Yeast Genetic Modeling of Pbp1/ATXN2-linked Neurodegenerative Diseases

by

Kirk Matthew James Szafranski

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Kirk Szafranski
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Laboratory Medicine and Pathobiology
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Abstract

The polyglutamine expansion of the human gene ATXN2 is associated with neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS) and Spinocerebellar Ataxia type 2 (SCA2). Work in our lab demonstrated that deletion of the *Saccharomyces cerevisiae* ATXN2 orthologue Pbp1 results in the accumulation of RNA-DNA hybrids. Here I demonstrate that hybrid accumulation in *pbp1Δ* cells triggers aberrant recombination within the ribosomal DNA (rDNA) repeats and shortening of replicative lifespan. These deleterious phenotypes can be rescued by caloric restriction. To better model disease I created a yeast genetic model of human ATXN2-linked diseases by generating cells expressing polyglutamine-expanded Pbp1. These cells also had shorter replicative lifespan. However, hybrid accumulation or aberrant recombination within the rDNA repeats was unaffected. Instead, these strains demonstrate gross chromosomal rearrangement more substantial than those observed in *pbp1Δ* cells suggesting that replicative lifespan is impacted by distinctive mechanisms in strains lacking Pbp1 or expressing polyglutamine-expanded Pbp1.
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List of Abbreviations

AD; Alzheimer’s disease

ADE2; Adenine requiring 2

AL; *Ad libitum*

ALS; amyotrophic lateral sclerosis

AOA2; apraxia with oculomotor ataxia

Asf1; Anti-silencing function 1

ATP; adenosine triphosphate

ATXN; Ataxin

BER; Base excision repair

BP; Base pair

Bre5; Brefeldin A sensitivity 5

*C9ORF72*; Chromosome 9 open reading frame 72

CHEF; Contour-clamped homogeneous electric field

ChIP; Chromatin immunoprecipitation

CLS; chronological lifespan

CR; Calorie restriction

DEPC; Diethylpyrocarbonate

DMSO; Dimethyl sulfoxide

Doa1; Degradation of Alpha 1
DSB; Double strand break

DTT; Dithiotheritol

EDTA; Ethylenediaminetetraacetic acid

EtBr; Ethidium bromide

EtOH; Ethanol

Fob1; Fork block 1

FTLD; Fronto-temporal lobular degeneration

FUS; fused in sarcoma

G4DNA; G-quadruplex DNA

GCR; gross chromosomal rearrangement

H3K9/K14; Histone H3 lysine 9 and lysine 14

H3K56; Histone H3 lysine 56

HRP; Horseradish peroxidase

IF; Immunofluorescence

IGS; Intergenic spacer

IP; Immunoprecipitation

LC-MS/MS; Liquid chromatography coupled to tandem mass spectrometry

lncRNA; Long non-coding RNA

Lsm12; Like-Sm 12

miRNA; MicroRNA
ncRNA; non-coding RNA

NER; Nucleotide excision repair

NHEJ; Non-homologous end joining

OD; optical density

Pab1; Poly-A binding protein 1

PBS; Phosphate buffered saline

PEG; polyethylene glycol

PFGE; Pulsed-field gel electrophoresis

Pbp1; Pab1p-binding protein 1

Pbp4; Pbp1-binding protein 4

PD; Parkinson’s disease

Pif1; Petite integration frequency 1

Pma1; Plasma membrane ATPase 1

PMSF; Phenylmethanesulfonfylfluoride

Pol; Polymerase

RAN; Repeat-association non-ATG

RCF; relative centrifugal force

rDNA; ribosomal DNA or ribosomal RNA genes

RISC; RNA-induced Silencing Complex

RNAP; RNA polymerase
Rnh1; Ribonuclease H1

Rnh201; Ribonuclease H201

RLS; Replicative lifespan

RT; Room temperature

RT-PCR; Reverse transcriptase PCR

Rtt109; Regulation of Ty1 transposition 109

SC; Synthetic complete

SCA2; Spinocerebellar ataxia type 2

SDS; Sodium dodecyl sulfate

SDS-PAGE; Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SETX; Senataxin

Sir2; Silent information regulator 2

SiRNA; Small interfering RNA

Sod1; Superoxide dismutase 1

Stm1; Suppressor of TOM1

TAP; Tandem affinity purification

TBS; tris buffered saline

TCA; trichloroacetic acid

TDP43; TAR DNA-binding protein 43

TEV protease; tobacco etch virus protease
tRNa; Transfer RNA

Ubp3; Ubiquitin specific protease

USCE; Unequal sister chromatid exchange

WT; Wild-type

YEP; Yeast extract peptone

YEPD; Yeast extract peptone dextrose
Chapter 1
Introduction

Portions of this chapter are modified from the following publications:


1 Introduction

1.1 Neurodegenerative diseases associated with RNA regulation

Neurodegenerative diseases are a group of debilitating neurological disorders typically associated with old age (Eacker et al., 2009). These conditions are characterized by the progressive loss of neurons within one or more regions of the central nervous system (CNS) (Eacker et al., 2009). Most neurodegenerative diseases are sporadic in origin, meaning that they arise in patients with no apparent family history or known environmental cause (Robberecht and Philips, 2013). These diseases are associated with a diverse spectrum of clinical presentations, brain pathologies, and health consequences. These include relatively well-known conditions like Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS) as well as a host of rare genetically linked conditions such as Friedrich’s Ataxia. Conditions, such as AD and Frontal Temporal Lobular Degeneration (FTLD) are primarily cognitive disorders and represent the two leading causes of dementia worldwide (Mattson, 2004). They are caused by progressive loss of neurons within brain regions responsible for reasoning, cognition, and memory. In contrast, ALS and PD are chiefly disorders of motor function, caused by the preferential loss of motor neurons and dopamine-producing neurons in the brain, respectively (Hardiman et al., 2011; Wirdefeldt et al., 2011). Although some conditions can be contained by pharmacotherapy and/or surgery, the majority of neurodegenerative diseases remain untreatable.

Basic research on potential disease mechanisms for these conditions has benefited greatly from studies using model organisms and/or novel experimental systems. Insights from such studies are providing mounting evidence that non-coding RNAs (ncRNAs) and ncRNA-regulatory processes are important players in the pathogenesis of neurodegenerative disease (Eacker et al., 2009; Esteller, 2011; Abe and Bonini, 2013). ncRNAs represent a functionally and structurally diverse class of RNA species that participate in a wide range of basic cellular processes including protein translation, mRNA splicing, chromatin organization, and the regulation of gene expression (Esteller, 2011). Several classes of ncRNAs (e.g. miRNAs, rRNAs, tRNAs, and many IncRNAs) fulfill discrete functions within cells. However, it is becoming clear
that a large proportion of the cellular transcript pool is comprised of ncRNAs that lack obvious function (Djebali et al., 2012; Palazzo and Gregory, 2014). These entities are thought to derive primarily from noisy transcription at intergenic sequences and are generally degraded rapidly within the nucleus (Djebali et al., 2012; Palazzo and Gregory, 2014). Recent studies suggest that under certain conditions, these ncRNAs can trigger processes that are toxic to cells. These processes include the sequestration of crucial RNA-binding proteins as well as the accumulation of genome-destabilizing R-loops, which are structures that can form when nascent RNA stably hybridizes with DNA (Aguilera and Garcia-Muse, 2012; Haeusler et al., 2014; Salvi et al., 2014; Santos-Pereira and Aguilera, 2015). Accordingly, cells have evolved a number of mechanisms to constrain both the amount and the ability of transcripts to engage in these detrimental processes (Palazzo and Gregory, 2014; Salvi et al., 2014).

Non-coding RNA regulatory mechanisms rely on the action of RNA regulatory proteins to splice, sequester, degrade, transport or otherwise process ncRNAs. Intriguingly, there are a diverse number of neurodegenerative diseases associated directly with mutations or localization defects in RNA regulatory proteins. For example, the RNA binding proteins FUS and TDP-43 are implicated in ALS and FTLD; the majority of ALS/FTLD patients harbour mutations and/or mislocalizations in these proteins (Neumann et al., 2006; Kabashi et al., 2008; Kwiatkowski et al., 2009; Vance et al., 2009; Mackenzie et al., 2010). Both of these proteins interact with the miRNA processing protein Drosha and regulate the development of the neural outgrowth factor miR-132 (Kawahara and Mieda-Sato, 2012; Morlando et al., 2012). In fact, siRNAs against TDP-43 decreased miR-132 production and inhibited neurite length in a manner that could be rescued by the reintroduction of miR-132 (Kawahara and Mieda-Sato, 2012). This result underlines the great importance of ncRNA regulation for proper neural health and development.

More and more research has focused on the role that aberrant mutations or localizations in RNAs themselves can have on neurodegenerative processes. For example, a hexanucleotide repeat expansion within the intron within the C9ORF72 gene has been demonstrated to be the most common known cause of ALS (DeJesus-Hernandez et al., 2011; Majounie et al., 2012). This mutation confers a toxic gain-of-function onto the C9ORF72 transcript: the repeat expansion itself when transplanted to another portion of the genome is sufficient to cause neurodegeneration in Drosophila melanogaster (Xu et al., 2013). This expansion causes abortive transcripts rich in G-quadruplexes, which are higher order structures built around hydrogen
bonded guanine tetrads (Sen and Gilbert, 1988). These G-quadruplexes bind to and sequester the RNA-binding protein nucleolin (Haeusler et al., 2014). With nucleolin distribution altered, cells show distinct signs of nucleolar stress (Haeusler et al., 2014).

In an interesting parallel with the *C9ORF72* hexanucleotide repeat expansion, there exist several RNA regulatory proteins that undergo a polyglutamine repeat expansion. One such protein is huntingtin which is causally linked to of Huntington’s Disease (Macdonald et al., 1993; Ross et al., 1997; Tsoi and Chan, 2013; Szafranski et al., 2015). These genes could therefore induce deleterious effects through a loss of RNA regulatory function or through a gain of toxic function. Gain of toxicity may occur due to the fact that CAG repeat RNA is able to bind to and sequester nucleolin (Tsoi et al., 2012; Tsoi and Chan, 2013). Additionally, CAG repeat RNA is capable of producing toxic proteins generated from translation initiated from within the CAG repeats – this is termed repeat associated non-ATG translation (RAN translation) (Koob et al., 1999; Zu et al., 2011). One such protein is Ataxin-2, whose orthologue Pbp1 is the focus of this thesis (Figure 1.1a).

### 1.2 Characterization of ATXN2

Ataxin-2 has been linked with the cerebellar degenerative disease known as spinocerebellar ataxia type 2 (SCA2) and ALS (Gispert et al., 1993; Pulst et al., 1996; Elden et al., 2010). Expansion of a naturally occurring polyglutamine tract in ATXN2 from 22-23 repeats to greater than 32 repeats causes SCA2 (Figure 1.1b) (Pulst et al., 1996). Length of the polyglutamine tract is inversely correlated with age of SCA2 onset (Hayes et al., 2000). The moderate expansion of 29-34 glutamine repeats in ATXN2 is associated with ALS (Figure 1.1b) (Elden et al., 2010). Notably, there is a small amount of overlap in polyglutamine tract length between these diseases and there have been rare reports of disease co-occurrence (Figure 1.1b)(Braga-Neto et al., 2011). Given the poor prospects for patients with these diseases, it is vital that we make progress in understanding the basic molecular mechanisms through which this expansion is causing neurodegeneration. Such an understanding would allow us to develop novel therapeutics for patients suffering from these devastating conditions.
Figure 1.1. ATXN2 conservation and disease association – (A) Protein domains of yeast Pbp1 and its ATXN2 orthologues in nematodes, fruit flies, mouse and human. There exists a CAG, site with a single glutamine in yeast/fruit fly/mouse and a poly-glutamine tract in human (yellow). Two RNA binding domains are highly conserved, the LSM (light blue), Like SM domain and LSMAD (dark blue), the LSM-associated domain. There also exists a conserved Pam2 motif (red) through which the proteins interact with conserved PABP proteins. Amino acid numbers are indicated (below proteins) and similarity percentages relative to human ATXN2 are shown on the right for LSM (top, light blue) and LSMAD (bottom, dark blue). N.S. – no significant similarity detected by amino acid BLAST (B) A spectrum exists in ATXN2 polyglutamine length and pathology. Expansions of 22-23 are most common, but tract length of anywhere between 15 to 28 glutamines has been demonstrated to have no pathological effect. Polyglutamine tract length between 28 to 33 glutamines is strongly associated with ALS, while longer polyglutamine expansions are associated with SCA2. There is a small amount of overlap in these ranges, but co-occurrence of ALS and SCA2 is rare.
ATXN2 appears to be a fairly conserved protein, showing over 98% homology between mouse and human and between 42 and 63% homology in its RNA binding LSM domains between ATXN2 and its yeast orthologue Pbp1 (Figure 1.1a) (Salvi et al., 2014). Thus, studies of ATXN2 function have been carried out in a wide variety of model systems.

As indicated in section 1.1, polyglutamine expanded ATXN2 may cause disease by loss of function and/or gain of toxic functions (van den Heuvel et al., 2014). On the toxic gain of function front, polyglutamine expanded ATXN2 may form cytoplasmic inclusions which induce the insolubility of some of ATXN2’s binding partners (Damrath et al., 2012). Many forms of ALS are characterized by inclusions of Fused in Sarcoma (FUS) – a protein important in regulating microRNA formation (Szafranski et al., 2015). ATXN2 has been shown to co-localize with FUS aggregations, which suggests that ATXN2 may mediate a toxic loss of FUS function (Farg et al., 2013). An even more common feature of ALS is the presence of ubiquitinated inclusions of TAR DNA Binding Protein 43 (TDP-43) (Neumann et al., 2006). Both yeast and drosophila studies have demonstrated that ATXN2 orthologues can enhance TDP-43 toxicity (Elden et al., 2010). Moreover, ATXN2 mutations both increase the expression of TDP-43 and increase cleavage of TDP-43 – a feature known to enhance TDP-43’s ability to aggregate (Elden et al., 2010; Hart and Gitler, 2012).

