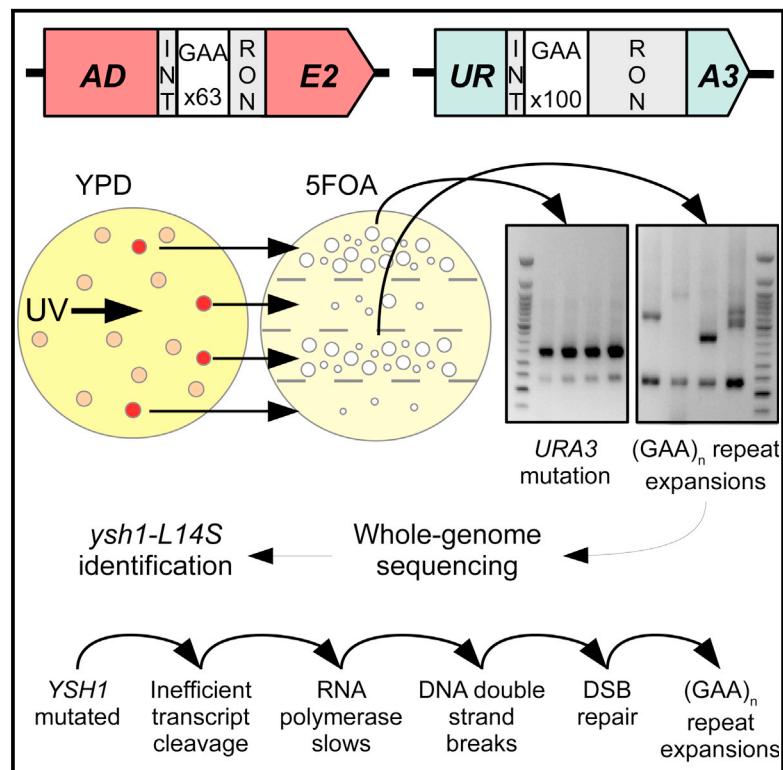


# A Defective mRNA Cleavage and Polyadenylation Complex Facilitates Expansions of Transcribed (GAA)<sub>n</sub> Repeats Associated with Friedreich's Ataxia

## Graphical Abstract



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## In Brief

McGinty et al. developed a genetic screen in *S. cerevisiae* to identify genes promoting expansions of (GAA)<sub>n</sub> repeats. The authors uncovered the unexpected involvement of essential RNA-processing gene, *YSH1*. Mutation in *YSH1* leads to slow transcription elongation, promoting DSBs, whose repair via HR causes repeat expansions.

## Highlights

- Genetic screen: UV mutagenesis → select for repeat expansions → genome sequencing
- Point mutants in essential gene *YSH1* increase the rate of GAA repeat expansions
- YSH1* mutation → slow transcription elongation → DSB → repeat expansions

# A Defective mRNA Cleavage and Polyadenylation Complex Facilitates Expansions of Transcribed (GAA)<sub>n</sub> Repeats Associated with Friedreich's Ataxia

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## SUMMARY

Expansions of microsatellite repeats are responsible for numerous hereditary diseases in humans, including myotonic dystrophy and Friedreich's ataxia. Whereas the length of an expandable repeat is the main factor determining disease inheritance, recent data point to genomic *trans* modifiers that can impact the likelihood of expansions and disease progression. Detection of these modifiers may lead to understanding and treating repeat expansion diseases. Here, we describe a method for the rapid, genome-wide identification of *trans* modifiers for repeat expansion in a yeast experimental system. Using this method, we found that missense mutations in the endoribonuclease subunit (Ysh1) of the mRNA cleavage and polyadenylation complex dramatically increase the rate of (GAA)<sub>n</sub> repeat expansions but only when they are actively transcribed. These expansions correlate with slower transcription elongation caused by the *ysh1* mutation. These results reveal an interplay between RNA processing and repeat-mediated genome instability, confirming the validity of our approach.

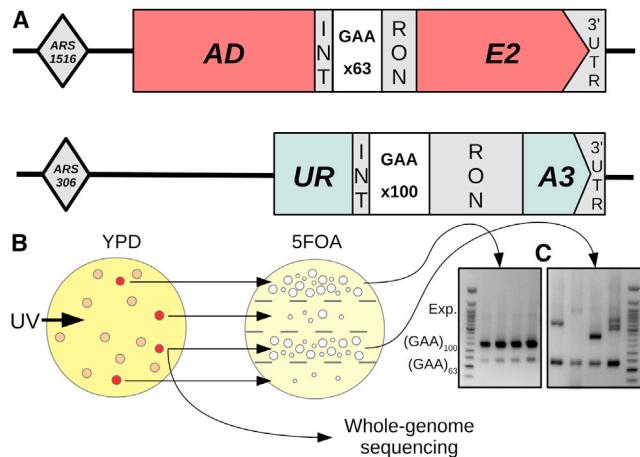
## INTRODUCTION

Expansions of DNA microsatellites are responsible for several dozens of hereditary diseases in humans, including fragile X syndrome (FXS), myotonic dystrophy (DM1 and DM2), Huntington's disease (HD), Friedreich's ataxia (FRDA), many spinocerebellar ataxias (SCA), the familial form of amyotrophic lateral sclerosis and frontotemporal dementia (ALS), and others (López Castel et al., 2010; McMurray, 2010; Mirkin, 2007). The scale of expansions differs depending on the location of the DNA repeat: they are relatively small scale when positioned in the protein-coding part of a gene or very large scale when in the non-coding

parts of a gene, such as 5' and 3' UTRs, or introns (Mirkin, 2007). Repeat expansions readily occur during intergenerational transmissions in human pedigrees, which accounts for the phenomenon of genetic anticipation that is characteristic for these diseases. In some somatic tissues, repeats continue expanding throughout life, which affects age of onset and disease severity (Kovtun and McMurray, 2008).

It is generally believed that the length of an expandable repeat is the key factor determining disease inheritance and development. Significant amounts of data, however, point to the existence of *trans* modifiers that can affect the likelihood of repeat expansions and, thus, disease progression. Whereas most of these data came from studying repeat expansions in model experimental systems (Usdin et al., 2015), the idea is also supported by human genetics data (Morales et al., 2012).

