

Characterizing the Role of the Rad5 Family of Proteins in DNA Repair

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ABSTRACT

Trinucleotide repeats (TNRs) pose as barriers to DNA transcription, replication, and repair, and have implications in several neurodegenerative diseases. CAG repeats are one form of TNRs that are particularly deleterious, as their ability to form non-B form DNA (hairpin loops) lead to many complications such as repeat breakage (fragility) and subsequent changes in tract length (instability) that contribute to disease exacerbation and anticipation—earlier age of onset—over generations. Many genetic factors such as replication, transcription, and repair have previously been shown to be important in CAG repeat fragility and instability. Rad5 is a protein that has been shown to impact recombination-mediated repair at CAG repeats. Interestingly, Rad5 is part of a larger family of proteins which are characterized by a *SWI/SNF2* helicase motif interrupted by a RING finger domain, thus conferring chromatin remodeling capability. One such member of the Rad5 family, Irc20, is an E3 ubiquitin ligase that was identified in a screen for CAG repeat fragility in a yeast artificial chromosome (YAC) system. Although additional studies failed to show a role for Irc20 in CAG repeat fragility and instability on its own, in turning attention to relationships between other Rad5 family members, we show that Uls1 and Rad16 have significant roles in preventing CAG repeat fragility and that Irc20 may have a compensatory relationship with Rad5 in certain model systems.

INTRODUCTION

I. Disease implications

The human genome is incredibly complex and requires faithful maintenance to prevent errors in DNA synthesis and repair. Microsatellites are repeats of one to six nucleotides that occur naturally throughout the human genome and often pose as barriers to DNA replication and repair, including post-replication repair (PRR). Trinucleotide repeats (TNRs) specifically have been implicated in a myriad of neurodegenerative diseases, in which TNR expansion above certain disease thresholds results in varying degrees of the disease phenotype. TNR expansion diseases are generally neuromuscular or neurodegenerative in character and often result in disease anticipation, which is progressively earlier onset of disease in subsequent generations (Paulson 2018).

CAG repeats in particular are implicated in nine diseases, including Huntington's disease and several types of spinocerebellar ataxia. Since the CAG codon codes for the amino acid glutamine, CAG repeat expansion diseases are also referred to as polyglutamine (polyQ) diseases (Mirkin 2007). Huntington's disease (HD) is an autosomal dominant and neurodegenerative disease prevalent in European populations (Bates 2005). It is caused by CAG repeat expansions in exon 1 of the Huntingtin (HTT) gene, categorized into normal, intermediate HD, HD with reduced penetrance, and HD depending on the number of expanded repeats. Repeat expansions can occur via various pathways during or outside of replication (McMurray 2010, Paulson 2018). Recent evidence suggests that the CAG repeat length itself and not the polyglutamine length drives disease onset, which has broad implications pertaining to the pathogenesis of these polyQ diseases and puts more emphasis on understanding the mechanisms of CAG repeat expansion (Lee et al. 2019).

II. Replicative mechanisms of CAG repeat fragility and instability

CAG (and CTG) repeats are barriers to DNA replication and repair due to their tendency to form stable, non B-form secondary structures; hairpin structures are the most common, in which intra-strand base pairing occurs between the cytosine and guanine bases. There is a correlation between expanded CAG repeats and repeat fragility and instability, suggesting that processes involving DNA breaks, such as DNA repair and Okazaki fragment processing, contribute to CAG repeat expansions (Callahan et al. 2003). This places emphasis on identifying mechanisms of CAG repeat expansions in respect to disease progression and future clinical applications.