It is also possible that it may actually be the ATXN2 RNA that is mediating some toxicity, either by sequestering RNA binding proteins or through the production of toxic dipeptides by RAN translation (Li and Bonini, 2010; Zu et al., 2011). However, it is unlikely that the low repeat level seen specifically in ATXN2 ALS cases would cause RAN translation as the efficiency of RAN translation is tract length dependent (Pearson, 2011). In fact, even in SCA2 cases with ATXN2 lengths of over 100 repeats, RAN translation was only detected when ATXN2 had a CMV promoter and not when its own endogenous promoter was present (Scoles et al., 2015). Evidence also exists that suggests that polyQ repeat RNA binds and sequesters nucleolin (Tsoi et al., 2012; Tsoi and Chan, 2013). However, these data also come from extraordinarily long CAG repeat RNA (78 or 128 repeats), so similar caveats must be taken with applying them to the lower levels of polyQ repeats seen in most ATXN2-linked pathologies (Tsoi et al., 2012; Tsoi and Chan, 2013).
A polyglutamine expansion of ATXN2 might also disrupt native functions of the protein and ATXN2 has many important functions. For example, ATXN2 is a key regulator of mRNA stability through its interaction with the polyadenylation factor PABPC1 (Yokoshi et al., 2014). Binding of ATXN2 to U-rich regions in 3’ UTRs promotes stability of ATXN2 bound mRNAs and this in turn promotes protein expression (Yokoshi et al., 2014). Additionally, the polyglutamine expansion of ATXN2 from 23 repeats to 31, 35 and 39 repeats reduces expression of target proteins in a polyQ tract-dependent manner in HEK293 and SH-SY5Y cells (Yokoshi et al., 2014). Additional data for the role of ATXN2 in translational regulation come from the drosophila orthologue Atx-2, which is essential for regulating circadian rhythm proteins (Lim and Allada, 2013; Zhang et al., 2013). Additionally, ATXN2 is essential for the formation of stress granules and is localized to P-bodies (Nonhoff et al., 2007; Kaehler et al., 2012; Nostramo et al., 2015). Both stress granules and p-bodies are important sites of RNA inhibition – stress granules pause translation until favourable conditions for translation return while P-bodies are sites of RNA decay (Nonhoff et al., 2007; Kaehler et al., 2012; Nostramo et al., 2015). Therefore, there is a wide variety of mechanisms through which ATXN2 may cause neurodegeneration (van den Heuvel et al., 2014). However, many of these links are tentative. Creation of a genetic model to investigate the molecular pathways of this protein would be very valuable in clarifying its function and role in pathology.

Our lab has demonstrated a novel role for the ATXN2 orthologue Pbp1 using a yeast \textit{pbp1Δ} model. My colleagues discovered that Pbp1 is an important regulator of structures known as RNA-DNA hybrids (Salvi et al., 2014). Could this finding have relevance to human ATXN2 disease?
1.3 Yeast as a model organism

Budding yeast has long been recognized as an important model organism as it is easy to genetically manipulate, has “short” generation times and shares many metabolic and genetic processes with mammals (Steffen et al., 2009). *Saccharomyces cerevisiae*, the organism used in this thesis, has an extremely active homologous recombination pathway which allows for ease and precision in knocking out or inserting genes endogenously into the yeast genome. Possessing a generation time of approximately 90 minutes also allows for a quick examination of the cell cycle progression or cellular senescence (Longo et al., 2012). This ease of manipulation has allowed for groundbreaking discoveries to be made in yeast which were later validated in multicellular eukaryotes. For example, the 2001 Nobel Prize was awarded for the discovery of cell cycle regulatory proteins; 2/3 of the laureates performed their work in yeast (Hartwell et al., 1970; Nurse et al., 1976). Similarly, Nobel prize winning work done on vesicular trafficking used yeast as the initial model organism (Novick et al., 1980).

Owing to its extremely short generation time and ease of micromanipulation, budding yeast is an ideal organism to study factors that increase cellular senescence. Many of the factors found to be important for yeast longevity have been validated as longevity factors in mammals (Kaeberlein, 2010). The measure of lifespan used in this thesis, replicative lifespan (RLS), is a measure of how many daughter cells a single yeast mother cell produces (Mortimer and Johnston, 1959). This is accomplished by micromanipulation – the smaller daughter cells can be removed using a dissection needle and counted for a single virgin mother cell. Studies of yeast RLS have found a number of highly conserved longevity factors. For example, mice lacking the S6 kinase S6K1 have an extended lifespan (Selman et al., 2009). This factor was initially discovered to regulate lifespan using the yeast orthologue Sch9 (Fabrizio et al., 2001). Similarly, work done in yeast, flies and nematodes established the importance of the nutrient signaling Target of Rapamycin (TOR) network in lifespan regulation before it was verified as a vital longevity factor (Vellai et al., 2003; Kapahi et al., 2004; Kaeberlein et al., 2005; Harrison et al., 2009). Exactly why cellular senescence is conserved from yeast to humans and which aging cell populations are most important in humans to overall health and lifespan are not fully understood yet. One model is that yeast RLS may be similar to some features of ageing mitotic cell populations such as stem cells (Figure 1.2) (Kaeberlein, 2010; Kwan et al., 2013). Stem cell
aging has emerged as a major contributor to general organismal aging (Sharpless and Depinho, 2007). In particular, declining neurogenesis function is a feature of aging (Molofsky et al., 2006). This could then contribute to the age-association of neurodegenerative diseases. In fact, a diverse array of neurodegenerative diseases are marked by a decline in neurogenesis (Winner and Winkler, 2015).

Figure 1.2. Similarities between yeast RLS and stem cell aging—A yeast mother cell produces a stochastic number of daughter cells that is nevertheless affected by various longevity factors. Such longevity factors might similarly affect stem cell populations such as neural stem cells which produce a set number of stem cells (self arrow) or progenitor cells before senescence. This could lead to depletion of stem cell populations which would inhibit adult neurogenesis. Alternatively, aging factors could limit the development of certain types of neural progenitor cells.

RLS is a model of ageing for mitotically active cells, so to better model the ageing of post-mitotic cells, a separate model, known as chronological lifespan (CLS), was devised. In this assay, yeast cells are kept in a non-dividing, quiescent-like state – usually through growth in carbon-depleted liquid media. The ability of cells to resume mitotic growth in fresh media after varying time points is assessed as the CLS of the culture (Parrella and Longo, 2008). Similarly to RLS, CLS studies have revealed a number of conserved longevity factors. For example, deletion of Sch9 in yeast similarly increases CLS and RLS, which translates to longevity in mice lacking the orthologue S6K1 (Fabrizio et al., 2001; Selman et al., 2009). Additionally, compounds such as Rapamycin (which reduces activity in the TOR pathway) and spermidine increase both yeast CLS and lifespan of nematodes and fruit flies (Powers et al., 2006; Stanfel et al., 2009). Interplay between CLS and RLS is still relatively understudied. It is known that chronologically aged cells have a reduced RLS (Ashrafi et al., 1999). One idea that would explain this is due to the fact that
chronologically aged cells have greater genomic instability which is known to reduce RLS (Qin et al., 2008; Kaeberlein, 2010). However, CLS studies have faced a major barrier in that during CLS assays, the growth medium gradually acidifies and pH buffering is sufficient to increase CLS (Burtner et al., 2009). Accumulation of acid in the surrounding cellular medium seems to be an unlikely cause of ageing in humans. Notably, pH buffering the medium does not affect RLS, suggesting that this only concerns yeast CLS (Wasko et al., 2013). Future studies that seek to buffer the pH of the cell medium should prove to determine even more conserved longevity factors by CLS.

This thesis will demonstrate a novel factor in yeast RLS longevity – the regulation of RNA-DNA hybrids. The next subsection will outline what RNA-DNA hybrids are and how their regulation could be important for maintaining lifespan.

1.4 RNA-DNA hybrids and neurodegenerative diseases

Nascent ncRNA transcripts are capable of hybridizing with their template DNA strands forming RNA–DNA hybrids (Figure 1.3) (Wahba et al., 2011; Aguilera and Garcia-Muse, 2012; Salvi et al., 2014). These RNA–DNA hybrids form structures known as R-loops, which can threaten genome integrity by inducing double stranded breaks and aberrant recombination when collisions occur between R-loops and advancing transcription/replication machineries (Aguilera and Garcia-Muse, 2012). Alternatively, R-loops can help regulate the expression of a diverse set of RNAs and are suspected to play a broader role across the genome than it is currently appreciated (Skourt-Stathaki and Proudfoot, 2014). For example, the formation of RNA-DNA hybrids is important for transcriptional induction of the galactose gene in yeast (Cloutier et al., 2016). Likewise, hybrid formation is important for centromeric silencing in S. pombe and flowering gene expression in Arabidopsis (Nakama et al., 2012; Sun et al., 2013).
Figure 1.3. RNA-DNA hybrids form genome destabilizing R-loop structures—Work done in yeast and humans has determined that neurodegeneration-linked proteins and their orthologues are responsible for RNA–DNA hybrid suppression. R-loop structures form when RNAPII is transcribing DNA and the lagging RNA strand (in red) hybridizes with the DNA strand (in black). SETX (implicated in AOA2 and ALS) has helicase activity and can unwind RNA–DNA hybrid structures. Pbp1 (yeast orthologue of human ATXN2 implicated in SCA2 and ALS) has an RNA-binding domain that inhibits hybrids in some other way, possibly by binding to unwound RNA to prevent it from re-hybridizing with its template DNA strand. R-loops can lead to double stranded breaks and genomic instability when they collide with advancing replication forks.

In several neurodegenerative disorders, aberrant R-loop formation impacts the fate of individual neuroprotective genes (Salvi and Mekhail, 2015). For example, in individuals with fragile X syndrome, a trinucleotide repeat expansion within the promoter of the FXTAS-linked FMR1 triggers the formation of R-loops, which cause the aberrant epigenetic silencing of this gene, thereby contributing to disease (Colak et al., 2014). Similarly, R-loops formed upon intronic trinucleotide repeat expansion within the Friedrich’s Ataxia gene FXN promote its silencing and are thought to contribute to the disease (Groh et al., 2014). R-loops are also responsible for the abortive transcription observed at the hexanucleotide repeat-expanded...
C9ORF72 locus (Haeusler et al., 2014). In addition, data from yeast and Arabidopsis point to R-loops as regulators of the expression of several types of long non-coding RNAs, but it remains unclear if R-loops regulate neurodegenerative disease-linked IncRNAs such as the AD-linked BACE1AS (Nakama et al., 2012; Sun et al., 2013).

Beyond individual genes, some neurodegenerative disorders may encompass broad defects in R-loop or RNA–DNA hybrid regulation. Early onset or juvenile ALS as well as the neurodegenerative disease ataxia with oculomotor apraxia type 2 (AOA2) are linked to mutations within SETX, which has emerged as a conserved RNA–DNA helicase from yeast to human (Skourtí-Stathaki et al., 2011; Yuce and West, 2013). Loss of SETX function leads to a drastic increase in the number of R-loops formed within cells (Skourtí-Stathaki et al., 2011; Yuce and West, 2013). Interestingly, R-loops were easily detected in dividing germ cells but not in post-mitotic brain cells (Yeo et al., 2014). One possibility may be that the level of R-loops driving neural degeneration is too small for detection using present methods. It is also possible that R-loop formation drives disease by disrupting support cells within the nervous system or certain cell types along the neural lineage including neural stem cells. Future work should clarify the intersection between SETX, R-loops and neurodegeneration.

Similar to SETX-ALS/AOA2 links, our lab has established a link between R-loops and ATXN2-associated neurodegeneration with a study conducted in the budding yeast S. cerevisiae. We demonstrated that deletion of Pbp1, the yeast orthologue of ATXN2, causes aberrant accumulation of hybrids harboring lncRNA and intergenic DNA within repetitive DNA loci including ribosomal DNA (rDNA) repeats and subtelomeric regions (Salvi et al., 2014). Similarly, studies done in human HeLa cell lines found that ATXN2 knockout increases RNA-DNA hybrid accumulation within human rDNA repeats (Abraham et al, under review). This thesis will outline the work that I have done in establishing the downstream consequences of this hybrid accumulation; including lifespan reduction and destabilization of the rDNA repeats in yeast (see Chapter 3).

Therefore, although R-loops can play positive/regulatory roles in the cell, their aberrant regulation may trigger pathological processes leading to neurodegeneration (Figure 1.3). Aberrant R-loop accumulation can lead to the accumulation of toxic abortive transcripts, suppress the expression of neuroprotective factors, or compromise overall genome integrity. The
emerging role of R-loops in various diseases may also point to novel avenues for therapeutic intervention. Notably, we have found that these hybrids were particularly enriched in the rDNA repeats within both yeast and human cells (Salvi et al, 2014; Abraham et al, 2016). In the next subsection, we will take a closer look at the rDNA repeats and examine how they’re vital to overall genome integrity and cellular lifespan.

1.5 rDNA repeats and genome stability/lifespan

Cells require a very large amount of rRNA transcripts to keep up with the demands of translation. Therefore, eukaryotic cells have large arrays of tandem repeats of rRNA encoding DNA. This repeat DNA forms the ribosomal biogenesis structure known as the nucleolus (Warner, 1990). The rDNA repeats of S. cerevisiae consist of approximately 190 rRNA genes tandemly arranged on chromosome XII (Mekhail and Moazed, 2010). RNAPI and RNAPIII transcribe the rRNA genes while RNAPII can mediate transcription from endogenous promoters within intergenic spacers (IGS1 and IGS2) flanking rRNA genes (Figure 1.4). Intergenic transcription within the rDNA repeats can pose a major threat to genome stability and therefore cells have several mechanisms to counteract transcription within the intergenic spacers (Mekhail and Moazed, 2010)
Figure 1.4. Structure of the rDNA repeats in *S. cerevisiae*—rDNA repeats on Chr. XII. Each unit yields RNAPI-transcribed 35S precursor rRNA (processed into 25S, 18S, and 5.8S) and RNAPIII-transcribed 5S rRNA. CEN, centromere; TEL, telomere; IGS, intergenic spacer; rightward red fork, replication fork block; red rectangle, intergenic promoter E-pro; blue arrows, RNAPII-dependent ncRNA transcription; red circle, DNA replication origin; black arrow, RNAPI-dependent rRNA transcription. Primers amplifying IGS regions (P1–P5) are shown.

One such mechanism is the action of the Sir2 histone deacetylase at IGS1, which promotes rDNA silencing. Sir2-dependent rDNA silencing involves the compaction of DNA onto histones which limits access to RNAPII and recombination machineries (Gottlieb and Esposito, 1989; Imai et al., 2000). Sir2 does this by removing acetyl groups from lysines on the tails of histone H3 and H4 proteins (Imai et al., 2000; Moazed, 2001). More specifically, *in vitro* experiments demonstrate that Sir2 acts on residues lysine 16 of H4 (H4K16) and lysines 9 and 14 of H3 (H3K9/K14) (Imai et al., 2000; Moazed, 2001). Sir2-dependent silencing is not absolute and thus there is a residual amount of RNAPII transcription originating from the intergenic promoters (Kobayashi and Ganley, 2005). To compensate for IGS origin activation, recruitment of the Nrd1-Sen1 complex to the rDNA IGS regions promotes early RNAPII termination (Vasilieva et al., 2008). Disruption of these transcription-repressing mechanisms, such as through deletion of Sir2 or Nrd1, increases RNAPII localization within rDNA IGS regions and abrogates rDNA silencing, resulting in the accumulation of ncRNA transcripts (Vasilieva et al., 2008).
To counteract residual RNAPII activity, cells can target already generated intergenic transcripts for polyadenylation and degradation (Houseley et al., 2007). This ncRNA degradation is mediated via cooperation between the TRAMP complex (composed of Trf4/Pap2, Air1/2, Mtr4, and Pap1) and the exosome (Houseley et al., 2007). Similar to the deletion of Sir2 or Nrd1, Trf4 deletion leads to IGS ncRNA accumulation (Houseley et al., 2007).

