the cell leaves the mitosis stage (130). Apc9 null mutant cells have been observed to accumulate cells in the G2/M phase of the cell cycle because the metaphase-anaphase transition is delayed. Mutants have also been observed to have a decreased lifespan (131). Our results are consistent with Pbp1 regulating cell cycle in that lacking Pbp1, Spr28, or Apc9 is not detrimental to cell growth, but lacking Spr28 or Apc9 in conjunction with Pbp1 causes severe growth defects.

Fun19 also displays a synthetic lethal phenotype with Pbp1. The *fun19* strain shows slightly stunted growth from the wildtype, but the *fun19pbp1* strain shows similar growth to other double knockouts. Fun19 is a paralog of YOR338W that arose through the whole genome duplication (132). Fun19 is a non-essential gene that is involved in chromatin binding and remodeling through regulating histone acetylation (133, 134), and is required for sporulation (135). The severe growth deficiencies shown in all four double knockout strains indicate that Pbp1 and each cell cycle protein have overlapping functions that are stunted when both proteins are lacking. Conversely the normal growth of single knockouts may indicate recovery functions between Pbp1 and its binding partners. In contrast, Bnr1 shows a slight synthetic lethality with Pbp1. Bnr1 belongs to the formin family, which are conserved proteins that promote the assembly of actin filaments; these critical filaments aid remodeling of the actin cytoskeleton (136). Bnr1 null mutants exhibit many abnormal traits related to cell cycle functions, like abnormal actin cytoskeleton, abnormal budding pattern, and decreased chromosome maintenance (137).

Further cell cycle analysis analogous to the above cell cycle data was attempted with these cell cycle protein strains, however, differences were not found to be statistically significant. Overall, trends show *pbp1* strains have lower G1/G2 ratios than wildtype, and samples with Pbp1

overexpression have ratios of G1/G2 higher than wildtype. *psk1psk2* strains exhibit higher G1/G2 ratios than wildtype, but PAS kinase overexpression has a similar effect as Pbp1 deficiency. Overexpressed PAS kinase samples have G1/G2 ratios resembling those of samples lacking Pbp1. These trends are likely not statistically significant because of the high variability seen in overexpression and double knockout strains.

3.4.4 Pbp1 sequesters Apc9 to stress granules

Previous studies have revealed colocalization of Pbp1 and Psk1 to stress granules (117), and Pbp1 is known to sequester TORC1 to stress granules (116). Thus, Pbp1 may affect cell cycle progression by sequestering cell cycle proteins to stress granules. To test this hypothesis, Apc9-GFP was visualized along with Pbp1-RFP. Stress granule formation was induced by glucose deprivation. Ptc6 colocalized to stress granules with Pbp1 (Figure 3-3), suggesting Pbp1 inhibits Apc9 activity through this sequestration.

3.4.5 Pbp1 sequesters mitochondrial phosphatase Ptc6 to stress granules

In addition to cell cycle effects, the role of Pbp1 in regulating Ptc6 was investigated due to the metabolic alterations seen in ALS patients, as well as the shared functions of Ptc6 with TORC1in mitophagy (138). Ptc6, a mitochondrial type 2C protein phosphatase, is required for mitophagy in stationary phase cells (139). To determine if Pbp1 also regulates activity of its binding partner Ptc6 via sequestration to stress granules, confocal microscopy was performed. Ptc6-GFP was used to visualize the location of Ptc6 and Pbp1-RFP was used to visualize the location of Pbp1. Stress granule formation was induced by glucose deprivation. Pbp1 and Ptc6 were shown to colocalize within the cell to cytoplasmic foci (Figure 3-4). This suggests that Pbp1 sequesters Ptc6 to stress granules when the cell is under cellular stress, thus regulating its activity.

3.4.6 Pbp1 deficient yeast exhibit increased mitophagy and mitophagy is restored to normal in Pbp1/Ptc6 deficient yeast

Mitophagy is the process of clearing damaged mitochondria from the cell. It is thought that improper mitophagy can lead to the accumulation of dysfunctional or damaged mitochondria, which contribute to neurodegeneration in ALS patients (140). Because Ptc6 has known involvement in mitophagy, Ptc6 was chosen for further investigation. Mitophagy assays were used to test our hypothesis that Pbp1 is inactivating Ptc6 by sequestration assays. If Pbp1 is absent from the cell, Ptc6 may not be sequestered to stress granules, allowing for mitophagy to occur.