Single-stranded breaks (SSBs) arise from DNA damage and incomplete repair processes, and structure formation from non-ligated Okazaki fragments or unprocessed 5' flaps from unresolved gap repair processes can result in repeat expansions (Usdin et al. 2015, Chatterjee and Walker 2017). CAG/CTG repeats are especially prone to double-stranded breaks (DSBs), which can arise from stalled replication forks, SSB processing into a DSB, and nuclease cleavage (Freudenreich et al. 1998, Usdin et al. 2015). The lesions are preferentially repaired by different processes depending on the phase of the cell cycle, such as DSB repair by HR during S and G2 phases (Branzei and Foiani 2008). SSBs and DSBs can be repaired by a variety of pathways during and outside replication including homologous recombination (HR), which is highly relevant to this paper.

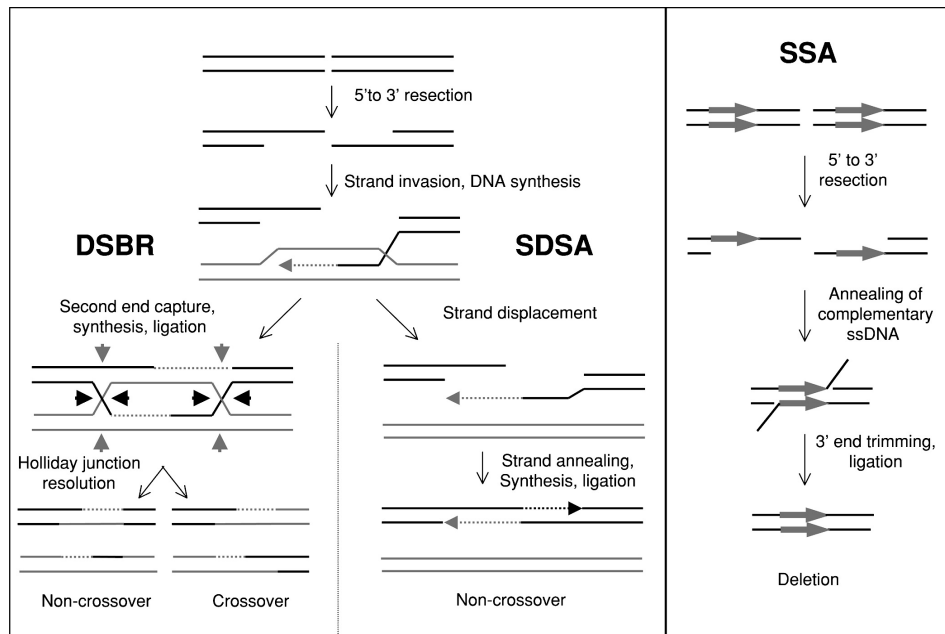


Figure 1. HR DSB repair pathways. (Krogh and Symington 2004)

HR, active in the S and G2 phases, has a general mechanism of DNA strand invasion and subsequent DNA synthesis off a homologous template (Krogh and Symington 2004) (Figure 1). Sub-categories of HR include single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA), break-induced replication (BIR), and sister chromatid recombination (SCR) (Negritto et al. 2010, Sreejith et al. 2018). Notably, sister chromatids are the preferred template for repair, and SCR is thought to occur via the SDSA mechanism (Kadyk and Hartwell 1992, Johnson and Jasin 2000). When DNA replication stalls due to breaks or lesions, post-replication repair (PRR) pathways are induced to mediate lesion bypass or replication restart, which rely on translesion (TLS) polymerases and HR-based mechanisms, respectively (Branzei and Szakal 2017, Polleys et al. 2017). This means that HR plays a central role to DNA repair during replication.