Expansions of (CAG)<sub>n</sub>, (CGG)<sub>n</sub>, (GAA)<sub>n</sub>, and (ATTCT)<sub>n</sub> repeats have been extensively studied in yeast experimental systems. These studies revealed that knocking out genes involved in DNA replication, repair, recombination, and transcription machineries can strongly elevate or decrease the rate of repeat expansions in dividing cells (Kim and Mirkin, 2013). Studies of (CAG)<sub>n</sub> repeat expansions in a *Drosophila* system showed that repeat instability was decreased when a fly homolog of the nucleotide excision repair gene XPG, *mus201*, was mutated (Yu et al., 2011). Mice models for repeat expansions demonstrated the critical role of mismatch repair genes in promoting repeat expansions during both intergenerational transmission and in somatic cells (Kovtun and McMurray, 2001; McMurray, 2008; Savouret et al., 2003, 2004). At the same time, mutations in the base excision repair machinery specifically prevented repeat expansions in somatic tissues (Kovtun et al., 2007). In a humanized mouse model of fragile X syndrome, the loss of the transcription-coupled DNA repair protein ERCC6/CSB led to a lower frequency of germ-line expansions and a reduction in the scale of somatic expansions (Zhao and Usdin, 2014). In cultured human cells, fork-stabilizing proteins Claspin (CLSPN), TIMELESS, and TIPIN were shown to counteract (CAG)<sub>n</sub> repeat expansions (Liu et al., 2012), whereas knockdown of the FANCJ protein resulted in the accumulation of double-stranded breaks



**Figure 1. Overview of Screening Method**

(A) Diagram of selectable *ADE2* and *URA3* cassettes. The *ADE2* marker contains a short artificial intron with only 63 (GAA) repeats, whereas the *URA3* marker contains a longer artificial intron with 100 (GAA) repeats.

(B) Screening procedure: cells are mutagenized and grown on complete (YPD) media. Colonies form, and those that turn red (*ADE2* inactivation) are spread on sections of a plate containing the selective drug 5FOA. For each strain with a high number of 5FOA-resistant colonies (*URA3* inactivation), four individual 5FOA colonies were tested via PCR for repeat length.

(C) Example PCRs for amplification of (GAA)<sub>n</sub> repeats in both cassettes. The *URA3* (GAA)<sub>100</sub> repeat consistently expands in strains containing genuine repeat expansion *trans* modifiers (right), while remaining at WT length in strains containing off-target modifiers (left). The *ADE2* (GAA)<sub>63</sub> repeat does not appear to expand in any strains.

(DSBs) and ectopic rearrangements at those repeats (Barthélémy et al., 2016). Finally, transcription-coupled repair was shown to trigger (CAG)<sub>n</sub> repeat contractions in human cells (Lin et al., 2010; Lin and Wilson, 2007).

Clinical genetics data, whereas more fragmentary and limited in scope, are generally in line with the conclusions of the model systems studies. In case of DM1, it was found that the rate of (CTG)<sub>n</sub> repeat expansions in the *DMPK* gene is a heritable trait in itself, pointing to the existence of *trans* modifiers throughout the genome (Morales et al., 2012). More recently, a polymorphism in the *MSH3* mismatch repair gene was specifically associated with the extent of somatic instability of (CTG)<sub>n</sub> repeats in the blood of DM1 patients (Morales et al., 2016). SNPs in genes involved in DNA replication, repair, and recombination have been associated with increased risk of repeat expansions in HD and SCA3 families (Genetic Modifiers of Huntington's Disease [GeM-HD] Consortium, 2015; Martins et al., 2014). It was also suggested that differential expression levels for replication and repair genes in various parts of the HD patient brains might determine the extent of somatic instability in the corresponding brain regions (Mason et al., 2014).

There exists, however, a serious gap between the model systems and human genetics data. The former primarily describe the effect of gene knockouts, i.e., an all-or-none scenario, whereas the latter deal with SNPs, i.e., much more subtle changes in gene functioning. In this study, we attempted to fill this gap by conducting a genetic screening to detect *trans* mod-

ifiers of repeat expansions in our yeast experimental system (Shah et al., 2012; Shishkin et al., 2009). The screening strategy involves mutagenesis and selection for repeat expansions, followed by whole-genome sequencing and identification of causal SNPs in the expansion process. Totally unexpectedly, this screening revealed mutations in *YSH1*, a gene central for RNA processing.

*YSH1* encodes a component of the cleavage and polyadenylation specificity factor complex (CPSF or CPF), which in concert with cleavage stimulation factor (CstF or CFIA) and cleavage factor I (CFI or CFIB) cleaves mRNA transcript at poly(A) signals (Chan et al., 2011; Millevoi and Vagner, 2010). This subsequently allows both the addition of the poly-A tail to the 3' end of the mRNA via Pap1 (poly-A polymerase), as well as the loading of Rat1 exonuclease to the 5' end of the transcript, leading to the transcription termination (Porrua et al., 2016).

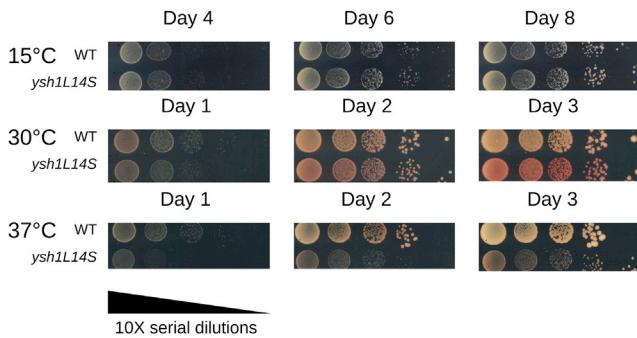
We found that in *Ysh1* mutants that came from our screen, inefficient transcript cleavage is accompanied by slowed transcription elongation and accumulation of DSBs within transcribed (GAA)<sub>n</sub> repeats followed by their expansions in a homologous recombination (HR)-dependent manner. These results reveal a totally unsuspected interplay between RNA processing and repeat-mediated genome instability, hence confirming the validity of our whole-genome screening approach. In the future, this approach can be used to identify *trans* factors for large-scale expansions of other repeats, such as (CGG)<sub>n</sub> repeats responsible for fragile X syndrome or (CTG)<sub>n</sub> repeats responsible for myotonic dystrophy type 1.

## RESULTS

### Screen Design and Implementation

In yeast, large-scale repeat expansions are rare events as opposed to repeat contractions (Kim and Mirkin, 2013). To detect rare large-scale expansion events, we have developed an experimental system in which expansions of the (GAA)<sub>100</sub> repeat within an artificial intron of the *URA3* gene (Figure 1A) inhibits its splicing, resulting in yeast growth on 5-FOA-containing media (Shah et al., 2012; Shishkin et al., 2009). Crossing this reporter cassette into various yeast knockout libraries helped us to identify numerous genes involved in DNA replication, repair, and transcription that affect repeat expansions (Zhang et al., 2012).