Mitophagy was induced through 48 hour-growth in YPL and mitophagy was quantified by the amount of degraded OM45-GFP, measured through western blot. ATG1 was included as a control, as it should block mitophagy pathways. The level of mitophagy seen in Ptc6 matched what has been reported in previous studies, with mitophagy being lowered in this strain. Pbp1 yeast showed significantly elevated levels of mitophagy, suggesting a novel role of Pbp1 in regulating mitophagy within the cell. In addition, Pbp1Ptc6 yeast showed mitophagy levels similar to wild-

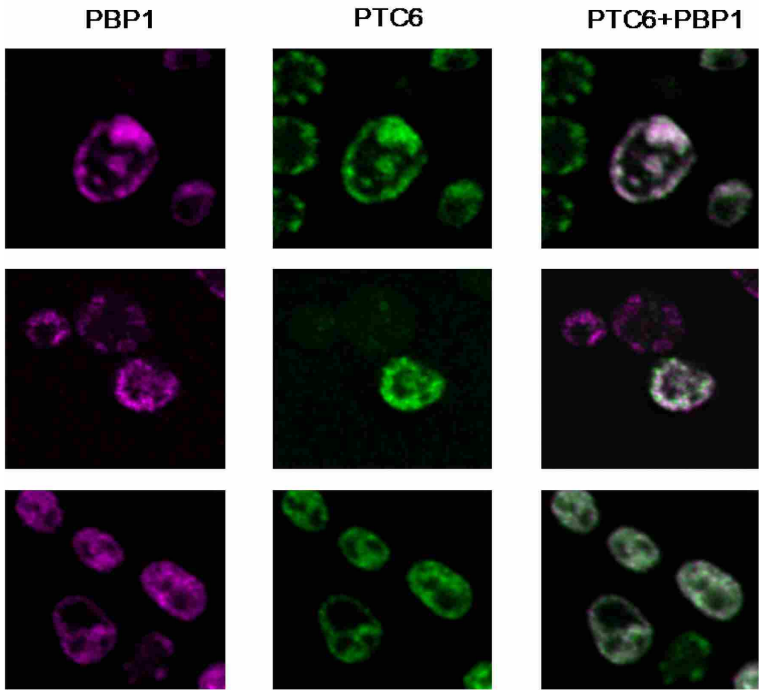

Figure 3-10: Colocalization of Pbp1 and Ptc6.
Ptc6-GFP fusion yeast (ThermoFisher) was transformed with Pbp1-RFP (JG1669), grown under glucose deprivation, and imaged using an Olympus Fluoview. confocal microscope.

type. This was unexpected as Ptc6 has been reported as an essential gene necessary for mitophagy. The remaining strains tested revealed no significant changes. One-way ANOVA was performed using JMP Pro14 (version 14.0) software (SAS Institute, Cary, NC, USA) with Tukey's post-hoc test.

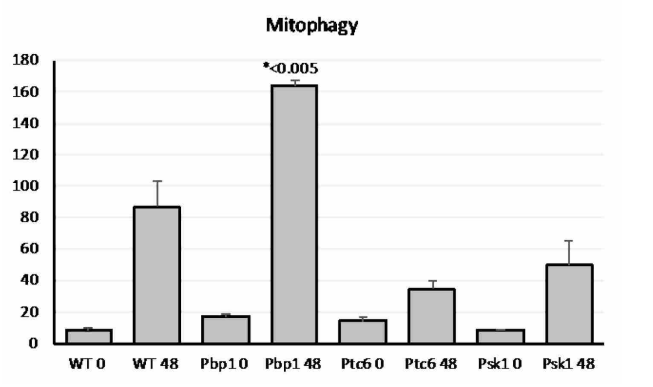

Figure 3-11: Pbp1 yeast display increased levels of mitophagy. Mitophagy assays were conducted using the OM45-GFP processing assay developed by Kanki et al. Mitophagy was induced through growth in YPL and the amount of degraded GFP was measured as a marker for mitophagy. Bands were quantified using ImageJ. Significance was determined by one-way ANOVA and Tukey's post-hoc test. n=2.