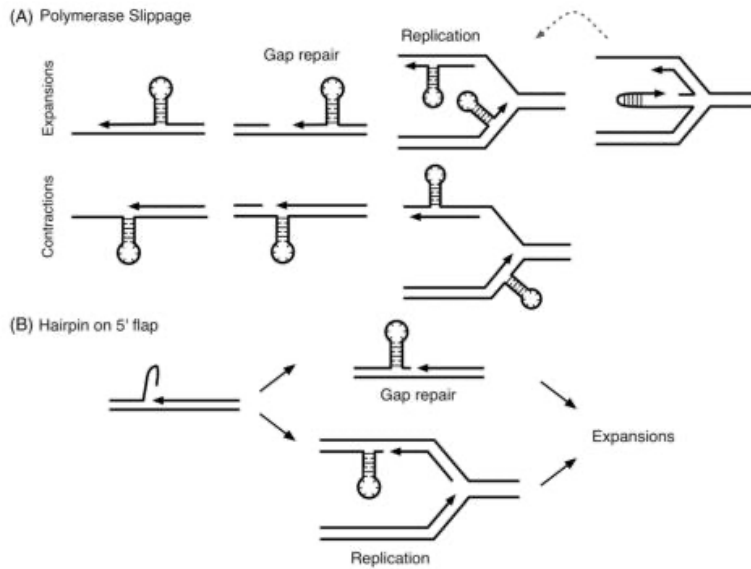


Figure 2. (A) Hairpin formation during DNA synthesis can cause polymerase slippage and subsequent instability. (B) Hairpin formation during gap repair or on an Okazaki fragment can result in expansions. (Usdin et al. 2015)

CAG repeat expansions in particular are thought to arise from a few distinct mechanisms during DNA replication. A simple strand slippage during replication can result in expansions (Figure 2A): the replicative polymerase encounters a repeat tract and stalls, temporarily dissociating from the template strand to allow the incomplete newly synthesized strand to detach from the template strand and pair with another repeat unit slightly upstream; the re-association of the replicative polymerase continues replication and re-inserts repeat units onto the newly synthesized strand (Lenzmeier and Freudenreich 2003, Usdin et al. 2015). Secondary structures can also form during gap repair or Okazaki fragment processing, which tend to result in expansions (Figure 2B).

Replication restart is another prominent model for TNR expansions during replication, in which the lagging strand encounters a repeat tract and stalls the replication fork. Dissociation of the opposite nascent strand from the leading strand for fork reversal and template switch allows the opportunity for secondary structure formation, triggering a variety of responses, including nucleotide excision repair (NER) machinery to resolve the structure (McMurray 2010, Usdin et al. 2015). Fork reversal and template switch can restart replication without resolution of the

secondary structure; bypassing the structure directly or DNA synthesis with the lagging strand as a template during fork reversal can produce contractions and expansions, respectively (Polleys et al. 2017). This mechanism is part of the PRR model, which will be discussed further in the context of Rad5.

III. *RAD5*

RAD5 has human orthologs HLTF (helicase-like transcription factor) and SHPRH (*SNF2* histone-linker PHD-finger RING-finger helicase) that share evolutionarily conserved domain structures. HLTF is most similar to Rad5 and is reported to act primarily as a DNA-binding protein, localizing to damaged DNA and stalled replication forks. The proposed tumor suppression ability of HLTF and SHPRH is particularly of interest; HLTF was shown to suppress colony growth in some colon cancer cell lines, and SHPRH was mapped to chromosomal region in which loss of heterozygosity was observed in several cancers. SHPRH is another ubiquitin ligase that helps PCNA (proliferating cell nuclear antigen) polyubiquitination, but contains a H15 domain and a PHD-finger domain not in Rad5 or HLTF that confer some role of SHPRH in chromatin remodeling (Unk et al. 2010). Therefore, HLTF and SHPRH are not thought to be redundant to each other.

The Freudenreich lab has previously looked at Rad5 in the context of CAG repeat instability. It was shown that Rad5 does play a role in CAG repeat maintenance, as House et al., (2018) showed a significant increase in expansions and contractions (4.3-fold and 1.8-fold over wildtype, respectively) in a *rad5Δ* mutant in the ADE2 URA3 CAG-85 YAC. However, Nguyen et al., (2017) were unable to show any statistically significant changes in their *rad5Δ* mutant's instability in the URA3 CAG-70 YAC. Their fold-change over wildtype were 1.9-fold and 1.8-

fold for expansions and contractions, respectively. There are some CAG repeat length-specific differences in frequency of instability changes, since longer repeats stall replication forks more severely, so the difference between 70 and 85 CAG repeats could cause differences in statistical analysis (Polleys et al. 2017). So in the absence of Rad5 in the ADE2 URA3 CAG-85 YAC, we expect an increase in fragility to mirror the increase in expansion and contraction frequencies.