Binding of the replication Fork block 1 (Fob1) protein at IGS1 (see Figure 1.4) creates torsion on rDNA and forms a structure that acts as a block to advancing replication forks (Kobayashi, 2003). Loss of Fob1 severely limits the amount of recombination within the rDNA repeats (Kobayashi et al., 1998; Kobayashi, 2003). This is thought to be due to the fact that advancing replication forks collide with fork blocks, causing recombination-inducing double stranded breaks (Kobayashi et al., 1998; Kobayashi, 2003). rDNA recombination can be beneficial as it allows for adaptive expansion or contraction of rDNA repeats under stress (Kobayashi and Ganley, 2005; Mekhail and Moazed, 2010). However, dysregulation of this process can lead to hyperactive or aberrant recombination events such as unequal sister chromatid exchange (USCE) within the repeats (Figure 1.5) (Kobayashi and Ganley, 2005). This can lead to devastating downstream effects in yeast, such as the shortening of replicative lifespan (Kaeberlein, 2010).
During rDNA replication, an advancing replication fork collides with the Fob1-mediated replication fork block (RFB) and results in a double stranded break. In most cases, DSB repair will occur by recombining between aligned sister chromatids leading to fidelity of the rDNA sequence. However, if the chromosomes are misaligned, it causes the aberrant recombination event known as unequal sister chromatid exchange. This can destabilize the rDNA repeats and lead to gain or loss of genetic material. Figure courtesy of Jayesh Salvi.

The stability of the rDNA array has been well-established as an essential determinant of yeast RLS (Kobayashi, 2011). For example, Sir2 prevents recombination within the rDNA repeats while Fob1 promotes recombination and accordingly, sir2Δ mutants have a shorter RLS while fob1Δ mutants are one of the few deletion mutants with an increased RLS (Defossez et al., 1999; Kaeberlein et al., 1999). Initially, aberrant recombination within the rDNA repeats was shown to promote the formation of “pop-out” extra-chromosomal rDNA circles (ERCs) specifically within dividing mother cells (Sinclair and Guarente, 1997). However, later work using a strain that cannot accumulate ERCs demonstrated that the rDNA instability in general is sufficient to reduce cellular lifespan (Ganley et al., 2009).

In yeast, the stability of the rDNA repeats has been shown to be intrinsically linked to its position in the nucleus. A protein complex known as cohibin interacts with the rDNA repeats and
anchors the complex to the inner nuclear membrane (Mekhail et al, 2008). Cohibin based tethering of the rDNA repeats stabilizes the repeats (as measured by USCE) at least partially through Sir2-independent tethering (Mekhail et al, 2008). Notably, tethering of the rDNA is required to maintain yeast RLS (Chan et al, 2011).

How exactly does rDNA stability mediate cellular senescence? It could be assumed that instability in the rDNA repeats would lead to fewer or poorer quality ribosomes which would then severely impair protein synthesis. However, decreasing ribosomal protein abundance actually increases cellular lifespan (Steffen et al., 2008). Therefore, a decreased quantity of ribosomes is unlikely to reduce lifespan in rDNA unstable mutants, although poor ribosome quality may still be a factor. Alternatively, the large size of the rDNA array means that instability in this locus will sequester DNA repair proteins, lowering the free concentrations of these repair proteins and ultimately resulting in global genetic instability (Kobayashi, 2011). This would then induce the DNA damage response checkpoint which arrests the cell cycle and stops the mother cell from producing subsequent daughter cells (Kobayashi, 2011).

If rDNA instability leads to a reduction in cellular lifespan, then therapies that reduce the amount of rDNA instability could be vital for treatment of age-associated illnesses such as neurodegenerative diseases. One method of stabilizing the rDNA repeats and increasing yeast RLS is through the overexpression of Sir2 (Kaeberlein et al., 1999). This pathway appears to be conserved from yeast to mammals, as mice that overexpress the Sir2 orthologue SIRT1 specifically within neurons have an extended lifespan (Satoh et al., 2013). A related and similarly conserved genome stabilizing longevity intervention, calorie restriction, is explored in the next subsection.

1.6 Caloric restriction promotes genome stability and lifespan

Caloric (or dietary) restriction (CR) has long been heralded as one of the most wide-ranging and potent anti-aging interventions known to science. As early as 1935, it was established that reduction of caloric intake relative to ad libitum (AL) results in an extension in the average and maximal lifespan of laboratory mice (McCay et al., 1935). Since then, similar findings have been observed in a diverse range of organisms including yeast, nematodes, fruit
flies, fish, rats, mice, and dogs, amongst others (Weindruch and Walford, 1988). The discovery of such a potent anti-aging intervention has set the stage for research into the biology of ageing, and caloric restriction has been linked to many molecular pathways which govern ageing (Kaeberlein, 2010). One of the great hopes of the study of conserved longevity pathways is that they will translate to human application and ultimately help us increase the health span of our population.

The existence of conserved longevity pathways seems counterintuitive from an evolutionary perspective. If reducing calories extends lifespan, why wouldn't natural selection prefer individuals with reduced appetites? A leading theory of the evolution of ageing, antagonistic pleiotropy, can explain this; it predicts that genotypes promoting reproduction earlier in life can accelerate the ageing process later in life (Partridge et al., 2005). As evolution selects for reproductive success rather than longevity per se, lifespan takes a backseat to offspring generation (Figure 1.6). Consistent with this, several species experience a decline in early reproductive rate under CR conditions (Partridge et al., 2005).
Figure 1.6. The evolution of aging and dietary effects on lifespan.— (A) Positive and antagonistic pleiotropy theories suggest that alterations conferring advantages in early life respectively trigger beneficial and deleterious effects at the post-reproductive age. (B) Generalized relationships between diets and lifespan.

CR modulates lifespan through many avenues. See Szafranski and Mekhail (2014) for more details. For the purpose of this section, we focus on how CR maintains overall genomic stability, which is critical to lifespan optimization.

We noted in Section 1.4 that controlling rDNA recombination is a key factor that can promote an increased lifespan. CR typically decreases recombination within the rDNA repeats and this extends lifespan (Riesen and Morgan, 2009; Smith et al., 2009). CR has been proposed to suppress recombination within rDNA repeats through multiple mechanisms including the repression of the nutrient-sensing TOR complex as well as activation of Sir2 (Riesen and Morgan, 2009; Smith et al., 2009; Salvi et al., 2013). More recently, CR has also been proposed to increase lifespan by suppressing the activity of rDNA origins of replication preventing them
from excessively competing with weaker replication origins elsewhere in the genome (Kwan et al., 2013). In human cells, the Sir2 homologue SIRT1 (Sirtuin 1) regulates rDNA silencing as a component of the eNoSC (energy-dependent nucleolar silencing complex) protein complex in a glucose-dependent manner (Murayama et al., 2008). SIRT1, which is also activated by CR, is linked to cell survival in human cells and brain-specific lifespan extension in mice (Cohen et al., 2004; Satoh et al., 2013). It is likely that SIRT1-dependent rDNA silencing increases cellular lifespan by decreasing deleterious recombination, similarly to yeast.

On another front, telomeres are linear DNA sequences that are located at the ends of linear chromosomes and are amplified to prevent excessive chromosome shortening and subsequent genome destabilization during cell division (Blackburn and Gall, 1978; Allsopp et al., 1992; Chan and Blackburn, 2002; Collado et al., 2007). Telomeres also help propagate Sir2-dependent silent chromosome structures along nearby regions on chromosome arms (Gottlieb and Esposito, 1989; Hoppe et al., 2002; Wellinger and Zakian, 2012). Importantly, it is clear that telomere length maintenance as well as downstream sirtuin-dependent silent chromatin assembly are both critical to lifespan maintenance (Allsopp et al., 1992; Dang et al., 2009; Chan et al., 2011; Heidinger et al., 2012). CR can promote subtelomeric silencing in yeast through Sir2 and this translates to a longer cellular lifespan (Salvi et al., 2013). SIRT6 (Sirtuin 6), which is another human Sir2 homologue, also promotes telomeric silencing in human cells (Tennen et al., 2011). As CR increases SIRT6 levels, this suggests that CR may promote mammalian lifespan in part by increasing telomeric silencing (Kanfi et al., 2008). Furthermore, in mice and rats, a CR diet helps maintain the length of telomeres over the lifetime of the animal (Pendergrass et al., 2001; Vera et al., 2013). Telomere length maintenance is a strong predictor of lifespan and it is thus likely that CR-dependent telomere length maintenance promotes lifespan (Allsopp et al., 1992; Heidinger et al., 2012). SIRT1 may also regulate telomere length and attenuate age-associated telomere shortening, suggesting that CR acts upstream of SIRT1 to regulate telomere length and promote lifespan (Palacios et al., 2010; Kim et al., 2012). However, TOR also appears to be important for telomere length maintenance and lifespan in yeast (Kwan et al., 2011; Ungar et al., 2011). Therefore, it is likely that CR acts through both sirtuins and TOR modulation in order to optimize lifespan-sustaining telomeric functions. It is important to note that additional CR-dependent processes maintaining rDNA/telomere stability may still exist as currently identified pathways only partly account for the beneficial effects of CR at these repetitive genomic loci.
Connections between CR and several DNA repair processes also exist. One of the DNA repair pathways influenced by CR is base excision repair (BER), which repairs small non-helical distorting lesions in DNA. BER is the most commonly used DNA repair pathway in mammals (Maynard et al., 2009). BER declines with age but caloric restriction prevents such age-associated declines in mice (Cabelof et al., 2003; Stuart et al., 2004). This is likely linked to the ability of CR to promote rate limiting factors in the BER pathway. For example, CR increases the enzymatic activity of apyramidine/apurinic endonuclease as well as the expression of DNA polymerase β (Cabelof et al., 2003; Kisby et al., 2010). Caloric restriction is also capable of activating nucleotide excision repair (NER), which repairs helical-distorting DNA damage caused by large bulky adducts (Guo et al., 1998). In mice, as with BER, NER rates decline with age but this decline is prevented by CR (Guo et al., 1998). In addition, NER dysfunction is linked to skin cancer development and the premature aging disease xeroderma pigmentosum (Lehmann et al., 2011). Another DNA repair pathway in which CR may be implicated is non-homologous end joining (NHEJ), which repairs the majority of DNA double-stranded breaks (DSBs) in mammalian cells (Heydari et al., 2007). The autoantigen Ku is a DNA binding protein in the NHEJ pathway (Jeggo, 1997). Interestingly, Ku expression declines with age in rats but CR can counteract this phenomenon (Um et al., 2003). Future work will show if CR impacts actual NHEJ rates and if this in turn directly affects lifespan. Taken together, CR may promote lifespan by promoting the lifelong maintenance of several DNA repair pathways.

All in all, CR impacts lifespan by promoting genomic stability. This is done by controlling the rDNA, maintaining telomere length and maintaining lifelong rates of DNA repair. Sustaining DNA repair rates over the lifespan may be vital for CR mediated lifespan extension in higher eukaryotes as an increase in cellular lifespan increases the need for DNA repair rates in older cells to prevent age-related diseases such as cancer. However, there are multiple pathways through which CR increases viability of older cells to counteract the increase in circulating aged cells, so this is not the complete story.
1.7 Rationale and hypothesis

My thesis focuses on two interconnected ideas. The first idea is whether or not the accumulation of RNA-DNA hybrids in pbp1Δ cells would affect the replicative lifespan of these mutants. The second was to attempt to replicate these findings in a more disease relevant Pbp1 polyglutamine mutant. These two goals represent a unifying question in this thesis: Do RNA-DNA hybrids affect cellular lifespan and can we create a yeast model of ATXN2 disease to determine if hybrids are a factor in ALS or SCA2?

Goal 1

Rationale: RNA-DNA hybrid accumulation within the rDNA repeats can cause aberrant recombination, which threatens genome integrity (Salvi et al., 2014; Szafranski and Mekhail, 2014). The maintenance of genome integrity is vital to maintaining replicative lifespan (Kaeberlein, 2010). Therefore, Pbp1 likely suppresses RNA-DNA hybrids to maintain genomic stability which in turn maintains replicative lifespan. Treatments that promote genomic repair processes, such as caloric restriction, might serve to increase genomic stability and lifespan in cells lacking Pbp1.

Hypothesis: Pbp1 maintains replicative lifespan and genomic stability by regulating RNA-DNA hybrid formation at the rDNA repeats. Treatments that suppress RNA-DNA hybrids independently of Pbp1 will rescue at least some of the lifespan and genomic stability defect in these cells.

Goal 2

Rationale: Polyglutamine expansion of the human ATXN2 gene can cause SCA2 and ALS (Pulst et al., 1996; Elden et al., 2010). Data from our lab indicate that loss of ATXN2 increases RNA-DNA hybrids, suggesting that this function of Pbp1 is conserved (Abraham et al, under review). Polyglutamine expansion of ATXN2 is known to impair at least some of its endogenous functions (Yokoshi et al., 2014). Therefore, polyglutamine expansion of Pbp1 could impair its
endogenous functions, including R-loop suppression. This would then lead to an increase in RNA-DNA hybrids and a decrease in genomic stability and lifespan; as seen in \( \textit{pbp1}\Delta \) cells.

**Hypothesis:** Polyglutamine expansion of \( \textit{Pbp1} \) will inhibit \( \textit{Pbp1} \)’s ability to suppress hybrids and thereby decrease genomic stability and replicative lifespan.
Chapter 2
Materials and Methods

Portions of this chapter are modified from the following publication:

2 Materials and Methods

2.1 Strains and materials

Yeast strains used in this thesis are listed in Table 2.1. Antibodies: anti-Actin (Fisher), anti-diAcH3-K9-K14 (abcam), HRP-conjugated anti-mouse IgG (GE), HRP-conjugated anti-rabbit IgG (GE), anti-RNA-DNA S9.6 (S. Leppla), anti-TAP-HRP [anti-peroxidase] (Sigma), Anti Histone H3K56ac (abcam), anti-Ubiquitin (abcam). Primers for ChIP, RT-PCR and copy number determination are listed in Table 2.2.

2.2 Yeast transformations

Yeast transformation was done using a standard lithium acetate protocol. Overnight cultures were diluted and grown to early log phase in 5mL media (OD ~ 0.5). Cells were resuspended in LiOAC mix (100mM lithium acetate pH 7.3, 10mM Tris-HCl pH 8.0, 1mM EDTA). 100 μL of these cells were incubated with 14μL of purified concentrated DNA generated from PCR, 30 μL of salmon sperm DNA and 700 μL PEG Mix (21 g PEG3350, 100 mM LiOAc pH7.3, 10 mM Tris-HCl pH8.0, 1 mM EDTA) at 30°C for 45 minutes. Cells were subsequently treated with 100 μL of DMSO and incubated at 42°C for 15 minutes in a water bath. Cells were pelleted, resuspended in SOS mix (1 M sorbitol, 1/3 v/v YEP media, 6.5 mM CaCl₂) and plated. For antibiotic resistance selection, cells were plated onto YEPD plates and transferred to antibiotic selection plates after 24 hours. For amino acid selection, cells were plated directly onto drop out media. Integration was confirmed by colony PCR.