3.4.7 PTC6 deficient yeast exhibit mitochondrial alterations

Due to the significant increase in mitophagy in Pbp1 deficient yeast and the significant decrease in mitophagy in Ptc6 deficient yeast, mitochondria health was measured in each of the strains tested for the mitophagy assay. First, mitochondria content was quantified using flow cytometry. Mitochondria were tagged with OM45-GFP and endoplasmic reticulum was tagged with RFP. Endoplasmic reticulum was used as a cellular control. Our results show that PTC6 deficient yeast contain significantly more mitochondria. However, PTC6/Pbp1 deficient yeast show levels similar to WT. One-way ANOVA was performed using JMP Pro14 (version 14.0) software (SAS Institute, Cary, NC, USA) with Tukey's post-hoc test.

This change in mitochondria content suggested that a change in respiration may also be seen due to the elevated mitochondrial content. Plate respiration assays were utilized to first determine if any noticeable respiratory differences could be detected. Each strain was grown up overnight and 1:10-fold serial dilutions were performed and plated 1:100-fold serial dilutions were performed from the overnight and plated onto both glucose (a fermentative carbon source)

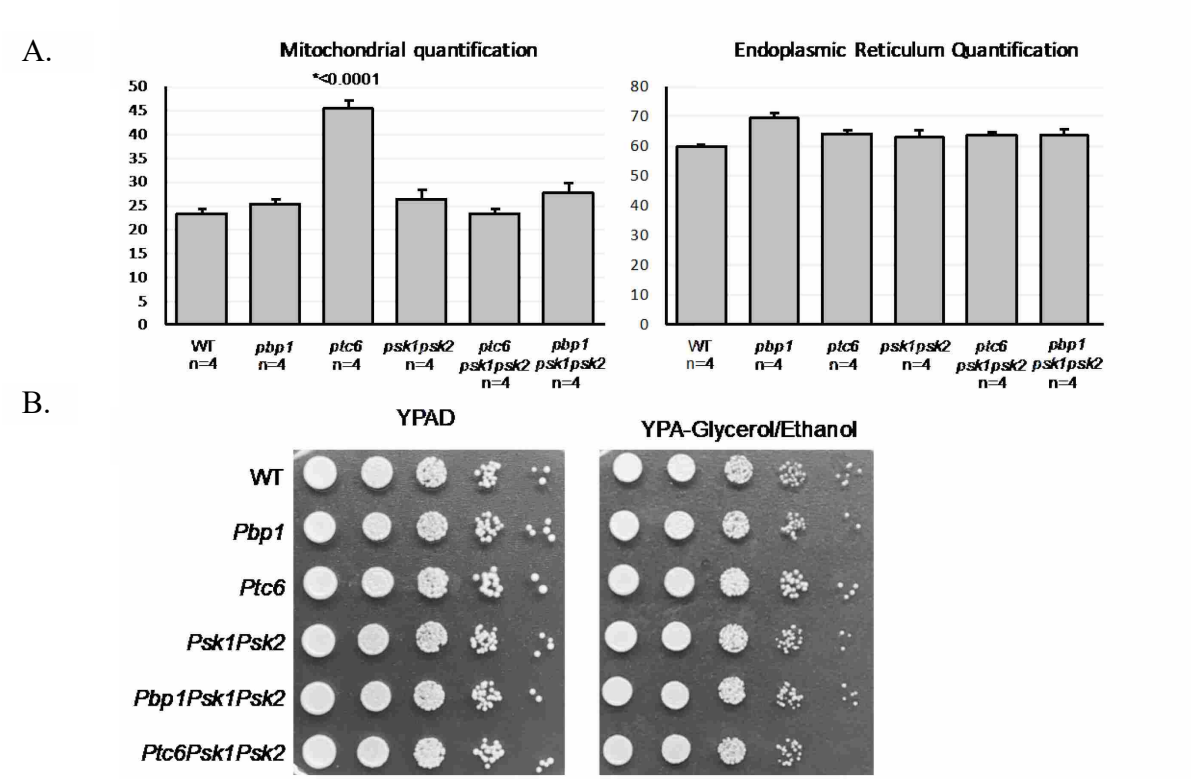

Figure 3-12: Evidence for Ptc6 regulation of respiration.
(A) Mitochondria was quantified using flow cytometry. OM45-GFP was used as a marker for mitochondrial content and the ER was quantified using Sec63-RFP, a protein in the ER. The ER was included as a control of overall cellular content. (B) Spot dilutions revealed increased respiration in Ptc6 yeast. Together, these results suggest the elevated mitochondrial content is contributing to elevated respiration.

and glycerol/ethanol (a respiratory carbon source). Following two days of growth, it was revealed that Ptc6 deficient yeast showed increased colony mass.