The current model of PRR requires Rad5 in both the error-free template switch (TS) pathway and the error-prone translesion synthesis (TLS) pathway for different roles (Choi et al. 2015, Friedl et al. 2001, Gao et al. 2017). These pathways are differentiated by the ubiquitination status of the PCNA DNA clamp, leading to recruitment of different repair factors (Kanao and Masutani 2017) (Figure 3).

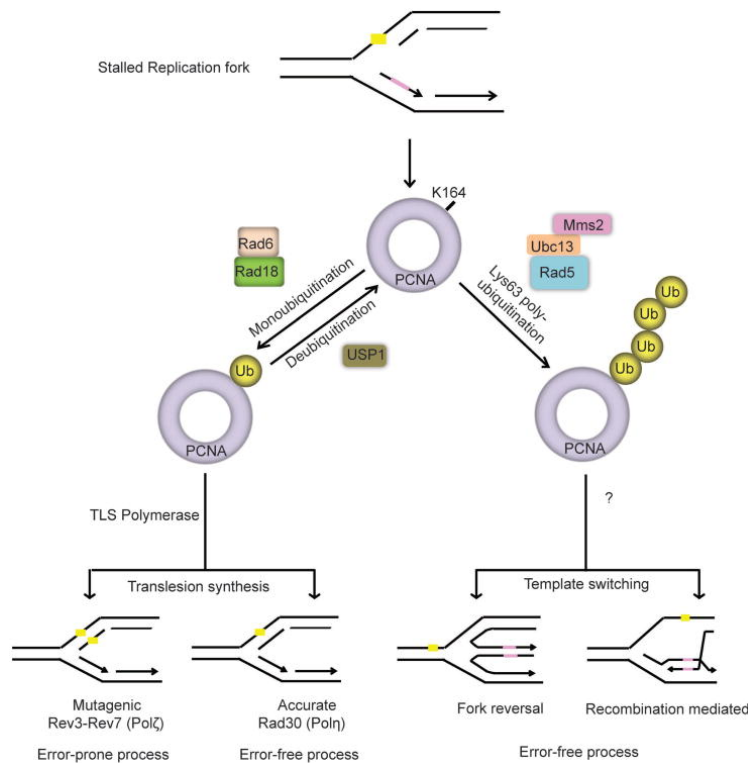


Figure 3. DNA damage tolerance (DDT) pathway, also known as the post-replication repair (PRR) pathway. A stalled replication fork caused by lesions (yellow squares) is funneled into either the TLS pathway or the TS pathway. Pathway choice depends in PCNA ubiquitination status. (Ghosal and Chen 2013)

In the template switch pathway, Rad5 complexes with the E2 ubiquitin conjugase complex (Ubc13-Mms2) to directly catalyze the polyubiquitination of PCNA (Bi 2015). This leads to DNA repair via a recombination-mediated or a fork reversal method, producing error-free DNA repair. The sister chromatid is used as the template for DNA synthesis in the TS pathway of PRR, which therefore also involves Rad51 and Rad52 (Polleys et al. 2017). These are HR proteins that facilitate both fork reversal and canonical HR (Rickman and Smogorzewska 2019, Symington 2002). Rad51 is a recombinase protein that facilitates strand exchange and homology