We were concerned, however, that gene knockouts are too blunt of a tool, particularly when it comes to essential genes, and thus wanted to assess the effect of subtler genetic changes on repeat expansions. To this end, we chose mild UV mutagenesis to induce point substitutions as opposed to gene deletions or gross-chromosomal rearrangements. Whereas we expected this approach to generate point mutations in genes affecting repeat expansion in our system, we were acutely aware that it might also lead to the accumulation of mutations in the body of our reporter or in other proteins involved in uracil biosynthesis. To minimize the latter prospect, we added another selectable cassette to make our screening a two-stage process. The second cassette, which contained the *ADE2* reporter with a (GAA)<sub>100</sub> repeat within its artificial intron, replaced the endogenous *ADE2* gene on chromosome XV (Figure 1A). Unexpectedly,



**Figure 2. Mutant *ysh1-L14S* Is Temperature Sensitive for Growth**

WT and *ysh1-L14S* mutant strains were serially diluted and grown on complete media at three different temperatures. No difference in growth rate is observable at 15°C, which is below the optimal temperature for WT yeast. At the optimal temperature of 30°C, the *ysh1* mutant displays slightly reduced growth, best observable after 1 day of growth. Red pigment is observable after 3 days, due to inactivation of the *ADE2* cassette. Incubation at 37°C severely slows the growth of the *ysh1* mutant.

however, the presence of even the starting length repeat within this intron completely inactivated the *ADE2* gene, making yeast colonies red. Thus, we shortened the (GAA)<sub>n</sub> run down to 63 repeats (Figure 1A) to keep the reporter active. Notably, the presence of 63 repeats in the *ADE2* intron already decreased the reporter's expression sufficiently that the resultant strain had a borderline ADE+ phenotype and pink colonies (see also below).

The strain carrying both selectable cassettes was irradiated with UV light followed by a two-step selection protocol (Figure 1B): identification of red colonies (step 1: *ADE2* inactivation), which were then analyzed individually for 5-FOA resistance (step 2: *URA3* inactivation). Nearly half of mutagenized red colonies gave rise to augmented papillae growth on 5-FOA-containing media. For roughly half of them, PCR analysis of those 5-FOA-resistant colonies revealed large-scale expansions in the *URA3* cassette. Unexpectedly, however, repeat expansions in the *ADE2* cassette were not detected in any of them (Figure 1C). As shown later, the *ADE2* inactivation is likely due to reduced expression of the *ADE2* mRNA in these mutants. In summary, our screen revealed new genetic *trans* modifiers that repress the reporter gene carrying a short (GAA)<sub>63</sub> repeat, while simultaneously promoting expansions of longer (GAA)<sub>100</sub> repeats.

#### Identification of Mutations in the *YSH1* Gene

We conducted whole-genome sequencing of sixteen UV-mutagenized strains that simultaneously showed *ADE2* inactivation and high rate of repeat expansions in the *URA3* gene. In brief, genomic DNA was isolated from these strains, and barcoded libraries were generated and sequenced using Illumina GAII with 100-bp paired-end reads. This gave an average coverage of ~80x per strain. Reads were then aligned to the S288C reference genome using Bowtie (Langmead et al., 2009), and mutant variants were called using the SAMtools software (Li et al., 2009). This analysis revealed that our mutagenesis strategy resulted in the accumulation of ~10 mutations per yeast strain.

To assess which of these multiple mutations could potentially be causative, they were further analyzed using snpEFF (Cingolani et al., 2012) and PolyPhen2 (Adzhubei et al., 2013) tools. Remarkably, two out of sixteen sequenced strains contained missense mutations in the same essential gene, *YSH1*, which encodes a cleavage and polyadenylation factor subunit (Garas et al., 2008; Zhao et al., 1997). Furthermore, these mutations (*ysh1-L439S* and *ysh1-L14S*) affected highly conserved amino acids. The yeast L14S and L439S substitutions correspond to L17S and L427S in the human cleavage and polyadenylation factor CPSF-73 (Chan et al., 2011; Millevi and Vagner, 2010). Both mutations are outside of the enzyme's catalytic center (Figure S4). The L14 residue appears to reside on the surface of the protein and could potentially affect the stability of the CPF complex, whereas the L439 residue resides internally, but not in the active site.

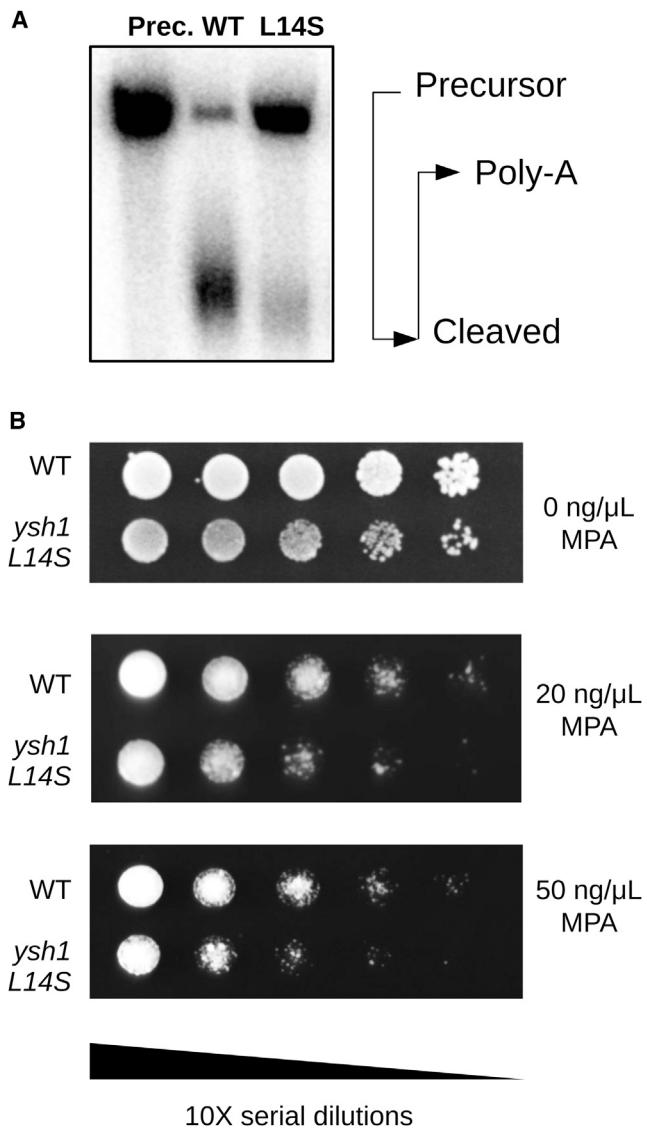
Because two independent mutational hits appeared in conserved parts of the *YSH1* gene, we hypothesized that these mutations could be causative for the observed phenotype of increased repeat expansions and gene inactivation. To validate this hypothesis, we made two strains containing individual *ysh1-L439S* and *ysh1-L14S* mutations (see [Experimental Procedures](#)) along with the two repeat-containing cassettes.

#### Characterization of the *YSH1* Mutant Strains

We first looked at the growth characteristics of the strains with individual *ysh1-L439S* and *ysh1-L14S* mutations. These strains readily turned red, indicating that the *ysh1* mutations are indeed responsible for inactivating the *ADE2* cassette. Both mutants grew more slowly than the wild-type, and this slow growth was exacerbated at higher temperatures and rescued at lower temperatures. The *ysh1-L14S* mutant appeared to be the stronger of the two mutants in each test we conducted, and it had a clear-cut temperature-sensitive growth phenotype (Figure 2). Consequently, this mutant was chosen for all further analyses.