2.3 Time course experiments

To generate longer generation times, cells were cultured on YEPD plates. Briefly, cells were streaked out and then a single colony was re-streaked onto a new YEPD plate every 2 days to avoid nutrient depletion. Approximately 2 weeks of time passed for the 200 generations time point. Generation times were estimated by assuming a duplication to occur approximately every
1.6 hours, as is typical for *S. cerevisiae* (Longo et al., 2012). Standard growth rates were confirmed in all mutants by determining doubling time in liquid culture.

### 2.4 Cloning

Cloning was performed according to NEB protocols. Purified PCR-generated inserts and vectors were digested with restriction enzymes according to supplier’s instructions. Next, 50 ng of vector was incubated with insert at a 1:3 vector to insert molar ratio alongside 1X T4 DNA Ligase Buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT), 400 units T4 DNA Ligase (New England Biolabs) and sterile water to 20 μL. The reaction was incubated in a thermocycler for 25°C for 30 minutes, 16°C for 10 hours and 12°C overnight. 200μL of DH5α cells were thawed on ice and incubated with 4.5μL of ligation reaction for 30 minutes. Cells were heat shocked for exactly 45 seconds, cooled on ice for 2 minutes and then incubated alongside 800μL of LB media (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C for 1 hour. Cells were plated onto pre-warmed LB+amp plates and incubated overnight. Successful transformants were confirmed via colony PCR.

### 2.5 Creation of Pbp1 polyQ plasmids

The vector pRS303 was used as a template. First, a TAP + Trp tag was cloned into the vector using the restriction enzymes PdiI and XhoI (Fisher). Next, a full length Pbp1 + promoter PCR product was generated from W303 genomic Pbp1 + promoter (460BP upstream of Pbp1 start site) using Phusion (NEB) and cloned into the TAP tagged vector using PdiI and PauI (Fisher). To generate the polyglutamine plasmids, Pbp1 polyglutamine sequence plasmids (460BP upstream to ClaI cut site within Pbp1 downstream of polyglutamine site) were ordered from ThermoFisher. These plasmids were digested with ClaI and PauI (Fisher) and the Pbp1 polyglutamine sequence was gel purified and ligated into the full length Pbp1 + promoter plasmid. All plasmids had sequence fidelity confirmed by Sanger sequencing using the sequencing primers listed in Table 2.3.
2.6 Unequal sister chromatid exchange

Assays were performed essentially as described (Kaeberlein et al., 1999; Mekhail et al., 2008). Cells were grown to OD600 = 0.4–0.8, sonicated for 5 seconds at 5% amplitude, and spread (approximately 400 cells per plate) on thick plates (5 mg/l adenine). Incubation was at 30°C for 3 days, 4°C for 3 days, then RT for 1 day. Rates were obtained by dividing the number of half-sectored colonies by the total number of colonies excluding completely red colonies. For calorie restriction USCE experiments, cells were grown overnight under calorie restriction conditions before re-diluting and further growing to OD600 = 0.4–0.8, and then plating on low adenine media.

2.7 Replicative lifespan

Replicative lifespan of yeast strains were determined by micromanipulation as previously described (Kaeberlein et al., 1999). The number of times that a new mother cell buds was measured and the Wilcoxon rank-sum test was conducted for statistical analysis. For each genotype, two independent clones (20 cells for each) were analyzed and data was pooled from two to four separate experiments. Only simultaneously counted isogenic strains were compared to each other on graphs and within our statistical analyses.

2.8 Western blotting

Proteins were isolated by the trichloroacetic acid-coupled (TCA) method (Mekhail, K. et al. 2008). Overnight cultures were diluted and 2.5 x 10^7 cells were grown to log phase (OD_{600} ≈ 1.0). Cells were spun down and resuspended in 500 μL of ice-cold water. Sequential addition of 75 μl of alkali/β-mercaptoethanol (1.85 M NaOH, 1.065 M β-mercaptoethanol) and 75 μl of 50% trichloroacetic acid solutions respectively were each followed by 10 minute incubation on ice. After centrifugation (14,000 RCF at 4 °C for 10 min), pellets were suspended in loading buffer (1 × standard loading buffer, 1.42 M 2-mercaptoethanol, 83.2 mM Tris-HCl pH 8.8), boiled for six minutes, and supernatants were saved. 12μL of lysate was run on a 12% SDS-PAGE gel and
transferred to a membrane. Membranes were probed at 1:1000 dilutions with anti-TAP-HRP (anti-peroxidase) or mouse anti-Actin in TBS with 0.1% Tween-20 and 5% skim milk. For secondary actin antibody, HRP-conjugated anti-mouse IgG was used at 1:2500 dilution in TBS with 0.1% Tween-20 and 5% skim milk. Membranes were transferred to autoradiography films and developed.

2.9 Genomic DNA isolation and copy number determination

Cells were grown to saturation, centrifuged and washed with 500 μl sterilized water. 200 μl of Genomic Lysis Solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA), 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1), and 300 μl of glass beads were added to cells followed by bead-beating for 8 min at RT. The samples were centrifuged (16,000 rcf, 4°C, 5 min) and DNA was precipitated by adding 1/10 volume of 3 M NaOAc pH 5.2 and 2 volumes of cold 100% EtOH and centrifuged (16,000 rcf, 4°C, 15 min). The resultant DNA pellet was washed with 2 volumes of cold 70% EtOH and let dry overnight. The dried DNA pellet was resuspended in 200 μl TE (10 mM Tris-HCl pH 8.0, 1mM EDTA) and treated with 3 μl RNase (10 mg/ml) at 37°C for 10 min. For copy number determination, genomic DNA was serially diluted to 0.05ng/μL. Quantitative real-time PCR was performed using the Biorad CFX Connect Real-Time system with triplicates. A 10 μl qPCR reaction contained 2X SsoAdvanced SYBR Green Supermix (Bio-Rad), 1 μM each forward and reverse primer, and 4 μl of 0.05 ng/μl genomic DNA. PCR parameters were 1 cycle of 95°C for 35 sec, 60°C for 30 sec, followed by 39 cycles of 95°C for 5 sec, 60°C for 30 sec, and the final melt curve at 65°C to 95°C in 0.5°C increment at 5 sec/step. ACT1 was used as a single-copy reference gene (REF). Copy number was then determined by the formula Copy Number = 2^(-ΔCt) where ΔCt = Ct (Gene) - Ct (ACT1).

2.10 Chromatin immunoprecipitation

Experiments were performed as described with modifications (Huang and Moazed, 2003; Mekhail et al., 2008). For standard ChIP assays, yeast cultures (50 ml) were grown to an OD600
of 1.8 and cross-linked with 1% formaldehyde at room temperature (RT) for 30 min. The reaction was quenched with glycine at a final concentration of 125 mM for 5 min at RT. Cells were washed with cold TBS (20 mM Tris-HCl pH 7.6 and 150 mM NaCl). Cell pellets were resuspended in 400 μl of lysis buffer [50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, complete protease inhibitor (Roche)]. Cells were bead-beated with an equal volume of glass beads 2 × 30 s with intermittent 2 min incubation on ice. Lysates were sonicated 3 × 20 s at 40% amplitude with intermittent incubations on ice. Lysates were clarified by 2 consecutive rounds of centrifugation at 16,000 rcf for 5 and 15 min. For antibody IP, 150 μl of lysate was incubated at 4°C for 2 h with 2.0 μg of polyclonal antibody and further incubated with 30 μl of 50% slurry of pre-washed Protein A-Sepharose beads at 4°C for 1 h. Beads were washed 3 × with lysis buffer, 1 × with LiCl buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and 1 × with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and eluted with 50 mM Tris-HCl pH 8.0, 10 mM EDTA, and 1% SDS. The eluant was incubated at 65°C overnight. RNase was added at a final concentration of 0.2 μg/μl and incubated at 37°C for 30 min, followed by proteinase K (to 0.2 μg/μl) and glycogen (to 0.03 μg/μl) treatment for 2 h at 37°C. Dilutions for IP and input DNA were 1:8 and 1:4000, respectively. QPCR Relative fold enrichments were obtained according to the following calculations: \[
\frac{\text{rDNA}_{\text{IP}}}{\text{CUP1}_{\text{IP}}} \div \frac{\text{rDNA}_{\text{Input}}}{\text{CUP1}_{\text{Input}}}.
\]

2.11 RNA extraction

Total RNA was prepared from logarithmically growing cells (OD₆₀₀ ≈ 1.0) via hot phenol extraction. Cells were centrifuged and resuspended in 400 μl of AE buffer (50 mM NaOAc pH 5.3 and 10 mM EDTA in 0.1% DEPC). 40 μl of 10% SDS and 440 μl of acidic phenol (pH 4.5) was added to each sample and incubated at 65°C for 4 min. The samples were rapidly chilled in a dry ice/EtOH bath until phenol crystals appeared. The samples were then centrifuged for 2 min at 16,000 rcf at 4°C, and the upper phase was transferred to fresh tubes. One volume of phenol:chloroform (pH 4.5) was added to each sample, followed by centrifugation and transferring of the upper phase to a fresh tube. 40 μl of 3 M NaOAc (pH 5.3) and 2.5 volumes of cold 100% EtOH was added to each tube prior to centrifugation to precipitate the RNA. The
resultant RNA pellet was washed with 2.5 volumes of cold 80% EtOH. The pellet was left to dry and then resuspended in 0.1% DEPC and quantified. 1 μg of total RNA was treated with 1 unit DNase I (Invitrogen) to further remove genomic DNA contaminations.

2.12 Semi quantitative RT-PCR and quantitative PCR

Semi quantitative reverse transcriptase PCR was performed as described with modifications (Xu et al., 2007). A 20 μl RT reaction was carried out using 10 mM dNTPs, 50 μM random nonamers (Sigma), 500 ng total RNA, 5X First-Strand Buffer (Invitrogen), 100mM DTT, 40 U/μl RNaseOUT (Invitrogen), and 200 U/μl M-MLV reverse transcriptase (Invitrogen) at 23°C for 10 min, 37°C for 60 min, and 70°C for 15 min. From this RT reaction, 1 μl was used in the subsequent PCR amplification. Primer sequences are listed in Table S7. Quantitative real-time PCR was performed using the Biorad CFX Connect Real-Time system. A 10 μl qPCR reaction contained 2X SsoAdvanced SYBR Green Supermix (Bio-Rad), 1 μM each forward and reverse primer, and 1 μl of 10 ng/μl genomic DNA. PCR parameters were 1 cycle of 95°C for 35 sec, 60°C for 30 sec, followed by 39 cycles of 95°C for 5 sec, 60°C for 30 sec, and the final melt curve at 65°C to 95°C in 0.5°C increment at 5 sec/step.

2.13 CHEF electrophoresis

1ml of saturated yeast culture was washed and suspended in 300 μl of EDTA/Tris (50 mM EDTA, 10 mM Tris-HCl pH 7.5). Cells were lysed with 2ul Zymolyase (20 μl/ml in 10 mM Na2HPO4 pH 7.5). 500 uL of low melting-point Seakem agarose (1% in 125 mM EDTA pH 8.0, 42 °C) was subsequently added to cultures which were poured into plug moulds and solidified at 4 °C. Plugs were incubated overnight sequentially in 1 ml of 10 mM Tris-HCl pH 7.5, 500 mM EDTA at 37 °C and 1 ml of 2 mg/ml proteinase K in 10 mM Tris-HCl pH 7.5, 500 mM EDTA, 10 mg/ml N-lauroylsarcosine at 50 °C. Plugs were washed three times with EDTA/Tris (4 °C, 1 h per wash) and stored in 2 ml of EDTA/Tris (4 °C). Plugs and ladder (standard S. cerevisiae; Bio-Rad) were cut to 5 mm × 3 mm × 1.5 mm and resolved on a 0.8% Seakem Gold agarose gel in 0.5 × TBE using the CHEF-DR-II apparatus (Bio-Rad). CHEF running conditions were 24 h,
6.0V/cm, 45 – 95 s, 14°C for whole chromosome analysis and 68 h, 3.0 V/cm, 300-900 s, 10°C for chromosome XII analysis. Gels were EtBr-stained and imaged.

2.14 Southern blotting

CHEF gels were transferred to a Hybond nylon membrane according to standard 20X SSC capillary transfer protocol (Mekhail et al., 2008). DNA was nicked by incubating gels with 250mL of 0.25% HCl for 20 minutes followed by denaturation (20 minutes gentle rocking with 0.5% NaOH) and neutralization (20 minutes gentle rocking with 0.5M Tris pH 7.5, 1.5M NaCl). Gels were then briefly rinsed with deionised water before being primed for 15 minutes with 10X SSC (3M NaCl, 0.3M trisodium citrate dihydrate, pH 7.0). Gels were transferred to membranes in 20X SSC for 16-24 hours using standard capillary assembly (Mekhail et al., 2008). Membranes were UV-cross linked for 5 minutes at blank % and primed in GE Rapid Hybridization buffer for >20 minutes. An IGS1 probe was created using P1 FP and P2 RP (Table 2.2) and α-32P labelled using the GE megaprime system. Primed membranes were then incubated with the radiolabeled probe overnight at 65°C. Membranes were washed with 2X SSC 0.1% SDS, 1X SSC 0.1% SDS for 20 minutes at 65°C sequentially followed by washes with 0.1X SSC 0.1% SDS until membrane radioactivity was below 1.8 Bq/cm². Nylon membranes were transferred to autoradiography films in a phosphorimager cassette with intensifying screens at -80°C for 1-3 days and developed.

2.15 Protein purification

2L of saturated cells were grown at 30°C to late log phase (optical density at 600 nm (OD600) of ~4.0) in yeast extract peptone glucose (YEPD) media with 2% glucose, 1% adenine, and 1% tryptophan. Cells were harvested by centrifugation, washed with ice cold water, and flash frozen using liquid nitrogen. Frozen cells were mechanically ground and thawed in 15 ml of 2X buffer L [12 mM Na2HPO4, 8 mM NaH2PO4·H2O, 0.2% NP-40, 300 mM NaCl, 4 mM EDTA, 2 mM EGTA, 100 mM NaF, 0.2 mM Na3VO4, 40 mM -mercaptoethanol, 2 mM PMSF, and 1 tablet of Complete protease inhibitor (Roche)] for 45 min at 4°C, then 5 ml of 1X buffer L for another 30
min at room temperature. All subsequent steps were performed at 4°C unless stated otherwise. One volume of 0.5 mm diameter cold glass beads (Biospec Products Inc., Cat. # 11079105, Bartlesville, Oklahoma) was added to the cells, and the mixture was subjected to ten pulses of 10s each on a bead-beater (Biospec Products Inc., Cat # 1107900-105, Bartlesville, Oklahoma) equipped with a 50 ml chamber (Biospec Products Inc., Cat # 110803-50, Bartlesville, Oklahoma). The extract was centrifuged at 30,000 RCF for 25 min, and the supernatant was nutated with 300 μl of a 50% slurry of pre-washed IgG-sepharose beads (GE) for 2 h. Beads were transferred to a Poly-Prep chromatography column (Bio-Rad Laboratories) and subjected to three washes with buffer W (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, and 1mM DTT), followed by a 10 ml wash using TEV-C buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 5% glycerol, and 1mM DTT). Beads were washed with 200 μl of TEV-C buffer containing 5 μg/ml TEV protease (Sigma), followed by an overnight incubation with 1 ml of TEV-C buffer containing 5 μg/ml TEV protease (Sigma). After cleavage, the eluate was transferred to a new Poly-Prep column and combined with two 1 ml TEV-C buffer washes of the IgG-sepharose beads. To the combined eluate and washes, 6 ml of binding buffer CAM-B (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% NP-40, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl2, 5% glycerol, and 10 mM -mercaptoethanol), 9 μl of 1M CaCl2, and 250 μl of a 50% slurry of pre-washed calmodulin-sepharose beads (GE) was added and nutated for 3 hours. Three washes with 1.5 ml of CAM-B buffer were performed and beads were eluted into five 250 μl fractions with elution buffer CAM-E (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% NP-40, 1 mM magnesium acetate, 1 mM imidazole, 10 mM EGTA, 5% glycerol, and 10 mM -mercaptoethanol). Half of the purified material was precipitated in 20% TCA on ice for 20 min and centrifuged at 16,000 RCF for 20 minutes. The pellet was washed with cold (-80°C) acetone, centrifuged for 30 min, air-dried at room temperature, digested with trypsin, and analyzed by LC-MS/MS.