3.5 Discussion

There are approximately 16,000 Americans living with ALS at any given moment. With a typical survival time of only 2-5 years, ALS is a grim diagnosis with few treatment options. Fortunately, advances in technology have allowed for a more in-depth understanding of the pathways involved in the disease and potential drug targets (141). Recently, ataxin-2 has been identified as a promising therapeutic target for ALS. It has been shown that intermediate-length polyglutamine expansions in the ataxin-2 gene increases the risk of ALS. Furthermore, it has

been shown that by lowering the levels of ataxin-2, it is possible to mitigate disease caused by TDP-43 proteinopathy in yeast, flies, and mice (142). This study focuses on identifying proteins that interact with Pbp1, the yeast homolog of ataxin-2, to elucidate the molecular pathways that may be affected during neurodegeneration.

A large-scale yeast interactome study identified 32 novel putative binding partners through yeast-two hybrid (seven binding partners) and mass spectrometry (24 binding partners). This has largely expanded the role of Pbp1 in yeast, which includes six proteins that are involved in RNA processing, a known role for Pbp1, five proteins involved in protein transportation, eight cell cycle proteins, and 13 proteins with other unknown functions. In addition, 21 of the 32 identified binding partners have a human homolog, suggested a possible conserved function between yeast and man.

Eight cell cycle-related binding partners were found for Pbp1, comprising one-fourth of the newly identified 32 binding partners. Due to this large number of cell cycle proteins identified in the interactome study, the effects of Pbp1 on cell cycle was analyzed. Flow cytometry was utilized to visualize the cell cycle in strains of interest. Single knockout strains of Pbp1 and PAS kinase were assessed alongside wildtype (see Figure 3-3). This revealed that Pbp1 deficiency cause more cells to remain in G2 phase, giving a lower ratio of G1/G2. PAS kinase deficiency caused fewer cells to remain in G2 phase, giving a higher ratio of G1/G2. These opposing effects are likely explained by the individual protein's unique binding partners' functionalities being altered.

PAS kinase is known to regulate carbohydrate storage and translation through activation of its substrates, so PAS kinase deficient cells lack a key activator of proteins that contribute to the growth of the cell (143, 144); therefore, PAS kinase deficient cells likely require more time

to pass through the restriction point of G1 phase, causing a higher G1/G2 ratio. Pbp1 is known to be involved in mRNA stability and R-loop regulation in addition to regulation through sequestration to stress granules (58). Pbp1 deficient cells may progress through G1 phase quicker because Pbp1 is not in the cell to sequester growth and proliferation proteins to stress granules. A quicker progression through G1 phase would lower the G1/G2 ratio of Pbp1 deficient cells. This prompted an investigation into the binding partners of Pbp1 that are associated with the cell cycle.

Of the eight cell cycle-associated Pbp1 binding partners identified, four were not lethal null mutants, and these were assessed through growth assays in conjunction with Pbp1 deficiency. Overall, none of the single knockout strains assessed (*pbp1 apc9, spr28, fun19, bnr1*) had significant growth deficiencies, however, when strains were Pbp1-deficient in addition, severe growth deficiencies were apparent (see Figure 3-4). This indicates that Pbp1 is a crucial activator of proteins that can recover lost functions of these investigated cell cycle proteins. Apc9 was investigated further and shown to colocalize with Pbp1 at stress granules, where its activity may be inhibited (see Figure 3-3).