*Ysh1* is the endonuclease responsible for cleavage of the nascent mRNA transcript during 3' end processing (Mandel et al., 2006; Ryan et al., 2004). It has no other known enzymatic functions, though its presence in the CPF complex facilitates related processes, including polyadenylation and splicing (Chanfreau et al., 1996; Garas et al., 2008; Zhao et al., 1999a). *Ysh1* mutants were shown to be defective in both cleavage and polyadenylation in vitro (Chanfreau et al., 1996; Garas et al., 2008; Zhao et al., 1999a). Therefore, we performed in vitro cleavage and polyadenylation assays for the wild-type and *ysh1-L14S* mutant as described (Zhao et al., 1999b). In brief, cell extracts from both strains were incubated with the full-length <sup>32</sup>P-labeled GAL7-1 RNA in the presence of ATP, and the reaction products were separated on a denaturing polyacrylamide gel and visualized via phosphorimager (Figure 3A). The *ysh1-L14S* mutation causes a strong decrease in the efficiency of RNA cleavage and polyadenylation at the non-permissive temperature. The individual steps of cleavage and poly(A) addition are also compromised in the mutant when uncoupled from each other (Figure S1).

It was previously reported that mutants defective in the CFIA cleavage/polyadenylation factor are characterized by a slower rate of transcription elongation (Tous et al., 2011), but such



**Figure 3. The *ysh1-L14S* Mutant Is Defective for mRNA 3' End Processing and Transcription Elongation**

(A) *In vitro* 3' end processing reaction. A precursor RNA is combined with cell extracts derived from WT or *ysh1*-L14S mutant yeast, which were grown at 30°C and shifted to 37°C for 1.5 hr. The precursor RNA is shortened by the cleavage reaction and then lengthened by the addition of the poly-A tail (positions indicated). For *ysh1*-L14S mutants, less of the precursor RNA is converted to the polyadenylated form. Cleaved products are not observed, suggesting that the cleavage step is rate limiting. See also Figure S1.

(B) *ysh1-L14S* is sensitive to the transcription elongation inhibitor mycophenolic acid (MPA). WT and *ysh1* L14S mutant strains were serially diluted and grown on synthetic media lacking uracil and containing the indicated MPA concentrations. Plates were incubated for 3 days at 30°C. *ysh1-L14S* strains display a pronounced growth inhibition under MPA treatment.

effects from mutation of CPF, the complex in which Ysh1 resides, have not been reported. We were curious whether the same is true for the *ysh1-L14S* mutant. To address this question, we studied its sensitivity to mycophenolic acid (MPA), an inhibitor of inosine monophosphate dehydrogenase (IMPDH), which

catalyzes the first committed step in guanosine monophosphate (GMP) biosynthesis. Transcription elongation mutants are hypersensitive to the depletion of guanosine triphosphate (GTP) pools in the presence of MPA (Desmoucelles et al., 2002). Figure 3B shows that the *ysh1-L14S* strain is hypersensitive to MPA as compared to the wild-type strain.

Because inactivation of the *ADE2* cassette in the *ysh1-L14S* mutant was not caused by the repeat expansions in its intron, we sought to determine to what extent 3' end-processing defects of *ysh1-L14S* affect its expression. We first compared the steady-state levels of mRNA for the normal and split *ADE2* gene in the wild-type and mutant strain using qRT-PCR. Owing to the concern that a polyadenylation mutant might affect any transcript used for normalization, we extracted DNA and RNA in parallel from an equal volume of cells, which allowed us to normalize qRT-PCR products to the total DNA. Figure 4A shows that the presence of a repeat-bearing intron within the *ADE2* gene decreased its expression 6-fold compared to the intronless gene, even in the wild-type strain. This result explains the borderline ADE+ phenotype in our starting strain used for mutagenesis. In the *ysh1-L14S* mutant, we observe an additional drop in the mRNA level in the selectable *ADE2* cassette ranging from 2-fold at 30°C to 5-fold at 37°C.

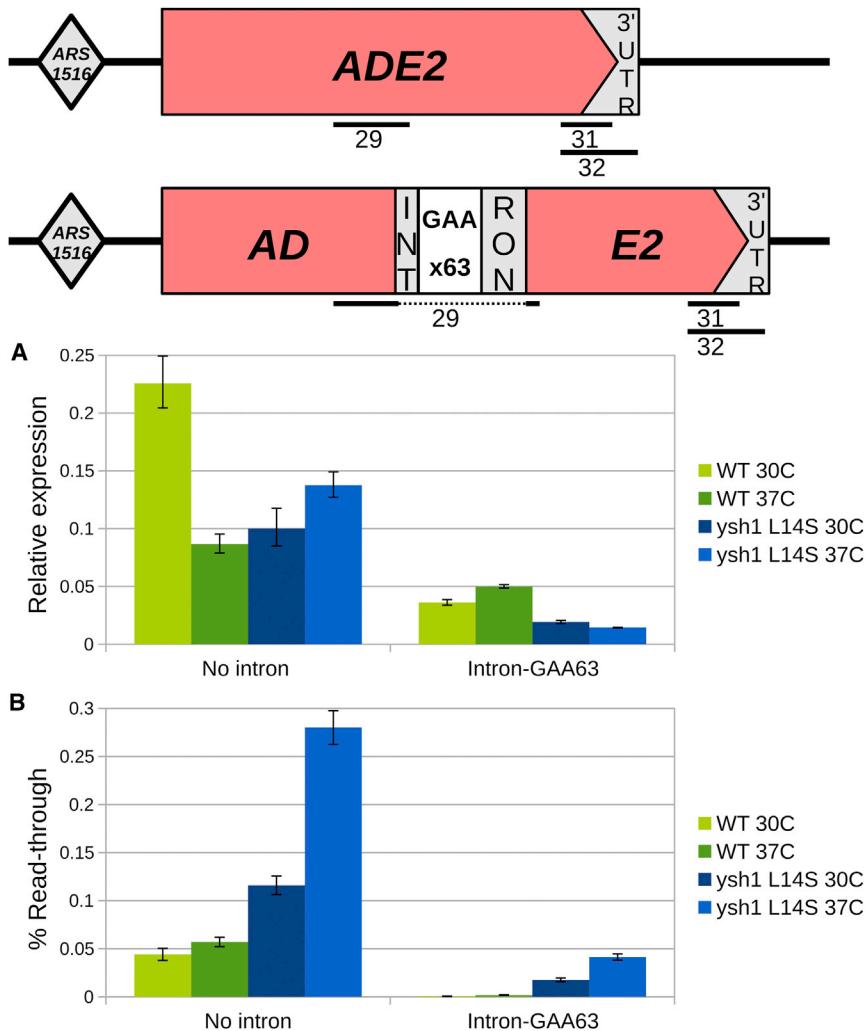
We then analyzed the usage of the main *ADE2* poly(A) site in the wild-type and mutant strain using qRT-PCR analysis with primers upstream or downstream of this site (Figure 4B). Read-through of the poly(A) site is drastically increased in the *ysh1-L14S* mutant, reaching ~20-fold more than wild-type (WT) at 37°C. We conclude, therefore, that the *ysh1-L14S* mutant is also defective for mRNA 3' end processing in vivo and cells with this mutation likely turn red due to decreased production of polyadenylated *ADE2* mRNA.