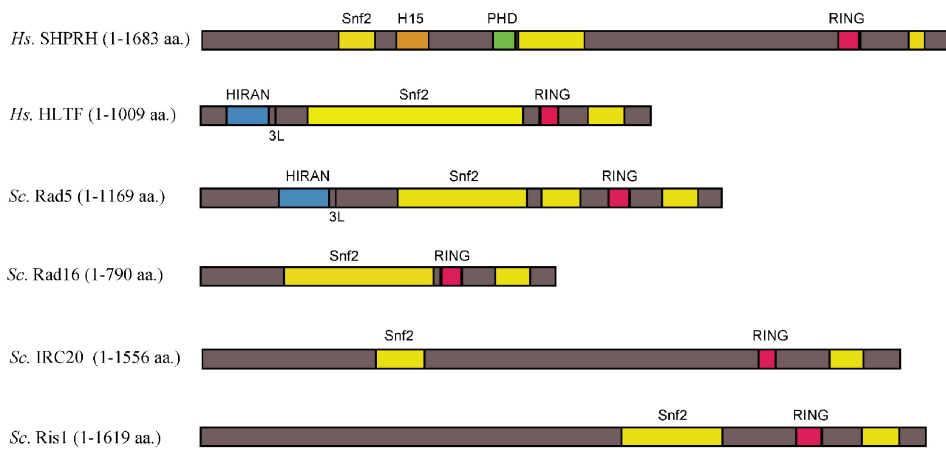


Figure 4. Domain analysis of yeast and human genes of the *SWI/SNF2* family, characterized by a RING finger domain embedded in the helicase motifs. Ris1 is an alias of *ULSI*. (Unk et al. 2010)

search in the first steps of HR repair (Chen et al. 2010). Rad52 facilitates Rad51 binding and is involved in two major Rad52-dependent recombination pathways, of which single-strand annealing (SSA) is Rad51-independent and minimally involves Rad5 (Coïc et al. 2008, Mott and Symington 2011).

Rad5 has three important domains that impart its involvement in the PRR pathways: a Snf2 helicase domain preceded by an N-terminal HIRAN domain containing a Walker B motif, and a RING finger E3 ubiquitin ligase domain (Choi et al. 2015, Gallo and Brown 2019) (Figure 4).

The Snf2 helicase and RING finger domains are required for PCNA ubiquitination in the TS pathway, and the HIRAN domain is specifically involved in the fork reversal mechanism (Unk et al. 2010, Gao et al. 2017). The Snf2 and RING finger domains also give Rad5 its ability to physically interact with Rad18 and PCNA in the translesion synthesis pathway, and is a quality shared by HLTf (Gallo and Brown 2019, Unk et al. 2010). More recent developments show that Rad5 also directly interacts with TLS polymerase Rev1 to recruit other TLS polymerases such as Pol ζ , and Pol η , and in support of this observation, a specific Rad5-Rev1 interaction via the N-terminus of Rad5 was identified (Fan et al. 2018). This suggests that beyond its helicase activity, Rad5's recruitment of TLS polymerases is also crucial to DNA repair and the PRR pathway (Gallo et al. 2019). Rad5 plays important roles in both PRR pathways.

IV. Other *S. cerevisiae* Rad5 family members

IRC20

Domain analysis by Unk et al., (2010) identified Irc20 as a member of the Rad5 family of proteins with helicase activity and chromatin remodeling abilities (Figure 4). Miura et al., (2012) also identified Irc20 as DNA/RNA helicase whose mutation reduced SDSA to a similar degree as a *srs2 Δ* knockout did. Since Srs2 is a helicase that is essential to SDSA, Irc20 could be functionally analogous to Srs2 if it affects SDSA to an equal degree. Characterization by Richardson et al., (2013) found physical interactions of Irc20 with Cdc48, an essential protein that can recognize SUMO (small ubiquitin-like modifier), and with SUMO as well. Cdc48 specifically mediates Rad51 binding to DNA damage and associates with a myriad of cofactors to modulate essential cell processes (Baek et al. 2015). These studies suggest that Irc20 could be a player in some DNA repair pathways.