Through the yeast two-hybrid assay we also identified Ptc6 as a novel interacting partner of Pbp1. Ptc6 (also referred to as Aup1 or PPP2 in yeast) is a serine/threonine phosphatase that is localized to the mitochondria. Ptc6 is necessary for efficient mitophagy and cell survival (139). This was validated through the OM45-GFP processing assay, as *PTC6* yeast demonstrated decreased levels of mitophagy. Interestingly, *PBP1* yeast exhibited significantly increased levels of mitophagy. This result combined with our colocalization assays creates a model for how Pbp1 is interacting with Ptc6. We hypothesize that when Pbp1 interacts with Ptc6 it sequesters it to the stress granule, effectively deactivating Ptc6 function in the cell and decreasing mitophagy

pathways (see Figure 3-7). When Pbp1 is absent from the cell, Ptc6 is not sequestered to the stress granules, resulting in an increase in Ptc6 function and increased mitophagy. This result is especially interesting when looking at the context of ALS. In ALS, mitophagy pathways are often blocked, allowing for the accumulation of damaged mitochondria in the cell and contributing to neurodegeneration (140). Elevated mitophagy in *Pbp1* yeast suggests yet another benefit of targeting Pbp1/ataxin-2 for ALS therapy, as lowering levels of Pbp1/ataxin-2 will result in increased mitophagy and decreased accumulation of damaged mitochondria.

This difference in mitophagy prompted a deeper understanding of the mitochondria in these yeast strains. Flow cytometry assays revealed elevated mitochondria in *PTC6* yeast. This is supported by the mitophagy assays, as an inability to perform mitophagy would result in elevated levels of mitochondria in the cell. Furthermore, *PTC6* yeast appear to have increased respiration as shown by plate assay. As such, further characterization of the mitochondria is needed to determine if the yeast is accumulating damaged and dysfunctional mitochondria in the cell. This includes viewing mitochondrial morphology via confocal microscopy and measuring mitochondrial respiration via the Seahorse XF Cell Mito Stress Test.

In addition to the mitochondrial phenotypes seen in *Ptc6* yeast, *Ptc6* yeast has been shown to be sensitive to rapamycin. This is very interesting because TORC1 is also sensitive to rapamycin. It is known that Pbp1 sequesters TORC1 to stress granules, but it is unknown how that happens. It is possible that Pbp1 is working through Ptc6 to sequester TORC1 to stress granules. This would also suggest that the *Ptc6* sensitivity to rapamycin could in part be due to it sequestering TORC1 to stress granules. To test this theory, TORC1-GFP could be transformed into *Ptc6* yeast with Pabp1-RFP allowing for sequestration of TORC1 to stress granules to be monitored. If Ptc6 is responsible for sequestration, then TORC1 will not colocalize with Pab1-