In contrast to the *ADE2* cassette, the *ysh1-L14S* mutation did not decrease the RNA level for the repeat-bearing *URA3* cassette (Figure S2A). Whereas we do not know why the *URA3* cassette behaves differently from the *ADE2* cassette, our preliminary data are indicative of a peculiar interplay between slower transcription elongation (see Figure 6A below) and higher splicing efficiency of the long-repeat-containing intron in the *URA3* cassette (Figure S2), similarly to what was discussed in Moehle et al. (2014). Whatever the reason, the lack of *URA3* repression necessitated that 5-FOA-resistant clones originating in the *ysh1-L14S* mutant background arose as a result of expansions of the (GAA)<sub>100</sub> repeat.

## Effects of the *ysh1-L14S* Mutation on (GAA)<sub>n</sub> Repeat Expansions

To study the effects of *ysh1-L14S* mutation on repeat instability, we first compared the expansion rates for the (GAA)<sub>100</sub> repeat within the *URA3* cassette (Figure 5A) in the WT and mutant strain using the fluctuation test approach conducted as described previously (Shah et al., 2012). The results shown in Figure 5B show that, even at the semi-permissive temperature (30°C), *ysh1-L14S* mutation elevates the expansion rate ~4-fold, whereas in cells pre-grown at 37°C, it was up ~10-fold as compared to the WT.

A mutation in the cleavage and polyadenylation factor complex likely affects expression of numerous yeast genes. We were concerned, therefore, whether its effect on repeat



**Figure 4. RNA Analysis of *ADE2* Cassette Transcripts**

(Top panel) Diagram of the *ADE2* gene and *ADE2*-(GAA)<sub>63</sub> cassette, indicating the position of primer pairs used for RNA analysis.

(A) Results of qRT-PCR using primer pair no. 29, which is specific to spliced mRNA in the split *ADE2* cassette. Comparing the two versions of *ADE2*, the presence of the intron reduces mRNA expression in both the WT and *ysh1*-L14S mutant background. With the split *ADE2* cassette, the *ysh1*-L14S mutant shows decreased levels of spliced *ADE2* mRNA at both 30°C and 37°C. Reverse transcription was performed using oligo-dT primers. Error bars represent the SD of qPCR technical replicates. See also Figure S2.

(B) Calculation of readthrough transcription levels based on qRT-PCR using primer pairs before (pair no. 31) and after the annotated poly-A site (pair no. 32). In both versions of the *ADE2* gene, *ysh1*-L14S mutants show increased levels of readthrough at 30°C, with a further increase at 37°C. Reverse transcription was performed using random hexamer primers. Error bars represent the SD of four qPCR technical replicates.

transcribed regions. However, the results below suggest a direct role for Ysh1.

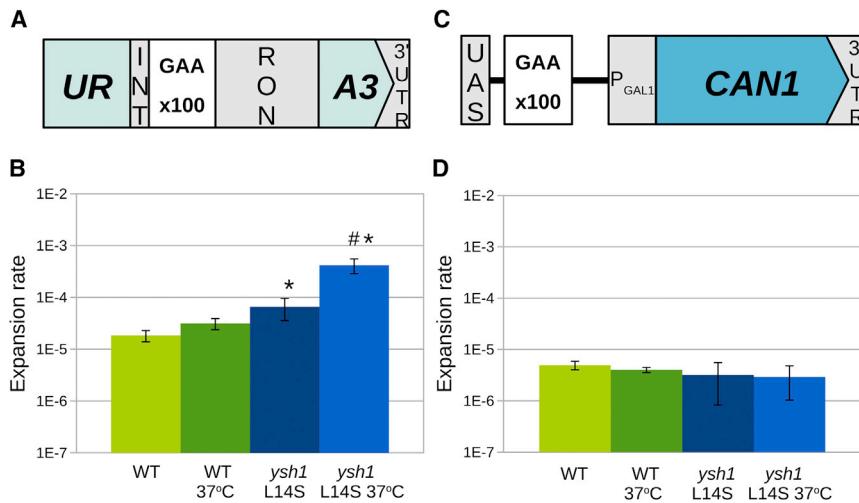
Ysh1p plays a critical role in co-transcriptional 3' end formation and in RNA polymerase II (RNAP II) transcription termination (Garas et al., 2008; Schaugency et al., 2014). In addition, the CPF complex in which Ysh1 resides is affiliated with actively transcribed chromatin (Kim et al., 2004) and the *ysh1*-L14S mutant is sensitive to the MPA inhibitor of elongation (Figure 3B). These observations raise

the possibility that transcription of the (GAA)<sub>n</sub> repeats might be important for expansion induced by the *ysh1* mutation.

Given that *ysh1*-L14S mutation specifically elevates instability of transcribed DNA repeats, we next compared transcription elongation through the *URA3* cassette in this mutant compared to the WT strain using an RNA polymerase clearance assay (Mason and Struhl, 2005). To this end, we replaced the *URA3* promoter in our selectable cassette with the inducible *GAL1-10* promoter. To analyze the transcription elongation rate, cells were grown in the presence of galactose, transcription was shut down by the addition of glucose, and RNAP II distribution along the body of the cassette was measured by chromatin immunoprecipitation (ChIP). Figure 6A shows the normalized (glucose/galactose) values for Pol II occupancy, i.e., the fraction of Pol II, which failed to clear the cassette following glucose repression. One can see that only 20% of RNA Pol II remains associated with promoter-distal parts of the *URA3* cassette in the WT strain, which is indicative of a robust transcription elongation and efficient cassette clearance. In mutant cells, in contrast, the clearance rate appears to be much slower: up to 50% of all RNAP II remain bound to the cassette after glucose

expansions could be mediated by a change in expression of a gene(s) involved in repeat expansions. If this were the case, one would expect *ysh1*-L14S to affect expansions of both transcribed and non-transcribed repeats to a similar extent.