2.16 Protein silver staining

50% of the purified protein mixtures were subjected to 12% SDS-PAGE and silver stained. The gel was incubated for 10 min at RT with 50% methanol, 5% methanol, 32 μM DTT, and 0.1%
silver nitrate sequentially, and developed with 280 mM sodium carbonate, 0.05% formaldehyde. The reaction was quenched by the addition of citric acid and gels were imaged.

2.17 Protein immunoprecipitation

Overnight cultures were diluted and 50mL were grown to late log phase (OD$_{600}$ ~ 1.6). Cells were washed with 20mL wash buffer (50mM Tris-HCl pH7.5, 150mM NaCl). Cells were lysed with 400μL of Lysis Buffer (50mM HEPES KOH pH7.5, 150mM NaCl, 10% glycerol, 0.5% NP-40, 1mM EDTA, 1mM PMSF, 1 complete tablet per 30mL). An equal volume of glass beads was added and cells were bead beat ed two times for 30s at 4°C with 2 minutes rest between cycles. Supernatant was separated with a 5min 4°C spin and 110μL was combined with 330μL of Lysis Buffer. Input was prepared by diluting to 1:4 with western loading dye and lysis buffer. Immunoprecipitated protein was prepared by adding 400μL of sample to 100μL of packed IgG beads. Beads were nutated with solution for 2h at 4°C. Beads were subsequently washed 3X with 1mL of Lysis Buffer at 4°C. Protein was eluted from beads by adding 40μL 1X loading buffer and boiling at 95°C for 5 minutes. Hot supernatant was transferred to fresh tubes. Inputs and IPs were then subjected to standard western blotting. Quantification of band intensities was done using ImageJ.
Table 2.1. List of yeast strains used in this thesis

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### Table 2.2. List of primer pairs used for ChIP analysis, RT-PCR and copy number determination.

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### Table 2.3. List of sequencing primers

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Chapter 3
Pbp1 Suppresses RNA-DNA Hybrids to Promote Replicative Lifespan and rDNA Stability

Statement of Contribution:

I performed all of the experimental work described in this Chapter except for indicated hybrid levels which were determined and assessed by Jayesh Salvi.

Portions of this chapter are modified from the following publication:

3 Pbp1 Suppresses RNA-DNA Hybrids to Promote Replicative Lifespan and rDNA Stability

3.1 Introduction

Our lab began to investigate the yeast orthologue of ATXN2, Pbp1, when we discovered that it interacts with ribosomal DNA (rDNA) tethering complexes (Mekhail et al., 2008; Chan et al., 2011). These tethering complexes are important for preventing aberrant hyper-recombination in the highly repetitive rDNA repeats (Mekhail et al., 2008). Therefore, we sought to analyze whether or not Pbp1 impacts hyper-recombination.

My colleagues discovered that the loss of Pbp1 does trigger aberrant recombination at the rDNA repeats (Salvi et al., 2014). Unexpectedly, Pbp1 loss had no effect on rDNA tethering or other factors known to prevent aberrant recombination such as silent chromatin assembly or degradation of non-coding RNA (ncRNA) (Moazed, 2001; Houseley et al., 2007; Mekhail et al., 2008; Vasilieva et al., 2008; Salvi et al., 2014). In fact, loss of Pbp1 was associated with decreased levels of ncRNA (Salvi et al., 2014). This was puzzling, given that it has been established that ncRNA degradation is important for maintaining rDNA stability (Vasilieva et al., 2008). This contradiction was resolved when it was discovered that loss of Pbp1 increased levels of genome-destabilizing RNA-DNA hybrids which would then reduce the amount of free ncRNA (Fig 1) (Salvi et al., 2014). RNA-DNA hybrids can form structures known as R-loops which threaten genome integrity by inducing double stranded breaks and aberrant recombination (Aguilera and Garcia-Muse, 2012). My colleagues discovered that loss of Pbp1 preferentially forms R-loops at sites within the genome that contain the tertiary DNA structure known as G-quadruplexes (G4DNA), although some hybrids are found at non-G4 sites as well (Salvi et al., 2014). Interestingly, Pbp1 interacts with Stm1, a factor that binds to and stabilizes G4DNA providing further evidence of the importance of G-quadruplexes in R-loop formation (Salvi et al., 2014). Overall, this presents a model wherein Pbp1 maintains genomic stability by resolving RNA-DNA hybrids, particularly at G4DNA sites. These hybrids form R-loops that constitute obstacles to advancing replication forks and can trigger aberrant recombination events (Fig 3.1) (Salvi et al., 2014).
Figure 3.1. Model for Pbp1-dependent RNA-DNA hybrid accumulation. Pbp1 suppresses RNA-DNA hybrid accumulation, especially at G4-DNA containing sites in the genome. Loss of Pbp1 increases hybrids, which are amplified when Stm1 binds to and stabilizes G4DNA. This leads to R-loop structures, which constitute deleterious obstacles to advancing replication forks and can lead to double stranded breaks (DSBs).

Preventing aberrant recombination is known to be important for maintaining yeast replicative lifespan (RLS) (Chan et al., 2011). RLS is a measure of the number of progeny that a single yeast mother cell produces (Kaeberlein, 2010). Studies of yeast RLS have found a number of highly conserved longevity factors and yeast RLS is thought to be analogous of the ageing of mitotic cell populations such as neural stem cells (Kaeberlein, 2010; Kwan et al., 2013). The loss of the C. elegans ATXN2 orthologue has been implicated in loss of stem cell proliferation (Ciosk et al., 2004). Thus, we wondered if the loss of Pbp1 would decrease RLS. More importantly, we wondered if Pbp1 maintained lifespan through the suppression of RNA-DNA hybrids and if there are factors that can resolve these hybrids in the absence of Pbp1.
3.2 Results

3.2.1 Pbp1 promotes replicative lifespan by suppressing RNA-DNA hybrids

We examined how Pbp1 impacts cellular lifespan by determining the yeast replicative lifespan (RLS) of pbp1Δ mutants using micromanipulation. Cells lacking Pbp1 have a shorter RLS, demonstrating a role for Pbp1 in maintaining replicative lifespan (Figure 3.2). My colleagues discovered that deleting the yeast protein Stm1 could suppress hybrids and decrease aberrant recombination in a pbp1Δ setting (Salvi et al., 2014). Stm1 can bind to and stabilize G-quadruplex-DNA (G4DNA) structures, which can be found within RNA-DNA hybrid-containing R-loop structures. (Frantz and Gilbert, 1995; Van Dyke et al., 2004). These R-loops are known to be present within rDNA repeats, and can generally be resolved by the Pif1 helicase (Hershman et al., 2008; Paeschke et al., 2011; Paeschke et al., 2013). The deletion of Stm1 in WT cells mildly decreased lifespan (Table 3.1), likely reflecting the fact that many native and conserved G4DNA structures have functions such as transcriptional regulation (Capra et al., 2010; Bochman et al., 2012). However, the deletion of Stm1 robustly extended the lifespan of pbp1Δ cells concurrently with reducing the amount of RNA-DNA hybrids (Fig. 3.2; Table 3.1 for p-values). In the pbp1Δ setting, in contrast to the WT setting, G4DNA stabilization provides further stabilization of R-loop structures formed by RNA-DNA hybridization (Figure 3.1). These data strongly suggests that Pbp1 maintains replicative lifespan by preventing RNA-DNA hybrids. We note that Stm1 deletion did not fully rescue the pbp1Δ lifespan defect, suggesting that Pbp1 may maintain replicative lifespan by regulating RNA-DNA hybrids at non-G4 sites or through other functions.
Figure 3.2. *pbp1Δ* cells have lower replicative lifespan that can be partially rescued through G4DNA disruption. Replicative lifespan plots with mean lifespan and relative RNA-DNA hybrid levels indicated. See Table 3.1 for p-values. Arrows for hybrids indicate approximately a drastic increase in hybrids (approximately 40 fold at rDNA) which is abolished by approximately 75% with Stm1 deletion at G4 sites.
Table 3.1. Replicative lifespan analysis of Pbp1 deletion system

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<tr>
<td></td>
<td>WT (23.3, n=98)</td>
<td>*sir2Δ CR (13.8, n=80)</td>
<td>2.5x10^-18</td>
</tr>
<tr>
<td>3.4B</td>
<td>WT (23.3, n=98)</td>
<td>*pif1Δ (20.9, n=80)</td>
<td>5.3x10^-3</td>
</tr>
<tr>
<td></td>
<td>*pif1Δ (16.7, n=80)</td>
<td>*pif1Δ (20.9, n=80)</td>
<td>6.2x10^-3</td>
</tr>
<tr>
<td></td>
<td>*pif1Δ (20.9, n=80)</td>
<td>*pif1Δ *pif1Δ (16.6, n=80)</td>
<td>2.9x10^-3</td>
</tr>
<tr>
<td></td>
<td>*pif1Δ (16.7, n=80)</td>
<td>*pif1Δ *pif1Δ (16.6, n=80)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>*pif1Δ *pif1Δ (16.6, n=80)</td>
<td>*pif1Δ *pif1Δ CR (20.1, n=98)</td>
<td>6.7x10^-4</td>
</tr>
<tr>
<td></td>
<td>*pif1Δ CR (20.5, n=88)</td>
<td>*pif1Δ *pif1Δ CR (20.1, n=98)</td>
<td>0.58</td>
</tr>
<tr>
<td>3.4C</td>
<td>WT (23.3, n=98)</td>
<td>*rnh1Δ rnh201Δ (16.2, n=80)</td>
<td>1.2x10^-11</td>
</tr>
<tr>
<td></td>
<td>*pbp1Δ (16.7, n=80)</td>
<td>*rnh1Δ rnh201Δ (16.2, n=80)</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>*rnh1Δ rnh201Δ (16.2, n=80)</td>
<td>*pbp1Δ *rnh1Δ rnh201Δ (12.5, n=83)</td>
<td>2.9x10^-3</td>
</tr>
<tr>
<td></td>
<td>*pbp1Δ (16.7, n=80)</td>
<td>*pbp1Δ *rnh1Δ rnh201Δ (12.5, n=83)</td>
<td>2.3x10^-4</td>
</tr>
<tr>
<td></td>
<td>*rnh1Δ rnh201Δ (16.2, n=80)</td>
<td>*rnh1Δ rnh201Δ CR (15.5, n=80)</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>*pbp1Δ *rnh1Δ rnh201Δ (12.5, n=83)</td>
<td>*pbp1Δ *rnh1Δ rnh201Δ CR (13.4,0.34)</td>
<td>n=81</td>
</tr>
<tr>
<td></td>
<td>*pbp1Δ CR (20.5, n=88)</td>
<td>*pbp1Δ *rnh1Δ rnh201Δ CR  (13.4,1.3x10^-10)</td>
<td>n=81</td>
</tr>
</tbody>
</table>

* All strains are isogenic with W303a and mean lifespan followed by the number (N) of cells analyzed are listed in parenthesis. For each genotype, two independent clones (~20 cells for each) were analyzed per experiment and data was pooled from three to six separate experiments. Only simultaneously counted isogenic strains were compared to each other on graphs and within our statistical analyses. Assessing the difference in differences in lifespan between WT and *pbp1Δ* and *fob1Δ* and *fob1Δ* *pbp1Δ* was done by compiling three simultaneously counted pairs of sets, analyzing the difference in lifespan between each pair and performing a student’s t test to determine significance.

£ CR, caloric restriction.
3.2.2 Pbp1 maintains lifespan through rDNA dependent and independent processes

RNA-DNA hybrid levels were highest in the rDNA intergenic spacers and these hybrids increased aberrant recombination at this loci (Salvi et al., 2014). Furthermore, rDNA stability is well known as a key determinant of yeast RLS (Oberdoerffer and Sinclair, 2007). We therefore asked if hybrid accumulation is decreasing lifespan by causing aberrant recombination in the rDNA. To test this, we used a fob1Δ strain, as Fob1 is required for rDNA recombination (Defossez et al., 1999). An ADE2 marker was inserted into the rDNA repeats and the loss of this marker indicates aberrant recombination by giving colonies a red colour on low adenine plates (Fig 3.3a, right). Half-sectored colonies (Fig 3.3a right, outlined in yellow) indicate the loss of the ADE2 marker in the first division after plating and thus allow for quantification of the rate of marker loss. As predicted, we show that loss of Pbp1 in a fob1Δ strain did not increase rates of rDNA recombination; in fact, recombination levels remained depressed relative to wild-type cells (Fig 3.3a). Loss of Pbp1 in a fob1Δ strain resulted in a lower percentage decrease in lifespan as determined using a student’s T-test (Fig 3.3b; Table 3.1). This demonstrates that Pbp1 must increase lifespan by maintaining rDNA stability. However, lifespan of our fob1Δpbp1Δ strain was still lower than the fob1 Δ strain (Fig 3.3b), indicating that Pbp1 must maintain lifespan through rDNA independent functions as well. This could be due to the fact that Pbp1 suppresses hybrids within telomeric heterochromatin as well as in G4-rich coding regions in the genome (Salvi et al., 2014).
Figure 3.3. Pbp1 maintains replicative lifespan through rDNA dependent and independent processes. (A) Relative rates of loss (±SD) of ADE2 marker gene from rDNA repeats. Representative images are shown. See Table 3.2 for associated information (B) Replicative lifespan plot with mean lifespans and the differential effect of pbp1 deletion indicated. See Table 3.1 for p-values.
Table 3.2. Rates of ADE2 marker loss from rDNA repeats.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ADE2 loss</th>
<th>P value b</th>
<th>Half-sectored over total c</th>
<th>Rate relative to wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of loss x10^-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.4 (± 0.8)</td>
<td>N/A</td>
<td>14/9,821</td>
<td>1</td>
</tr>
<tr>
<td>pbp1Δ</td>
<td>7.4 (± 1.0)</td>
<td>&lt;0.01</td>
<td>96/12,954</td>
<td>5.3</td>
</tr>
<tr>
<td>fob1Δ</td>
<td>0.2 (± 0.2)</td>
<td>&lt;0.01</td>
<td>2/9,517</td>
<td>0.1</td>
</tr>
<tr>
<td>fob1Δ pbp1Δ</td>
<td>0.2 (± 0.3)</td>
<td>&lt;0.01 (&lt;0.01)</td>
<td>2/7,382</td>
<td>0.1</td>
</tr>
<tr>
<td>Pbp1-TAP</td>
<td>1.1 (± 0.4)</td>
<td>0.46</td>
<td>5/4,350</td>
<td>0.8</td>
</tr>
<tr>
<td>Pbp1(1-50)-TAP</td>
<td>3.8 (± 1.1)</td>
<td>&lt;0.01 (&lt;0.01)</td>
<td>47/12,364</td>
<td>2.7</td>
</tr>
<tr>
<td>Pbp1(1-297)-TAP</td>
<td>0.9 (± 1.0)</td>
<td>0.23 (&lt;0.01)</td>
<td>11/11,847</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a Standard deviations are shown between brackets.
b P calculated using Student’s t-test. Values outside brackets obtained via comparison to WT cells.
c Total number excludes completely red colonies.
d Value in brackets obtained via comparison to pbp1Δ.
e Values in brackets obtained via comparison to fob1Δ.