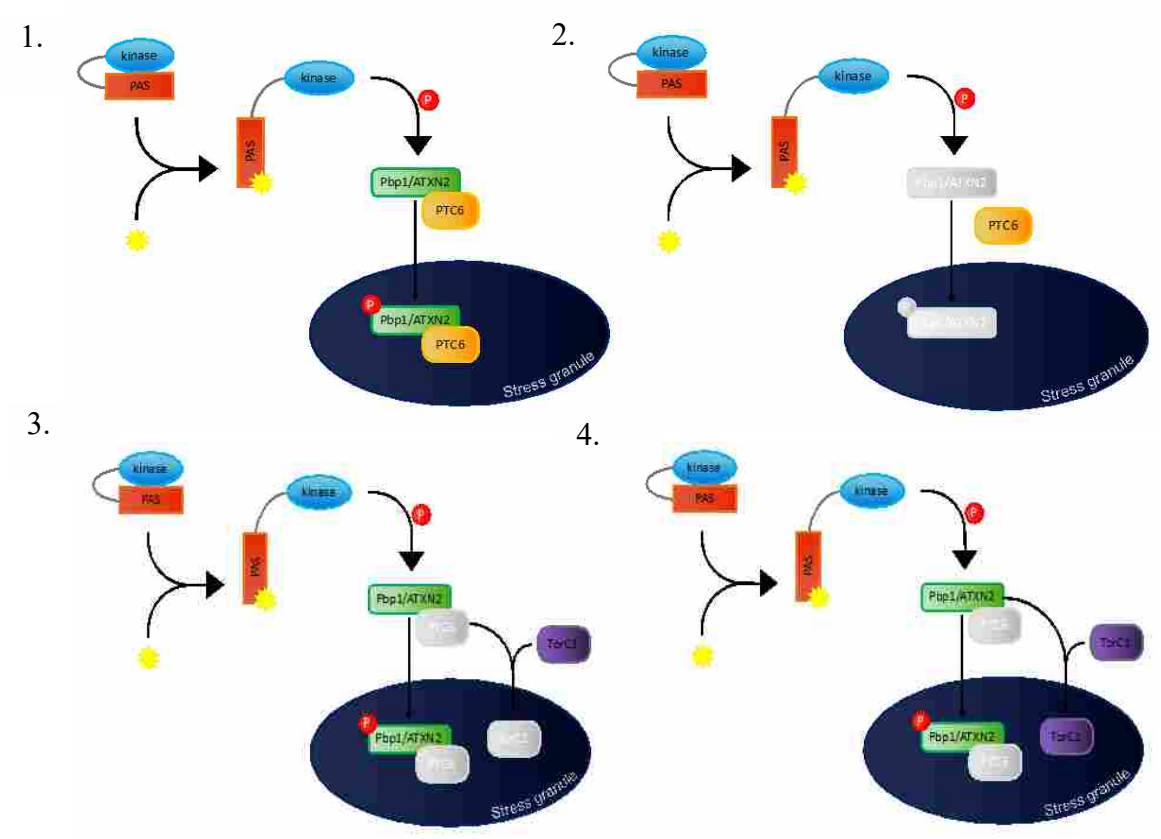

Figure 3-13: Possible models of Pbp1/ataxin-2 sequestration of Ptc6 to stress granules.
(1) Pbp1 activation by PAS kinase results in sequestration of both Pbp1 and Ptc6, thus decreasing the function of Pbp1. (2) When Pbp1 is absent from the cell, Ptc6 is no longer sequestered to stress granules, remaining functional and possibly contributing to the increased mitophagy seen in Pbp1 yeast. Possible models of Ptc6 dependent sequestration of TORC1 to stress granules. (3) Ptc6 is required for TORC1 sequestration to stress granules. In its absence, TORC1 is not sequestered to stress granules. (4) Ptc6 is not required for TORC1 sequestration to stress granules.

RFP in the stress granules. If TORC1 does colocalize to the stress granules, it would suggest that Pbp1 is regulating the Ptc6 and TORC1 pathways separately.

Through the identification of these 32 novel interacting partners of Pbp1 and the characterization of two identified ways Pbp1 affects cell health—through cell cycle proteins and Ptc6—, we have further solidified the importance of understand the role of Pbp1/ataxin-2 in ALS. By understanding both its upstream and downstream pathways, we can understand how to best utilize it as a therapeutic target to help individuals with the disease.

CHAPTER 4: Concluding Remarks and Future Directions

4.1 Ataxin-2 is an important cellular regulator involved in disease

Ataxin-2 is a diverse protein with multi-faceted functions that is conserved through eukaryotic organisms, form *S. cerevisiae* to *M. musculus* and *H. sapiens.* The diverse functions of ataxin-2 can be divided into two categories; RNA control and physiological functions. Ataxin-2 regulates R-loops and aids in mRNA stability and translation (7). It also helps form stress granules by recruiting proteins to the phase-separated granule (30). Physiologically, ataxin-2 has been shown to help maintain circadian rhythmicity and regulate proteins like TORC1 (8). When these functions go awry, cells experience impaired stress responses, metabolic dysfunction, or genome instability. Additionally, ataxin-2 has been linked to Amyotrophic Lateral Sclerosis. Understanding the pathways in which ataxin-2 is involved will elucidate the ways in which ataxin-2 contributes to neurodegeneration and how this can be exploited to treat disease.

Ataxin-2 is activated by PAS kinase (Figure 1-3), a serine/threonine kinase with evolutionary conservation in eukaryotes. While the activation relationship between PAS kinase and ataxin-2 is known, the effects of deregulation of PAS kinase on ataxin-2 and its functions are not known (8). Although both PAS kinase and ataxin-2 have yeast homologs, making *S. cerevisiae*, a good model, ALS is a neurodegenerative disease, which calls for an organism model with neurons for more complete investigation.