To distinguish between these possibilities, we studied the influence of the *ysh1*-L14S mutation on expansions of (GAA)<sub>n</sub> repeats within a different selection cassette, in which they are located between the galactose promoter and its upstream activating sequence (UAS<sub>GAL</sub>) (Figure 5C), a region that is practically non-transcribed. Large-scale repeat expansions shut off transcription of the *CAN1* reporter, which results in the appearance of canavanine-resistant colonies (Shah et al., 2014). Figure 5D shows that, in contrast to transcribed repeats, *ysh1*-L14S mutation has no effect on the expansion of the non-transcribed (GAA)<sub>100</sub> repeat at either 30°C or 37°C. We conclude, therefore, that transcription is required for expansion of the repeat in the *ysh1* mutant. Furthermore, the mutation is probably not affecting activity of a protein that directly represses repeat expansion. There remains the possibility that the mutation of Ysh1 affects the expression of a gene that promotes expansions solely within



(D) The *ysh1-L14S* mutant shows no change in the rate of repeat expansion in the non-transcribed setting. Error bars represent 95% confidence intervals of two trials. \*, significantly different from WT; #, significantly different from *ysh1-L14S*.

repression. Elongation defects were also observed in the *ysh1-L14S* mutant for the *YLR454*, *GAL10*, and *GAL1* genes that do not have (GAA)<sub>n</sub> repeats (Figure S3). We conclude, therefore, that transcription elongation rate is strongly decreased in the *ysh1-L14S* mutant.

Slow transcription elongation is known to stimulate R-loop formation at various sequences, including (GAA)<sub>n</sub> repeats (Butler and Napierala, 2015; Groh et al., 2014). It was foreseeable, therefore, that increased R-loop formation at (GAA)<sub>n</sub> repeats in the *ysh1-L14S* mutant could ultimately promote repeat expansions. RNase H is known to efficiently resolve R loops by hydrolyzing their RNA component (Hamperl and Cimprich, 2014). Thus, to evaluate a possible role of R-loop formation in our mutant, we knocked out both RNase H1 and RNase H2 in the *ysh1-L14S* strain and measured the rate of (GAA)<sub>n</sub> repeat expansions in the *URA3* selectable cassette described above (Figure 5A). We found that double RNase H knockout has no effect on the rate of repeat expansions in the *ysh1-L14S* mutant (Figure 6B). Our alternative approach was to overexpress RNase H1, which is known to counteract R-loop formation in vivo (Wahba et al., 2011). We first introduced the plasmid-overexpressing human RNase H1 described in Wahba et al. (2011) into our *ysh1-L14S* strain followed by measuring (GAA)<sub>n</sub> repeat instability. It appeared that RNase H1 overexpression had little if any effect on the repeat expansion rates. The caveat of these experiments, however, was that strains carrying the RNase H1-expressing plasmid appeared to be fairly sick. Thus, we used a different approach based on the regulation of RNase H1 expression under the control of the inducible *MET25* promoter (Janke et al., 2004). To this end, the promoter of the endogenous *RNH1* gene in our *ysh1-L14S* strain was replaced with the *MET25* promoter as described in the *Supplemental Experimental Procedures*. Figure 6B shows that the rate of repeat expansions was quantitatively the same whether the expression of RNase H1 was low (in the presence of methionine) or high (in the absence of methionine). Altogether, we conclude that the elevated expansion

**Figure 5. Mutation in *YSH1* Gene Increases Expansions of Transcribed (GAA)<sub>n</sub> Repeats**

(A) Selective system to assess large-scale (GAA)<sub>n</sub> repeat expansions in a transcribed setting. Repeats are placed within an artificial intron in the *URA3* counterselectable marker. This distance is length constrained, with an expansion inhibiting splicing of the intron. Fluctuation tests were performed to determine the large-scale (GAA)<sub>n</sub> expansion rate for WT and *ysh1-L14S* mutant strains.

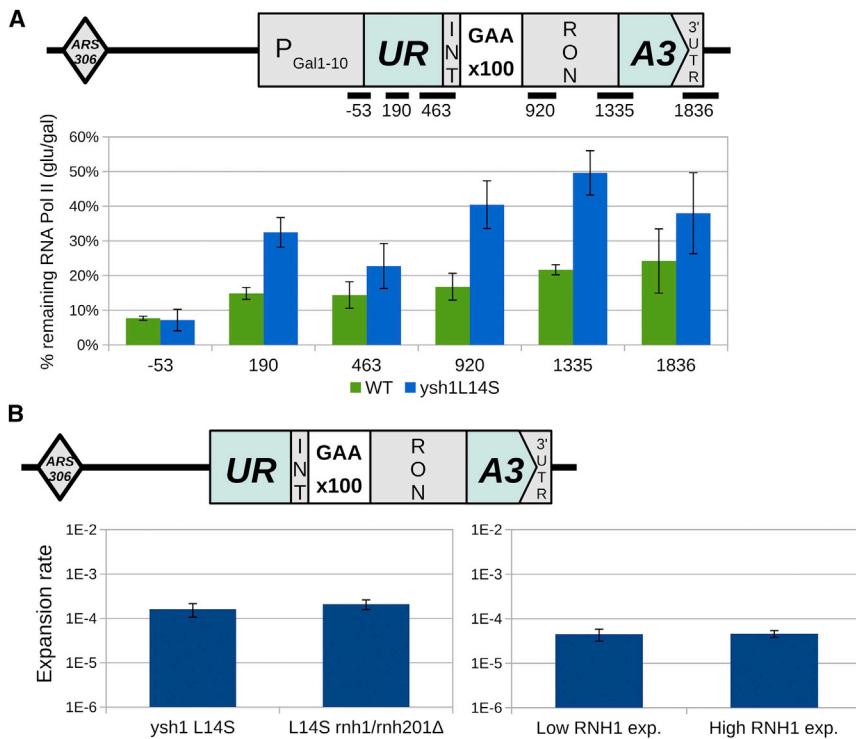
(B) The *ysh1-L14S* mutant shows increased rates of repeat expansion, which increase further under temperature-sensitive conditions (left side of graph).

(C) Selective system to assess expansions in a non-transcribed setting. Repeats are placed between the galactose promoter and its upstream activating sequence. This distance is length constrained, with an expansion shutting off expression of the *CAN1* marker.

sion rate of transcribed (GAA)<sub>n</sub> repeats in the *ysh1* mutant is unlikely to be caused by R-loop formation.

We have previously shown that (GAA)<sub>n</sub> repeats cause chromosomal fragility in yeast (Kim et al., 2008). Compromised transcription elongation is also known to promote the formation of double-strand breaks (Dutta et al., 2011; Nudler, 2012). It is foreseeable, therefore, that slow transcription through the repeat in the *ysh1-L14S* mutant could result in the formation of double-strand breaks, ultimately resulting in expansions. To test this hypothesis, we moved our *URA3* selectable cassette to the non-essential arm of chromosome V, centromere proximal to the endogenous *CAN1* marker gene (Chen and Kolodner, 1999). In this setting, breakage at the (GAA)<sub>n</sub> repeats could lead to a loss of the whole chromosomal arm containing both *CAN1* and the *URA3* reporters—an event that is easily detectable on selective media containing canavanine and 5-FOA. Figure 7A shows that the rate of arm loss is indeed significantly elevated in the *ysh1-L14S* mutant at 37°C. Thus, *ysh1-L14S* mutation indeed promotes breakage of the (GAA)<sub>n</sub> repeat.