3.2.3 Caloric restriction increases the rDNA repeat stability and lifespan of pbp1Δ mutants through the activation of RNase H enzymes not the helicase Pif1

Caloric restriction (CR) is generally linked to lifespan extension in various organisms and can increase genomic stability (Szafranski and Mekhail, 2014). Given that SCA2 and ALS are age associated diseases; we asked if CR can affect RNA-DNA hybrid levels in pbp1Δ cells. Overall, we found that CR decreased levels of RNA-DNA hybrids in Pbp1-deficient cells (Salvi et al., 2014). This was accompanied by a decrease in rDNA recombination and an increase in lifespan that was not seen in cells deficient in the CR-mediating histone deacetylase Sir2 (Figure 3.4a). The loss of the helicase Pif1, which maintains genomic stability through G4DNA helicase activity, decreased CR-mediated hybrid suppression in pbp1Δ cells (Paeschke et al., 2013; Salvi et al., 2014). The conserved RNaseH enzymes have specific RNA-DNA hybrid resolving activity and we found that pbp1Δ cells lacking the yeast RNaseH enzymes (Rnh1 and Rnh201) had reduced hybrid suppression under CR (Huertas and Aguilera, 2003; Salvi et al., 2014). Given this, we asked if CR was acting through either Pif1 or Rnh molecules to suppress rDNA USCE and increase lifespan. Despite the fact that the loss of either Pif1 or Rnh1 and Rnh201 can impair CR-mediated hybrid decrease, we found that only the Rnh enzymes were significantly necessary.
to maintain genomic stability or lifespan under CR (Fig 3.4b-d). This suggests that there may be lack of phenotypic penetrance in a *pbp1Δpif1Δ* CR setting.

Figure 3.4. Caloric restriction increases the rDNA repeat stability and lifespan of *pbp1Δ* cells through the activation of RNases not helicase Pif1. (A,C,D) Replicative lifespan plots with mean lifespan and relative RNA-DNA hybrid levels indicated. See Table 3.1 for p-values (B) Relative rates of loss (±SD * P<0.01; n.s. not significant) of ADE2 marker gene from rDNA repeats. Rnhs = *rnh1Δrnh201Δ*. See Table 3.3 for p values and counts. Two arrows indicate a drastic increase in hybrids (*pbp1Δ*; approximately 40 fold at rDNA IGS1) or slight increase in hybrids (*rnh1Δ, pif1Δ, rnh3Δ*; approximately 10 fold at rDNA IGS1). This increase is then either fully abolished by CR (95%+ reduction) in the single mutants, or only partially reduced in the *pbp1Δpif1Δ* and *pbp1Δrnh3Δ* mutants (30-70% reduction).
### Table 3.3. Effect of caloric restriction on rates of ADE2 marker loss from rDNA repeats.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ADE2 loss</th>
<th>Rate of loss ( \times 10^{-3} )(^a)</th>
<th>( P ) value (^{b,c,d})</th>
<th>Half-sectored over total (^e)</th>
<th>Rate relative to non-CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>1.3 (± 1.0)</td>
<td>N/A</td>
<td>35/27,704</td>
<td>1</td>
</tr>
<tr>
<td>WT CR</td>
<td></td>
<td>0.6 (± 0.4)</td>
<td>0.01</td>
<td>19/32,419</td>
<td>0.46</td>
</tr>
<tr>
<td>( pbp1\Delta )</td>
<td></td>
<td>6.2 (± 0.3)</td>
<td>&lt;0.01</td>
<td>272/43,632</td>
<td>1</td>
</tr>
<tr>
<td>( pbp1\Delta ) CR</td>
<td></td>
<td>2.9 (± 1.1)</td>
<td>&lt;0.01 (&lt;0.01)</td>
<td>101/34,320</td>
<td>0.47</td>
</tr>
<tr>
<td>( sir2\Delta )</td>
<td></td>
<td>6.8 (± 2.1)</td>
<td>&lt;0.01</td>
<td>163/23,995</td>
<td>1</td>
</tr>
<tr>
<td>( sir2\Delta ) CR</td>
<td></td>
<td>3.3 (± 1.0)</td>
<td>&lt;0.01 (&lt;0.01)</td>
<td>90/26,906</td>
<td>0.49</td>
</tr>
<tr>
<td>( pbp1\Delta ) ( sir2\Delta )</td>
<td></td>
<td>10.4 (± 3.2)</td>
<td>&lt;0.01</td>
<td>251/24,151</td>
<td>1</td>
</tr>
<tr>
<td>( pbp1\Delta ) ( sir2\Delta ) CR</td>
<td></td>
<td>9.5 (± 2.9)</td>
<td>&lt;0.01 (0.45)</td>
<td>284/29,854</td>
<td>0.91</td>
</tr>
<tr>
<td>( pif1\Delta )</td>
<td></td>
<td>2.8 (± 0.9)</td>
<td>&lt;0.01</td>
<td>39/14,138</td>
<td>1</td>
</tr>
<tr>
<td>( pif1\Delta ) CR</td>
<td></td>
<td>1.1 (± 0.3)</td>
<td>0.52 (&lt;0.01)</td>
<td>14/12788</td>
<td>0.47</td>
</tr>
<tr>
<td>( pbp1\Delta ) ( pif1\Delta )</td>
<td></td>
<td>9.2 (± 1.3)</td>
<td>&lt;0.01</td>
<td>103/9706</td>
<td>1</td>
</tr>
<tr>
<td>( pbp1\Delta ) ( pif1\Delta ) CR</td>
<td></td>
<td>6.0 (± 1.6)</td>
<td>&lt;0.01 (&lt;0.01)</td>
<td>52/8360</td>
<td>0.65</td>
</tr>
<tr>
<td>( rnh1\Delta ) ( rnh201\Delta )</td>
<td></td>
<td>1.5 (± 0.7)</td>
<td>0.55</td>
<td>19/12,642</td>
<td>1</td>
</tr>
<tr>
<td>( rnh1\Delta ) ( rnh201\Delta ) CR</td>
<td></td>
<td>0.9 (± 0.4)</td>
<td>0.13 (0.07)</td>
<td>11/12824</td>
<td>0.57</td>
</tr>
<tr>
<td>( pbp1\Delta ) ( rnh1\Delta ) ( rnh201\Delta )</td>
<td></td>
<td>8.4 (± 2.1)</td>
<td>&lt;0.01</td>
<td>103/12,321</td>
<td>1</td>
</tr>
<tr>
<td>( pbp1\Delta ) ( rnh1\Delta ) ( rnh201\Delta ) CR</td>
<td></td>
<td>6.5 (± 2.6)</td>
<td>&lt;0.01 (0.09)</td>
<td>83/12690</td>
<td>0.77</td>
</tr>
</tbody>
</table>

\(^a\) Standard deviations are shown between brackets.

\(^b\) \( P \) calculated using Student’s t-test.

\(^c\) Value outside brackets obtained via comparison to WT non-CR cells.

\(^d\) Value in brackets obtained via comparison to corresponding non-CR cells.

\(^e\) Total number excludes completely red colonies.
3.2.4 Pbp1 is not acting through its binding partner Pab1 to increase rDNA stability.

Pbp1 can bind to the polyadenylation factor Pab1 and can influence the polyadenylation of a few mRNAs (Mangus et al., 1998). Therefore, we wondered if Pbp1 might be acting through Pab1 to increase genomic stability. A Pbp1 (1-297) truncation mutant which harbours the RNA-binding LSM domains but cannot bind Pab1 showed no increase in rDNA recombination (Fig 3.5a,b). Therefore, Pbp1 is not stabilizing the rDNA through Pab1 binding.

![Figure 3.5. Pbp1 is not acting through its binding partner Pab1 to increase rDNA stability.](image)

(A) Coimmunoprecipitation analysis examining interactions between Pbp1, Pbp1(1–297), and Pbp1(1–50) with Pab1. (B) Relative rates of loss (±SD * P<0.01; n.s. not significant) of ADE2 marker gene from rDNA repeats. See Table 3.2 for associated information.

3.3 Discussion

Here we show that loss of the yeast ATXN2 orthologue Pbp1 promotes G4DNA/Stm1-stabilized RNA-DNA hybrids. The resulting R-loop structures, along with Fob1-dependent fork blocks, trigger aberrant recombination events, and shorten replicative lifespan.
Interestingly, CR counteracts Pbp1 loss by relying on RNaseH to degrade the RNA of hybrids and on Pif1 to unwind G4DNA allowing nontemplate single stranded DNA to compete with hybrids. However, only the RNaseH molecules are required for CR-mediated lifespan increase in *pbp1Δ* cells. This indicates that not all R-loops are created equal and highlights the importance of redundancy in RNA-DNA hybrid suppressing mechanisms. Recent work in our lab has demonstrated that magnesium can mirror the lifespan extending effects of CR in *pbp1Δ* mutants, but only the loss of both Pif1 and the Rnh molecules abolishes its lifespan-extension effects (Abraham et al, under review). This demonstrates that there are multiple pathways through which CR acts and the redundancy of these hybrid suppressing pathways are important to genomic maintenance. Additionally, we provide a new method through which CR works to increase genomic stability by upregulating RNA-DNA hybrid suppression factors.

Similar to yeast Pbp1, we expected roles for Ataxin2 proteins in the suppression of RNA-DNA hybrids and maintenance of cellular lifespan in other organisms. Indeed, our lab has demonstrated that loss of ATXN2 leads to the accumulation of nucleolar RNA-DNA hybrids in human HeLa and HEK293 cells (Abraham et al, under review). Dysfunction of the human *ATXN2* gene can lead to the severely debilitating neurodegenerative diseases SCA2 and ALS (Pulst et al., 1996; Elden et al., 2010). If excessive R-loop formation does indeed underlie one or more of these diseases, caloric restriction and its mimetic drugs may lead to viable therapeutic approaches. Such interventions may also be beneficial in other clinical settings as accumulating evidence points to genetic links between R-loop suppressors and human disease (Santos-Pereira and Aguilera, 2015). For example, the RNA-DNA helicase Senataxin is mutated in the neurodegenerative diseases ataxia with oculomotor apraxia and juvenile ALS (Chen et al., 2004; Moreira et al., 2004). BRCA1 or BRCA2 deficient cells also accumulate R-loops and subsequently genome instability and mutations in BRCA1 or BRCA2 are common causes of breast or ovarian cancer (King et al., 2003; Bhatia et al., 2014). Additionally, defects in the conserved human PIF1 helicase family are linked to premature aging and increased cancer risk (Paeschke et al., 2013).

All in all, we unearth roles for the yeast ATXN2 orthologue Pbp1 in genome and replicative lifespan maintenance and identify caloric restriction as an activator of processes counteracting the deleterious effects of Pbp1 deficiencies.
Chapter 4
Polyglutamine Pbp1 Cells Promote Gross Chromosomal Rearrangement and Reduced Lifespan

Statement of Contribution:

I performed all of the experimental work described in this Chapter except for Figure 4.7 which was performed by Ruchen Guo and analyzed by me, Figure 4.5B which was performed with assistance from Nancy Liu, Figure 4.5C which was performed with assistance from Ruchen Guo and 4.11B which was performed with assistance from Hongbo Guo.
4 Polyglutamine Pbp1 Cells Promote Gross Chromosomal Rearrangement and Reduced Lifespan

4.1 Introduction

As outlined in Chapter 1, ATXN2 is one of a diverse array of RNA regulatory proteins that have connections to neurodegenerative diseases. Polyglutamine expanded ATXN2 has been proposed to cause ALS or SCA2 by both loss of function and gain of toxic functions (van den Heuvel et al., 2014). In chapter 3, I demonstrated that Pbp1 deletion results in a loss of yeast replicative lifespan (RLS) concurrent with the loss of the stability of the rDNA repeats. This is presumably due to the R-loop suppressing function of Pbp1 discovered by my colleagues. For example, the deletion of the R-loop promoting Stm1 resulted in a miniscule drop in RLS in wild-type cells, but increased lifespan in cells lacking Pbp1 (Salvi et al., 2014). Similarly, caloric restriction was only able to rescue pbp1Δ RLS in the presence of the hybrid degrading RNaseH enzymes.

However, this work was done entirely with cells that lack Pbp1. Given that patients with ATXN2-linked diseases have a polyglutamine mutation in ATXN2, we sought to create a yeast genetic model of ATXN2 disease by inducing the expression of a polyglutamine expanded version of Pbp1. Such a model could be used for high throughput drug discovery tests. This chapter outlines the work that I’ve done in establishing and characterizing this yeast model system.