4.2 PAS kinase deficiency reduces ataxin-2 abundance in mice

While the interaction between PAS kinase and ataxin-2 (Pbp1) was established in a yeast model, ALS model mice were used to observe this interaction in a mammalian model. Mice that lacked PAS kinase were utilized to detect the effects of PAS kinase deficiency in mammals. As seen in Figure 2-4, no observable differences of general ataxin-2 abundance were observed in

PAS kinase-deficient mice who consumed a normal diet, but high-fat, high sugar dieting PAS kinase-deficient mice demonstrated significantly lower levels of ataxin-2. This indicates that inhibition of PAS kinase can reduce the amount of ataxin-2 in cells, especially in cells under stress caused by Western-style diets. This makes PAS kinase a potential alternative target to ataxin-2 for therapeutics. The question, however, of what these manipulations may cause in the cell arises.

4.3 PAS kinase and Pbp1 cause deviation from wildtype cell cycle

Flow cytometric analysis of the cell cycle (Figure 3-2) reveals that Pbp1 and PAS kinase deficiencies confer opposite effects on the overall cell cycle. Because proteins are synthesized in the G1 and G2 phases of the cell cycle, we compared ratios of G1 phase cells to G2 phase cells across the strains. Pbp1 deficiency decreases the ratio of G1 to G2 cells compared to wildtype, while PAS kinase deficiency increases the ratio. Analysis of the effects of cell-cycle related binding partners show variability too high to conclude definitive changes. Although PAS kinase deficiency has been shown to reduce Pbp1 abundance, and, therefore, confer similar phenotypic outcomes as Pbp1-deficiecncy, the proteins each have unique binding partners that are affected by their loss. PAS kinase is known to regulate carbohydrate storage and translation through activation of its substrates, so PAS kinase deficient cells lack a key activator of proteins that contribute to the growth of the cell (143, 144); therefore, PAS kinase deficient cells likely require more time to pass through the restriction point of G1 phase, causing a higher G1/G2 ratio.

Pbp1 is known to be involved in mRNA stability and R-loop regulation in addition to regulation through sequestration to stress granules (58). Pbp1 deficient cells may progress through G1 phase quicker because Pbp1 is not in the cell to sequester growth and proliferation

proteins to stress granules. A quicker progression through G1 phase would lower the G1/G2 ratio of Pbp1 deficient cells. To investigate the cause of these changes in Pbp1 deficient cells, we identified and analyzed cell cycle-related binding partners of Pbp1.

4.4 Pbp1 affects the cell cycle through its binding partners

To investigate the downstream effects of ataxin-2 reduction, binding partners of Pbp1 were identified through a yeast two-hybrid and mass spectrometry. Since the health of cells with deregulation of PAS kinase and ataxin-2 was of interest, Pbp1 binding partners with functions related to the cell cycle were analyzed—Spr28, Fun19, Apc9, and Bnr1. After construction of double knockout strains, growth assays were performed to assess the overall growth abilities of the strains, as seen in Figure 3-3. Double knockout strains show synthetic lethality while single knockout strains grow comparably to wildtype, indicating that Pbp1 shares vital roles with cell cycle proteins of various functions.

4.5 Future directions

The relationship between PAS kinase downregulation and the consequential effect of ataxin-2 reduction has been observed in yeast and mice, but this should also be considered in a human cell model. Due to the low levels of expression of these proteins, detection of successful knockdown was inconclusive, and previous attempts using embryonic kidney cells were, therefore, unsuccessful. Using a neuronal cell type would likely improve success.

Conclusive flow cytometric analysis of the cell cycle protein knockouts was unsuccessful because the samples showed high variability in the percentages of cells in each stage of the cell cycle. This is could be caused by high RNA content in the cells that could not be removed with RNase digestion, or the samples could truly have very different outcomes to loss of cell cycle proteins. To test if the variability stems from high RNA content interfering with analysis,

samples that showed high variability should be revisited using a DNA-specific stain, like DAPI. Additionally, Northern Blot analysis could be used to quantify relative RNA amounts compared to wildtype.

These downstream effects of manipulation of the PAS kinase-ataxin-2 activation pathway causes observable changes in the functions of the cell, however, these changes are not, by themselves, observably detrimental to the cell. When manipulating PAS kinase or ataxin-2, however, mutations in binding partners should be avoided to maintain functional cells.

Understanding the pathways in which ataxin-2 is involved is crucial to understanding the ways in which ataxin-2 is involved in neurodegeneration and degenerative disease.

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