In yeast, double-strand breaks are preferably repaired via HR. Misalignment of the repetitive runs in the process of recombination could ultimately result in repeat expansions (Kim et al., 2017). Thus, we decided to assess the role of the key HR proteins, Rad51 and Rad52 (Symington, 2002), on repeat expansions in the *ysh1-L14S* genetic background. To this end, we compared repeat expansions between a double *ysh1-L14S*, *rad52Δ* mutant and a single *rad52Δ* mutant, as well as a double *ysh1-L14S*, *rad51Δ* mutant and a single *rad51Δ* mutant. Because the *ysh1-L14S*, *rad52Δ* double mutant grew very slowly at 37°C, we were only able to generate reliable expansion data at the semi-permissive temperature (Figure 7B). Clearly, knocking down Rad52 brings the rate of repeat expansions in L14S mutant down to the WT level. In contrast to *rad52Δ*, knocking out *rad51Δ* did not affect the rate of repeat expansions in the WT or *ysh1-L14S* genetic backgrounds (Figure 7B). We believe, therefore,



**Figure 6. *Ysh1*-L14S Mutant Exhibits Slow Transcription Elongation, but Expansions Are Not Affected by R-Loop-Processing Enzymes**

(A) Diagram of the modified *URA3*-(GAA)<sub>100</sub> cassette placed under control of the *GAL1-10* promoter. This modified cassette was used to measure transcription elongation speed via RNA polymerase clearance assays. The *ysh1*-L14S mutant displays markedly slower elongation speed, especially downstream of the repeat tract, as indicated by a greater fraction of RNA Pol II remaining two minutes after glucose inhibition. Error bars represent SE of two trials. Primer pairs are numbered by the midpoint of the PCR product, with respect to the open reading frame (ORF) start position. See also Figure S3.

(B) Knockout of RNaseH1 (*rnh1Δ*) and RNaseH2 (*rnh201Δ*; left), which remove R loops, or over-expression of RNaseH1 (right) do not affect expansions in a *ysh1*-L14S mutant background. Fluctuation assays were performed using the *URA3* cassette located at ARS306, with cells grown at 30°C. Error bars represent 95% confidence intervals of two trials.

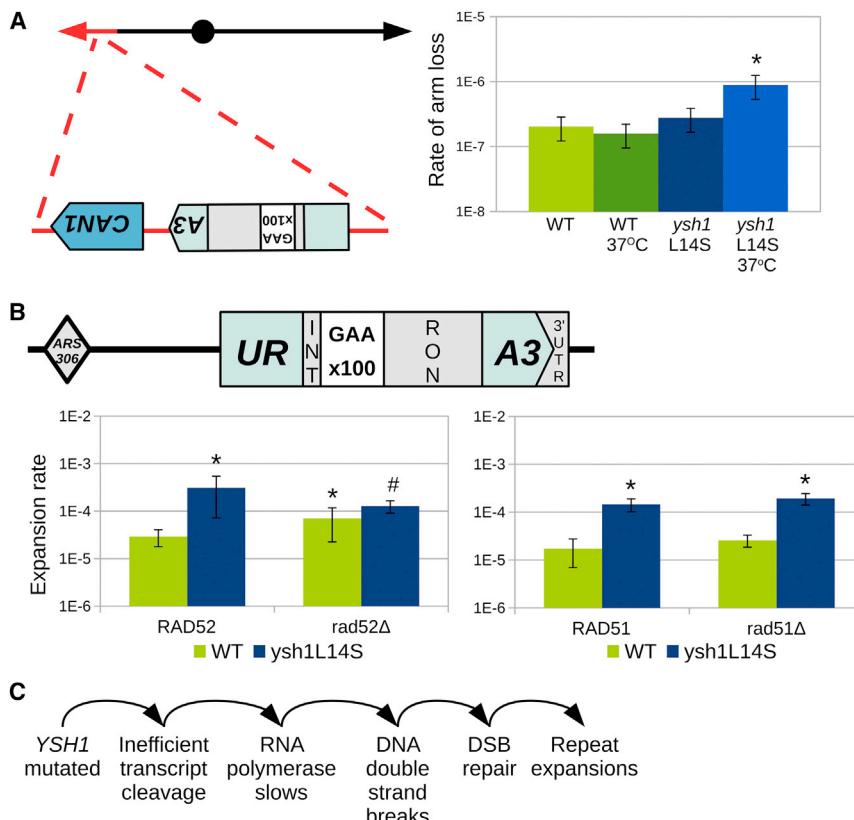
that a Rad51-independent sub-pathway of HR for DSB repair might be responsible for the elevated rate of (GAA)<sub>n</sub> repeat expansions in the *ysh1* mutant.

## DISCUSSION

Our screen revealed an unanticipated connection between RNA cleavage/polyadenylation and large-scale expansions of triplet DNA repeats in *S. cerevisiae*. The mechanisms responsible for this link are intriguing, as repeat expansions occur in the course of DNA, rather than RNA synthesis. That being said, there exists a substantial literature showing that transcription elevates triplet repeat instability. To give just a few examples, transcription of (CAG)<sub>n</sub> repeats increased their instability in cultured human cells in a transcription-coupled repair-dependent manner (Lin et al., 2009; Lin and Wilson, 2007). Changes in the chromatin structure during repeat transcription were also shown to promote expansions by making repeats more susceptible to inherent and external damage (Debacker et al., 2012; House et al., 2014; Shah et al., 2014; Yang and Freudenreich, 2010). Additionally, a number of studies implicated R loops in triplet repeat instability. R loops detected at triplet repeats (Groh and Gromak, 2014; Groh et al., 2014) were proposed to account for transcription-mediated repeat instability (Lin et al., 2010; Reddy et al., 2011, 2014). Similarly, R-loop formation and transcription-coupled repair protein ERCC6/CSB were implicated in CGG repeat expansions in a mouse model of the fragile X syndrome (Zhao and Usdin, 2014). None of these studies, however, investigated the role of co-transcriptional RNA processing.

In a separate development, recent genetic and molecular analyses began to identify RNA-binding proteins (RBPs) as important players in maintaining genome stability by preventing accumulation of harmful RNA/DNA hybrids and by regulating the DNA damage response (DDR) (Dutertre et al., 2014). In *S. cerevisiae*, seven essential subunits of the mRNA cleavage and polyadenylation machinery were implicated in DDR triggered by R-loops (Stirling et al., 2012). Knockout of the *TRF4* gene, encoding a non-canonical polyA polymerase involved in RNA surveillance, gave rise to a transcription-associated recombination phenotype (Gavaldá et al., 2013). Cleavage factor I was shown to contribute to genome integrity by preventing replication hindrance (Gallard and Aguilera, 2014). Similarly, *S. pombe* cleavage and polyadenylation factor Rna14 was implicated in the maintenance of genomic integrity (Sonkar et al., 2016). None of these studies, however, looked at triplet repeat expansions and/or fragility.