4.2 Results

4.2.1 Creation of Pbp1 polyglutamine system

To address whether or not polyglutamine mutation in Pbp1 would result in similar phenotypes to Pbp1 deletion, I generated plasmids expressing either non-mutated N-terminus TAP-tagged Pbp1 or a five, fifteen, thirty or forty glutamine expanded version of the protein along with the endogenous protein promoter (Figure 4.1a). See sections 2.3 and 2.4 for details.
about the cloning process. These plasmids were transformed into \textit{pbp1}\textDelta cells to create an episomal system which can demonstrate the difference in the rescue effect of wild-type Pbp1 versus polyglutamine expanded Pbp1. Expression was confirmed via western blotting against TAP (Figure 4.1b). Proteins expressed from plasmids may have different expression levels, post-translational modifications or localization compared to endogenous proteins (Al-Dosari and Gao, 2009). Thus, I sought to create chromosomal polyglutamine expanded proteins. To do this, a HIS selectable PCR product was generated from the plasmids and transformed into a strain already containing an endogenously TAP-tagged Pbp1. Fidelity of the insertion was confirmed via sequencing PCR products obtained from genomic DNA (Figure 4.1c). Unfortunately, the 40Q was not able to be transformed, possibly due to the highly repetitive nature of this PCR product. A 20 glutamine version of the protein was however confirmed via sequencing after a 30Q transformation, suggesting a random recombination event that produced a truncated insertion. Western confirmed the expression of these mutated proteins, with notable size shifts in the proteins beyond the 1-4 kDa shift expected due to the presence of the glutamine tract, suggesting that these proteins are perhaps undergoing different post-translational modifications (Figure 4.1c).
Figure 4.1. Creation of a Pbp1 polyglutamine system. (A) Sample plasmid map from the 30Q strain. (B,C) Western blotting against TAP confirms the expression of polyglutamine Pbp1 in both the (B) episomal and (C) chromosomal systems.
4.2.2 Polyglutamine expansion results in gross chromosomal rearrangement

Contour-clamped homogeneous electric field (CHEF) gels allow for separation of chromosomes and can determine if there are gross chromosomal rearrangements (GCR) – a devastating insult to genomic stability that underlie many oncogenic events in human disease (Stratton et al., 2009; Moore et al., 2012). I ran a CHEF gel using a 24 hour timeframe which allows for the separation of all 16 yeast chromosomes (Chung et al., 2015). Two independent runs confirmed that chromosome size shifts are seen in all polyglutamine mutants within the chromosomal system and within the 15Q and 30Q mutants in the episomal system; indicating gross chromosomal rearrangement (GCR) is a feature of these mutants (Figure 4.2a,b). In particular, the chromosomal system demonstrates recombination in smaller chromosomes such as XIV or XIII in Pbp1.5Q or XV or VII in Pbp1.20Q or Pbp1.30Q (Figure 4.2a). The episomal system showed aberrations only in chromosome XII (Figure 4.2b, Figure 4.3b). These chromosomal aberrations were not present in cells simply lacking Pbp1 (Figure 4.2a lane 2). This demonstrates a distinctive gain of toxicity mechanism within the polyglutamine mutants.
Figure 4.2. Polyglutamine expansion results in gross chromosomal rearrangement. (A,B) 24 hour CHEF gel demonstrating diverse changes in chromosomes of polyglutamine mutants in (A) chromosomal and (B) episomal systems respectively. Altered chromosomes are indicated by arrows to the right of the gel. Briefly, there is loss of double banding between Chromosomes XII and XVI in Pbp1.5Q (bottom arrow, left). Chromosomes XV and VII run as a single band normally but two bands are observed in Pbp1.20Q and Pbp1.30Q (middle two arrows on the left). Chromosomes IV and XII typically run as a single band but double bands are observed in Pbp1.15Q and pbp1Δ + 15Q and pbp1Δ + 30Q (top arrow left and right). Images filtered for ethidium bromide speckling.
4.2.3 Intermediate length polyglutamine expansions increase rDNA array size

Pbp1 is known to regulate the stability of rDNA repeats through RNA-DNA hybrid regulation (Salvi et al., 2014). The 24 hour CHEF gel does not allow for adequate separation of the rDNA-containing chromosome XII. Given that several chromosomes demonstrated altered sizes and the strong association of rDNA with lifespan, I sought to characterize both rDNA stability and size with a 68 hour CHEF gel. Strains with more destabilized rDNA repeats have very smeared or non-visible bands for chromosome XII due to frequent recombination events leading to highly variable chromosome size (Mekhail et al., 2008). Significant band smearing was not seen in Southern blots for chromosome XII in any of the strains (Figure 4.3a-b). However, there was a marked increase in the size of chromosome XII for both the 15Q and 30Q within both systems. This increase was confirmed by performing a copy number QPCR quantifying the difference in amplification of 25S rDNA versus the single copy ACT1 control from genomic DNA (Figure 4.3 c-d). Taken together, these data strongly suggest a mechanism through which intermediate length polyglutamine expansion activates factors that alter the size of the rDNA repeats.
Figure 4.3. Intermediate length polyglutamine expansions increase rDNA array size. (A,B) 68 hour CHEF gel of yeast chromosome plugs. Both ethidium bromide staining and IGS1 southern blotting are indicated for chromosomal (A) and episomal (B) systems. The black line indicates the wild type median Chr. XII size to facilitate comparison (C,D) Copy number QPCRs.
of 25S rDNA for chromosomal (C) and episomal (D) systems. Signal is normalized to single copy ACT1 gene. Student t-test results: * p<0.05 ** p<0.005. n=7 and 5 respectively
Another method to measure rDNA stability is examining the amount of unequal sister chromatid exchange via the loss of a single ADE2 marker inserted into the rDNA array (see Chapter 1.5 and Chapter 3 for more details). However, if there is a general trend towards increasing copy number, then the ADE2 reporter could randomly be duplicated in some instances. This would result in artificially low readings for the USCE ADE2 reporter assay, as there would need to be two marker loss events to create a half-sectored cell. Indeed, examining the ADE2 copy number by qPCR revealed that the ADE2 gene had on average doubled in the 15Q and 30Q strain for both chromosomal and episomal systems, with a significant subset of the 20Q strain having duplication events (Figure 4.4a-b). This correlated well with abnormally low USCE readings for these strains (Figure 4.5b-c). Strains that did not have increased rDNA size (5Q and 40Q) did not have increases in the rate of ADE2 marker loss. In the 5Q and 40Q strains we ascertain that rDNA USCE is not increased relative to basal rates, however this assay is unable report meaningful data about the amount of rDNA recombination in the 15Q and 30Q strains due to marker duplication.

Figure 4.4. Intermediate length polyglutamine expansions increase rDNA ADE2 marker copy number. (A,B) Copy number QPCRs of ADE2 gene normalized to single copy ACT1 control. Student t-test results: * p<0.05 ** p<0.005. n=7.
Figure 4.5. Intermediate length polyglutamine expansion produces artificially low rDNA ADE2 marker loss rates. (A) Loss of an ADE2 marker inserted into the rDNA array results in half-sectored cells (right, outlined in yellow) which indicate rDNA USCE. If the marker duplicates (Figure 4.4a,b) then two independent recombination events would need to occur to result in a half-sectored cell leading to artificially low readings (B,C) Dot plots demonstrating relative rate of loss of an ADE2 marker. Medians are indicated by a straight line. Student t-test results: * p<0.05 ** p<0.005 *** p<0.0005 N.S. Not significant
4.2.4 Increasing generation number promotes chromosome XII expansion but no additional gross chromosome rearrangements occur

Thus far I note that there is a diverse range of chromosomal rearrangements in the polyglutamine mutants in the chromosomal system and chromosome XII is larger in the 15Q and 30Q mutants for both the chromosomal and episomal systems. I wondered whether additional chromosome recombination would occur with successive generations, so I performed a time-course experiment with the chromosomal system to determine whether or not additional chromosome defects would occur with increasing generations. Chromosome IV and XII typically run as a single band, but they run separately in all strains at 200 generations (Figure 4.6A, indicated by the arrow). Separate banding is also observed at 100 generations in the 15Q and 30Q, and at 0 generations in the 15Q mutant – as noted in section 4.2.2 (Figure 4.6A). Southern blotting confirmed that the separate bands were all chromosome XII (Figure 4.6B). No other chromosomes demonstrated defects with increased generations (Figure 4.6A). This suggests that gross recombination of chromosomes other than chromosome XII may be a rare event that is unlikely to be captured after many generations. Alternatively, GCR could still be occurring commonly in the polyglutamine mutants but most GCR events are lethal and thus cells experiencing GCR would be selected out during culturing.

I wondered whether or not the double banding in chromosome IV and XII was due to chromosome XII hyperamplification. Therefore, I ran a chromosome XII resolving 68 hour CHEF gel. Here I note that all strains demonstrated increasing chromosome XII size with increasing generation (Figure 4.6B). This is consistent with previous reports that rDNA repeats are capable of increasing after successive generations (Kobayashi and Ganley, 2005). Previous work demonstrating accumulative rDNA repeat gain was done in a strain with an artificially low rDNA repeat array number and found that rDNA size stabilized after 150 generations (Kobayashi and Ganley, 2005). Thus, the wild type strain used in this study, W303 with an \textit{ADE2} marker inserted into the rDNA array, may have not been cultured for sufficient generations to establish rDNA array equilibrium after \textit{ADE2} insertion.
After 100 generations, the 15Q and the 30Q are both larger than the wild-type, consistent with the observation at 0 generations (Figure 4.6B). However, after 200 generations, while the 15Q is still relatively larger than the wild-type, the 30Q falls behind in repeat gain and subsequently has a smaller rDNA array than wild-type. Likewise, \textit{pbp1}Δ and 5Q – strains that are relatively similar or slightly larger than the wild-type at 0 generations – show exacerbating defects in rDNA repeat gain at 100 and 200 generations (Figure 4.6B). The 15Q, 20Q and 30Q strains all demonstrate a clear smear pattern at 100 generations suggestive of hyperactive recombination. Interestingly, smearing is not present in the \textit{pbp1}Δ strain despite higher rates of rDNA USCE (Figure 3.3a), suggesting that copy number in this strain is still relatively tightly regulated despite the increase in aberrant recombination. Perhaps \textit{pbp1}Δ cells only experience an increase in aberrant recombination despite overall recombination levels maintaining steady. After 200 generations, some smearing is present in all of these strains as well as the wild-type. All in all, the 15Q and 30Q strains are larger than wild-type during early time points. However, after 200 generations, \textit{pbp1}Δ, 5Q, 20Q and 30Q are no longer able to promote rDNA array expansion as efficiently as the wild-type or have reached an rDNA plateau earlier than wild-type. In contrast, the 15Q strain shows an expanded rDNA array relative to wild-type that persists through 200 generations. As well, the earlier smearing phenotype of the polyglutamine mutants suggests that these cells are more readily undergoing rDNA hyper-recombination. These data suggest that these strains might have a lowered CLS due to an earlier accumulation of genomic instability. However, I note that these cells were not cultured in a quiescent state as is typical of CLS studies (Parrella and Longo, 2008).
**Figure 4.6. Chromosome XII size increases with time.** (A) 24 hour CHEF gel after 0, 100 and 200 generations of culturing for the chromosomal system. The chromosome XII/IV band shows aberrations with greater generations (indicated by black arrows). No other chromosomes demonstrated noticeable size shifts. Southern blotting confirmed that these aberrations were chromosome XII (data not shown). (B) 68 hour CHEF gels after 0, 100 and 200 generations were run and Southern blotted against rDNA IGS1. All strains showed an increase in size relative to generation 0 (wild type generation 0 indicated by red line). After 100 generations, wild-type, 15Q and 30Q maintained their relative size differences with pnp1Δ, 5Q and 20Q falling behind. This is exacerbated at 200 generations at which point 30Q starts to fall behind as well.
4.2.5 Intermediate length polyglutamine expansions reduce lifespan

Cells with elevated rates of GCR have been shown to have much shorter RLS (Madia et al., 2008). Additionally, the relative stability of the rDNA repeats is known to tightly regulate lifespan, with mutations that stabilize rDNA repeats promoting lifespan increase while mutations that destabilize the rDNA repeats promote RLS reduction (Saka et al., 2013). Therefore, I examined whether or not polyglutamine expansion would impact replicative lifespan within the chromosomal system, particularly using the rDNA-unstable 15Q and 30Q strains. The episomal system was unable to be analyzed by RLS micromanipulation due to the necessity of amino acid selection in maintaining plasmid expression. Both the 15Q and 30Q expansion resulted in a drastic decline in lifespan; in fact, the 30Q lifespan is actually significantly shorter than the \( pbp1\Delta \) lifespan (Figure 4.7; Table 4.1). Given that the lifespan is actually shorter in 30Q cells than in \( pbp1\Delta \) cells, this further demonstrates that these strains have a gain of toxicity in addition to any possible deleterious effects from loss of Pbp1 function.

\[ \text{WT (23.85)} \]
\[ \text{\( pbp1\Delta \) (16.44)*} \]
\[ \text{Pbp1.15Q (14.86)*} \]
\[ \text{Pbp1.30Q (13.60)*} \]

**Figure 4.7. Intermediate length polyglutamine expansion reduces lifespan in chromosomal system.** Replicative lifespan plot with mean lifespan indicated for chromosomal mutants. WT= wild-type. *Significantly lower than wild-type. †Significantly lower than \( pbp1\Delta \). All stats are done using Wilcoxon rank-sum tests.
Table 4.1. Replicative lifespan analysis of polyglutamine system

<table>
<thead>
<tr>
<th>Figure</th>
<th>Strain A</th>
<th>Strain B</th>
<th>P value</th>
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<td>pbp1Δ (16.44, n=80)</td>
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<td></td>
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<td>Pbp1.15Q (14.86, n=79)</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>WT (23.85, n=80)</td>
<td>Pbp1.30Q (13.60, n=78)</td>
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* All strains are isogenic with W303a and mean lifespan followed by the number (n) of cells analyzed are listed in parenthesis. For each genotype, two independent clones (~20 cells for each) were analyzed per experiment and data was pooled from four separate experiments.

4.2.6 Polyglutamine expanded Pbp1 does not increase RNA-DNA hybrids at rDNA

Our previous findings indicated that Pbp1 could prevent RNA-DNA hybrids and this was important for RLS maintenance (Salvi et al., 2014). The most hybrid-enriched site was the intergenic spacer regions of the rDNA (Figure 4.8a) (Salvi et al., 2014). I sought to replicate this finding in the polyglutamine systems. In the episomal system I found no evidence of an increase in hybrids at either the IGS1 or IGS2 regions in strains expressing a polyglutamine expanded Pbp1 (Figure 4.8b-d). In contrast, the empty vector strain had a significant increase in hybrids at regions P1, P4 and P5 when compared to strains expressing wild-type Pbp1 from plasmid (Figure 4.8b-d). Similarly, there was an increase in hybrids at the IGS1 site P4 for pbp1Δ cells compared to cells expressing Pbp1TAP (Figure 4.8e). However, there was no significant increase in hybrids for cells expressing polyglutamine Pbp1 (Figure 4.8e). I note that the enrichment of hybrids for the endogenous system positive control was much lower than when using the plasmid system. This may reflect differences in antibody batches. All in all, I demonstrate that there is no evidence for polyglutamine expansion of Pbp1 increasing RNA-DNA hybrids within the intergenic spacer region of rDNA.
Figure 4.8. Polyglutamine expansions in Pbp1 do not increase RNA-DNA hybrids at rDNA. (A) Schematic demonstrating sites used to probe for rDNA hybrids. (B,C,D,E) Log transformed min max plots demonstrating effects of Pbp1 deletion or mutation on RNA-DNA hybrid levels as revealed by ChIP qPCR analysis employing the S9.6 anti-RNA-DNA hybrid antibody. (n=3, * p<0.05).
4.2.7 Polyglutamine Pbp1 does not affect rDNA silencing, E-Pro transcription or H3K56 acetylation

As hybrids appeared unaffected I sought to characterize if other changes at the rDNA was allowing for increases in array size in the 15Q and 30Q mutants. One method of increasing rDNA size is thought to be through USCE mediated by loss of cohesin. Sir2 establishes heterochromatin at the IGS regions of the rDNA through H3K9/K14 deacetylation and this prevents transcription originating within the P4 “E-Pro” region of IGS1 (Figure 4.8a) (Ide et al., 2013). Such transcripts can cause the dissociation of cohesin which coordinates equal sister chromatid exchange between rDNA repeats. Increased USCE then can serve to increase the size of the rDNA array (Kobayashi and Ganley, 2005; Ide et al., 2013). As the ADE2 marker loss assay results cannot reliably quantify USCE rates in my system, I examined other factors associated with increasing USCE. In the chromosomal system, I found no increase in H3K9/K14 acetylation within the IGS1 region of rDNA utilizing a H3K9/K14 antibody in ChIP for cells expressing polyglutamine expanded Pbp1 in contrast with sir2Δ cells (Figure 4.9a). Furthermore, I found no increase in the expression of E-Pro transcripts in chromosomal Pbp1 polyglutamine expressing cells in contrast with sir2Δ cells (Figure 4.9b,c). If anything, there appeared to be a subjective decrease in the amount of E-pro transcripts. These results suggest that the rDNA array is not being altered through unequal sister chromatid exchange.
Figure 4.9. Polyglutamine Pbp1 does not affect rDNA silencing or rDNA intergenic transcription in chromosomal system. (A) ChIP-qPCR analysis using an anti-H3K9K14 antibody within the IGS1 region of rDNA (P2). Log transformed values presented are relative to levels detected in wild-type cells (±SD; n = 3). (B,C) Log transformed min max plots showing relative RNA levels (n = 3; *p < 0.05) as revealed by reverse transcription coupled to quantitative PCR employing primer pairs amplifying various rDNA regions (as indicated in Figure 4.5a). Results are normalized to ACT1 control and values are presented relative to levels detected in wild-type cells.
Another pathway to rDNA recombination is through deletions in the H3K56 acetylation factors, including the proteins Asf1 and Rtt109 (Houseley and Tollervey, 2011; Ide et al., 2013). rDNA repeat amplification occurs independently of the canonical USCE/Sir2 based homologous recombination machinery; perhaps through so-called “rolling circle” amplification wherein a broken chromatid recombines with itself rather than with its sister chromatid (Houseley and Tollervey, 2011; Ide et al., 2013). Unexpectedly, rDNA amplification occurs if histone 3 is hyper- or hypo-acetylated (Ide et al., 2013). Therefore, given the initial increase seen in the size of chromosome XII in the polyglutamine mutants and the absence of evidence for canonical recombinatory mechanisms in promoting rDNA instability, I wondered if there could be changes in the amount of global H3K56 acetylation; similar to the rDNA recombinatory asfΔ1 and rtt109Δ mutants. In both the chromosomal and episomal systems (4.10a and 4.10b respectively) there were no gross changes in H3K56 acetylation. asf1Δ and rtt109Δ mutants are presented as controls for changes in H3K56 acetylation. Also of note is that the deletion of the Ubp3, a factor essential for the assembly of stress granules, did not result in changes in H3K56 acetylation levels (Figure 4.10a, fifth lane) (Nostramo et al., 2015). I note that in Figure 4.10b, there appear to be changes between the controls (1-3) and the episomal system (4-9). This is likely reflective of the fact that the controls were grown in synthetic complete media, while the episomal system was grown in selective synthetic complete media lacking histidine. Therefore, differences in growth medium have an effect on H3K56 acetylation while polyglutamine expansion of Pbp1 does not.
Figure 4.10. Polyglutamine Pbp1 does not affect H3K56 acetylation. (A,B) Western blotting against acetylated H3K56 reveals that polyglutamine Pbp1 mutants do not affect global H3K56 acetylation levels in the chromosomal (A) or episomal (B) systems. *asf1Δ* and *rtt109Δ* are presented as controls known to result in a global decrease in H3K56 acetylation and an increase in rDNA array size.
4.2.8 Polyglutamine Pbp1 mutants have changes in post translational modifications and protein-protein interactions

As my results so far suggest a polyQ gain of toxicity resulting in GCR and lifespan decrease, I wanted to see if protein-protein interactions were altered. To do this I used tandem affinity purification of the chromosomal expressed Pbp1 15Q and 30Q mutants, using chromosomal Pbp1TAP and untagged as controls. Silver stained gel confirmed that I had purified the protein complexes of interest (Figure 4.11a). My collaborator Hongbo Guo (Andrew Emili lab; University of Toronto) ran the liquid chromatography mass spectrometry to determine binding partners of the various proteins. Analyzing the results, I found that all proteins interacted with the known binding partners Pab1 and Stm1 (Figure 4.11b) (Salvi et al., 2014). However, Pbp1 binding partners Pbp4 and Lsm12, which are known to form a complex of unknown function were only detected in the wild type protein sample (Swisher and Parker, 2010; Salvi et al., 2014) (Figure 4.11b). Furthermore, I found that Pbp1 and the 15 and 30Q mutants interacted with the deubiquitinating complex composed of Bre5 and Ubp3 (Figure 4.11b) (Nostramo et al., 2015). This complex has recently been shown to be necessary for the formation of Pbp1-containing stress granules (Nostramo et al., 2015). Only one protein was shown to uniquely bind to both the 15 and 30Q mutants, Pma1 (Figure 4.11b). Pma1 is an essential proton ATPase (Baron et al., 2015). Recent literature suggests a negative genetic interaction between this ATPase and Sod1. Deletion of Sod1 combined with mutation of Pma1 is lethal to yeast cells as Sod1 is vital for protecting cells against the oxidative stress caused by low expression of the Pma1 mutant (Baron et al., 2015). Sod1 is another gene that is implicated in ALS, which highlights the similarities of ALS pathways (Salvi and Mekhail, 2015). Finally, I found that only the 30Q mutant interacted with Doa1, a protein involved in ubiquitin mediated proteolysis (Johnson et al., 1995). These data suggest possible alterations in the amount of ubiquitination of Pbp1 or its binding partners. Therefore, I immunoprecipitated Pbp1 and probed the IP with an antibody against ubiquitin. I note that 5Q failed to be pulled down – likely related to its low levels of expression in the chromosomal system (Figure 4.1C). By comparing the pull down of Pbp1 to the presence of ubiquitinated Pbp1, we see that PolyQ mutants have 2 to 5 fold more ubiquitinated Pbp1 (Figure 4.12c).
Figure 4.11. Polyglutamine Pbp1 mutants have changes in protein-protein interactions and post-translational modifications. (A) Silver-stained gel showing protein complexes used for Mass-Spec analysis. (B) Venn diagram of select protein hits from mass-spectrometry analysis. Only proteins with more than 2 peptides detected when corrected for background (untagged) noise were selected. (C) Immunoprecipitation shows an increase in the amount of ubiquitinated
Pbp1 in polyQ mutants. The band corresponding to the molecular weight of Pbp1 in the anti-ubiquitin western blot (indicated by the black arrow) was normalized to the band corresponding to the Pbp1 band in the anti-TAP pull down to generate ubiquitin signal. Note that 5Q failed to be pulled down, likely related to its low levels of expression in the chromosomal system (Figure 4.1C).

4.3 Discussion

Here we see that polyglutamine expansion of Pbp1 reduces yeast RLS in a manner distinct from deletion of Pbp1. Rather than increasing rDNA hybrids and rDNA USCE as with Pbp1 deletion; polyglutamine tract containing Pbp1 mutants have a hyper-recombinant rDNA array at early 100 generations, gross chromosomal rearrangements in various chromosomes and hyper-reduction of lifespan (Figure 4.12). Therefore, the polyglutamine expansion of Pbp1 must be conferring some sort of gain of toxic function. The precise mechanism causing this gain of toxicity remains to be fully elucidated. However, using some of the data presented herein, we can speculate about what the polyglutamine expansion is doing to the cell.
Figure 4.12. Pathways through which *pbp1Δ* and Pbp1 15-30Q decrease RLS. Pbp1 deletion and polyglutamine expansion both result in a decrease in replicative lifespan. However, in *pbp1Δ* cells, this lifespan decrease is cause at least in part due to hybrid accumulation within the IGS regions of rDNA which is accompanied by an increase in the amount of aberrant USCE recombination. Hybrid accumulation at other regions such as the telomeres is likely to affect lifespan as well. By contrast, polyglutamine Pbp1 strains experience gross chromosomal rearrangements through an unknown mechanism. This mechanism likely contributes to the rDNA hyper-recombinant phenotype seen over time and may contribute to a reduced lifespan independently of the rDNA. However, hyper-recombinant rDNA repeats over time likely contribute at least partially to the lifespan decrease seen in these mutants. Polyglutamine Pbp1.30Q also has a shorter RLS relative to *pbp1Δ*.

Gross recombination was seen in a diverse number of chromosomes in the chromosomal system (Figure 4.2a). One mechanism through which polyglutamine Pbp1 may promote an increase in chromosome recombination is through the hyper formation of stress granules and/or p-bodies. The polyglutamine expansion of the Pbp1 orthologue ATXN2 in mice causes insoluble aggregations of ATXN2 and its binding partners (Damrath et al., 2012). The formation of these insoluble aggregates could be due to an aberrant hyperactivation of stress granule and/or p-body
formation as ATXN2/Pbp1 promote the formation of both stress granules and p-bodies (Nonhoff et al., 2007; Li et al., 2013; Nostramo et al., 2015). Both of these structures bind mRNAs and inhibit translation, but stress granules allow for translation of sequestered RNAs after the cessation of stressful conditions while p-bodies are sites of mRNA decay (Nonhoff et al., 2007). If polyglutamine mutation of Pbp1 causes aberrant increases in cytoplasmic sequestration of RNAs, a diverse group of proteins and/or RNAs required for maintaining genomic stability could be repressed. This would cause a distinctive gain of toxicity separate from simple deletion of the protein. This thesis provides a hint that formation of these bodies is altered in polyQ mutants. Namely, polyglutamine Pbp1 is more prone to ubiquitination (Figure 4.11C). Stress granules stain strongly for ubiquitin and require the ubiquitin binding protein HDAC6 to form in human cells, so an increase in Pbp1 ubiquitination would be consistent with an increase in its association to stress granules (Kwon et al., 2007). Human p-bodies are also strongly associated with a number of ubiquitinating and deubiquitinating proteins (Zheng et al., 2011; Bett et al., 2013). Future work will determine whether or not stress granules are formed in Pbp1 polyglutamine cells. If polyglutamine expansion of Pbp1 is increasing the formation of stress granule it would underscore recent evidence that suggests that a variety of factors associated with the ATXN2 disease ALS are associated with stress granules – including TDP-43 and FUS (Li et al., 2013).

All in all, I have demonstrated that both Pbp1 deletion and polyglutamine mutation result in a decrease in yeast RLS. Given that RLS can function as a model for stem cell ageing, we provide corroboration for previous reports that indicate that ATXN2 might maintain stem cell populations (Ciosk et al., 2004; Kaeberlein, 2010). Interestingly, polyglutamine expansion of Pbp1 does not appear to reduce RLS through loss of endogenous Pbp1 hybrid suppressing function. Instead, it appears to mediate a gain of toxicity that causes recombination in a more diverse array of chromosomes. Using this yeast model of ATXN2 disease should allow for clarification of what the most important contributor is to cellular toxicity. Validating these results in human cell lines will allow for insight into ATXN2 disease and could pave the way for novel therapeutics and treatments for these devastating conditions.
Chapter 5 Future Directions
5 Future Directions

5.1 Novel methods for detecting RNA-DNA hybrids

In this thesis, I report using chromatin immunoprecipitation that polyglutamine mutation of Pbp1 fails to increase the amount of RNA-DNA hybrids in the IGS regions of the rDNA; in contrast to deletion of Pbp1 (See section 4.2.5). However, my hybrid detection method relied on the stability of the amount of hybrids within the control \textit{CUP1} gene which may be true for \textit{pbp1Δ} cells but not Pbp1 polyQ cells. Furthermore, if hybrids were increased stochastically throughout the genome it would be extremely hard to pick them up by using a single locus analysis method such as ChIP. Therefore, utilizing a more global method to detect hybrids would allow for a more definitive confirmation.

Recent work done by the Aguilera group has demonstrated that the hybrid-detecting S9.6 antibody can be used in immunofluorescence to detect RNA-DNA hybrids (Bhatia et al., 2014). Using IF is advantageous as it allows for a more global quantification of the amount of hybridization within the nucleus. Indeed, our lab group has recently reported that RNA-DNA hybrids are detected in ATXN2 knockout HeLa cells using IF – particularly within the nucleolus (Abraham et al., under review). Subsequent work done by my collaborator has failed to detect hybrids in polyQ strains using ChIP, but there is some preliminary data that hybrids accumulate by IF (Lauren Ostrowski, unpublished data). Completing the immunofluorescence dataset and verifying that hybrids are suppressed when RNaseH1 is overexpressed will give more definitive answers about hybrid regulation in Pbp1 polyglutamine mutants. If the IF data is robust then using IF might become the gold standard in RNA-DNA hybrid detection.
5.2 Stress granule accumulation in polyglutamine Pbp1 strains

As discussed in section 4.3, a promising explanation for the gain of toxicity seen in the polyQ strains is that these strains accumulate stress granules and/or p-bodies at aberrant rates which then inhibits the translation of a number of important mRNAs. Future work must evaluate the formation of stress granules and p-bodies to provide a complete assessment of the polyglutamine systems.

If Pbp1 is increasing the formation of these cytoplasmic bodies, we would expect an increase in cytoplasmic aggregations of Pbp1. This can be assessed using immunofluorescence. Indeed, previous studies have examined the formation of stress granules using mCherry-tagged Pbp1 as a marker (Nostramo et al., 2015). All of the strains in both the chromosomal and episomal system are tagged with TAP. Therefore, utilizing a fluorescent anti-TAP antibody, such as those manufactured by ThermoFisher (catalogue number: MA1-108-D680), should allow for the detection of cytoplasmic Pbp1 aggregates. Alternatively, tagging the mutants with mCherry, which is known to work with Pbp1 IF would provide a backup solution.

5.3 Validation of results in human cells

Complete characterization of the gain of toxicity in the polyglutamine system in yeast would provide a valuable model for high-throughput drug screens. To ensure relevance of any such screen to human disease, the polyglutamine system should be validated in human cells. To do so, our lab could replicate the episomal system in human cells. Our lab already has ATXN2 knockout cells generated using CRISPR-cas9 (Abraham et al, under review). Transfection with wild-type ATXN2 or polyglutamine expanded ATXN2 plasmids would allow for validation of many of these results in human cell lines. For example, IF is already validated in examining RNA-DNA hybrids in human cell lines, so polyglutamine-dependent hybrids could be tested by IF (Bhatia et al., 2014). Similarly, stress granules or ATXN2 aggregation foci could also be tested using IF. Gross chromosomal arrangements like those seen in Figure 4.2a could be analyzed using spectral karyotyping. By using 24 distinctive colours within fluorescent in-situ hybridization, every chromosome can be visualized. This allows for the accurate determination
of chromosome translocations, deletions or duplication events characteristic of GCR to be distinguished (Yu et al., 2000; Imataka and Arisaka, 2012).
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