Contrary to the above examples, we found that RNA/DNA hybrids are not likely to be involved in elevated repeat instability in the *ysh1*-L14S mutant background (Figure 6B). This difference may be due to the unique role that Ysh1 protein plays in RNA processing. Aguilera's group has proposed that mutations in RNA-binding proteins lead to their absence from the nascent RNA during transcription, which in turn allows this naked RNA to stably pair with its DNA template (Domínguez-Sánchez et al., 2011). We don't think that mutations in Ysh1 protein would result in the presence of naked RNA during transcription, as other members of the CPSF complex are still expected to be bound to RNA. At the same time, we have demonstrated that mutations in the Ysh1 protein significantly



slow down RNA polymerase progression (Figure 6A), likely because it remains bound to the transcript, but cannot cleave efficiently. It was also demonstrated by others that transient depletion of Ysh1p triggers transcriptional pausing downstream of known polyadenylation sites (Schaughency et al., 2014).

Our working model combines the above observations with the data from this study. A mutation in the Ysh1 protein, which was isolated from our repeat expansion screen, causes defects in transcript cleavage and polyadenylation (Figure 3). As this process occurs co-transcriptionally, we reasoned that the entire RNA-Pol-II-elongating complex may slow or stall at potential poly(A) sites on the DNA template when Ysh1 is not efficient. In our mutants, transcription elongation is significantly slowed down across the whole *URA3* cassette (Figure 6A). Transcription stalling and backsliding is known to trigger the formation of DSBs in DNA, owing to their collisions with replication machinery or other mechanisms (Mirkin et al., 2006; Nudler, 2012). We do see elevated fragility of the (GAA)<sub>n</sub> run in the *ysh1* mutant, which is consistent with DSB formation. When HR machinery attempts to repair the broken DNA ends, repetitive DNA strands can align out of register, resulting in repeat expansions after the next round of replication (Figure 7C). Supporting this reasoning, the increase in repeat expansions in the *ysh1*-L14S mutant fades when HR is completely shut down in the *RAD52* knockout. At the same time, repeat expansions in the *ysh1*-L14S mutant were not diminished in the *RAD51* knockout, indicating that a Rad51-independent sub-pathway of HR is either responsible for the

**Figure 7. *Ysh1*-L14S Mutation Leads to Double-Strand Breaks, which May Be Processed by HR into Repeat Expansions**

(A) Selective system for chromosomal arm loss at GAA repeats. The original *UR*-GAA100-A3 cassette was moved to the non-essential arm (marked in red) of chromosome V, just upstream of the endogenous *CAN1* marker gene. An unrepaired double-strand break at the repeats will confer resistance to both canavanine and 5FOA. Fluctuation assay shows an increase in the arm loss rate for the *ysh1*-L14S mutant, which becomes significant under temperature-sensitive conditions. Error bars represent 95% confidence intervals after two trials. \*, significantly different from WT.

(B) Knockout of *RAD52* (left) reduces expansions in a *ysh1*-L14S mutant background, whereas knockout of *RAD51* (right) does not affect expansions. Fluctuation assays were performed using the *UR*-GAA100-A3 cassette located at ARS306, with cells grown at 30°C. Error bars represent 95% confidence intervals after two trials. \*, significantly different from WT; #, significantly different from *ysh1*-L14S.

(C) Proposed chain of events leading to *ysh1*-L14S-driven repeat expansions.

expansions or can compensate in the absence of canonical Rad51-dependent HR. One possibility is the involvement of

the single-strand annealing pathway (SSA), which is known to act within repetitive regions and is not dependent on Rad51 protein (Downing et al., 2008). Another possibility is a Rad51-independent wing of the break-induced replication pathway (Ira and Haber, 2002).

Whereas our studies were performed in *S. cerevisiae*, they may have implications for Friedrich's ataxia in humans. An interesting repercussion from the transcription repeat breakage model is that expansions may pre-nucleate outside of the S phase. This phenomenon might therefore shed light on how repeat expansions can occur in non-dividing neural and cardiac cells (McMurray, 2010). It would be of prime interest to investigate whether FRDA patients carrying mutations in the *YSH1* homolog *CPSF-73* or other RNA-processing genes might be at higher risk for repeat expansions, accounting for the variation in disease severity and age of onset between different individuals. Even in the absence of germline mutations in the CPSF complex, transcription may already proceed more slowly through (GAA)<sub>n</sub> repeats (Krasilnikova et al., 2007). Transcriptional blocks at the (GAA)<sub>n</sub> repeat within the *FXN* locus could become prominent in specific cell lineages or arise transiently to produce large-scale expansions in non-dividing cells. This can hint at a potential therapy, if it becomes possible to prevent RNA polymerase stalling at the repeat (Gottesfeld et al., 2013; Soragni et al., 2014). Reducing transcription pausing at (GAA)<sub>n</sub> repeats may both reduce DNA breakage and rescue the poorly expressed mutant allele of the *FXN* gene.

## EXPERIMENTAL PROCEDURES

## Yeast Strain Construction

The list of our strains is presented in [Table S1](#). See [Supplemental Experimental Procedures](#) for further details.

## Fluctuation Assays

Fluctuation assays were performed as previously described (Shah et al., 2014; Shishkin et al., 2009). See [Supplemental Experimental Procedures](#) for further details.

## In Vitro 3' End Processing

Processing extracts were prepared as described (Zhao et al., 1999b) using strains SMY732 and RMG89, which were grown at 30°C and then shifted to 37°C for 1.5 hr. Extracts were incubated with ATP and full-length or pre-cleaved <sup>32</sup>P-labeled GAL7-1 RNA. Reaction products were run on a polyacrylamide urea gel and visualized via phosphorimager.

## Quantitative RNA Analysis

RNA levels were measured via qRT-PCR, employing a strategy wherein genomic DNA (gDNA) was extracted from an equal portion of the yeast culture used to extract RNA. See [Supplemental Experimental Procedures](#) for further details.

## RNA Pol II Elongation Assays

Assays were performed as previously described (Mason and Struhl, 2005). See [Supplemental Experimental Procedures](#) for further details.

## ACCESSION NUMBERS

The accession number for the genome sequencing data reported in this paper is SRA: SRP115438.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.08.051>.

## AUTHOR CONTRIBUTIONS

Conceptualization, R.J.M., A.Y.A., E.T.W., and S.M.M.; Software, R.J.M.; Investigation, R.J.M., F.P., A.Y.A., J.A.H., E.L.P., and E.T.W.; Resources, A.A.S.; Writing – Original Draft, R.J.M. and S.M.M.; Writing – Review and Editing, R.J.M., C.M., and S.M.M.; Visualization, R.J.M. and F.P.; Supervision, D.E.H., C.M., and S.M.M.; Funding Acquisition, A.Y.A., S.M.M., and C.M.

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