The Freudenreich lab utilizes a yeast artificial chromosome (YAC)-based assay containing CAG repeats of varying lengths as a measure of specific repeat breakage. This system is used as a genetic screen to find novel genes involved in CAG repeat breakage prevention. In one such screen, it was identified that loss of *Irc20* increased fragility compared to wildtype cells in the ADE2 URA3 CAG-85 YAC. This suggests that *Irc20* is involved in a repair pathway that significantly impacts CAG repeat maintenance. In support, Krijger et al., (2011) showed that in the absence of Rad5 in mice, PCNA polyubiquitination still occurs likely due to another E3 ligase redundant to Rad5, which they propose to be *Irc20*.

Since Rad5's role in the template switch pathway is dependent on its E3 ubiquitin ligase activity, this further supports that *Irc20* could have redundant function to Rad5 in the error-free (TS) PRR pathway as an E3 ligase (Unk et al. 2010) (Figure 4). It is less likely that *Irc20* acts in the error-prone PRR pathway, as it lacks the HIRAN domain that allows Rad5 to interact with TLS polymerase Rev1 (Fan et al. 2018). Further exploration showed that *Irc20* synergistically increases fragility in an *irc20Δrad5Δ* double mutant compared to a *rad5Δ* single mutant (OTN thesis), so it would be in our interest to consider possible overlapping roles these genes may have in the context of CAG repeat maintenance.

RAD16

RAD16 is involved in nucleotide excision repair (NER) and binds DNA in an ATP-dependent manner with Rad7p. Rad7 and Rad16 complex to form nucleotide excision repair factor 4 (NEF4), which binds with high specificity to UV-induced DNA damage and is essential to global genome NER (GG-NER) (Guzder et al. 1997). Furthermore, it was shown that GG-NER can act on transcriptionally-repressed regions of the genome via chromatin remodeling, which is supported by Rad16's homology with the Snf2 protein (Teng et al. 2008, Waters et al. 2015).

Several other DNA repair pathways have shown to contribute to CAG repeat expansions, including mismatch repair (MMR) and base excision repair (BER), so it is no surprise that mutation of NER and specifically, mutation of Rad16, can also cause CAG repeat instability (Koch et al. 2018). In non-dividing cells, TNR expansions have also been linked to repair processes such as BER and NER (McMurray 2010). Transcription-coupled NER (TC-NER), on the other hand, does not require Rad16, but has been linked to CAG repeat instability during transcription in human cells (Lin and Wilson 2007). Concannon and Lahue, (2014) also suggested that GG-NER functions with proteasome 26S to promote CAG expansions. This is related to *RAD16*'s role as part of the Elongin-Cullin-Socs (ECS) ligase complex, which polyubiquitinates GG-NER-specific factors to target for degradation by the proteasome and thus promote CAG expansions (Cherry et al. 2012).

ULS1

Uls1 has activity in suppressing NHEJ at telomeres and interacts with Rap1, an essential DNA-binding transcription regulator. Uls1 contains the *SWI/SNF2* helicase domain and the RING finger domain shared by the Rad5 family members, but importantly, is also a SUMO-targeted ubiquitin ligase (STUbL). It is also the only known STUbL with translocase activity, which has been hypothesized as support for its role in preventing NHEJ at telomeres (Cherry et al. 2012, Lescasse et al. 2013). Accumulation of telomere-telomere fusions were observed in the absence of Uls1, and recovery of normal telomeres occurs when no poly-SUMO chains are present. Rap1p normally binds telomeres to prevent NHEJ, but once SUMOylated, they can no longer prevent imprecise NHEJ. By its translocase activity, Uls1 can ubiquitylate SUMOylated Rap1 proteins to target them for degradation, thus preventing NHEJ fusions at telomeres (Marcomini et al. 2018).

Uls1 also has several other roles in DNA repair and replication stress response pathways, including modulation of the PRR pathway choice and fork restart. Prolonged S-phase progression has been observed in *uls1* Δ mutants that was amplified in *rad52* Δ *uls1* Δ double mutants, indicating an important role of Uls1 during replication stress potentially by directing repair (Cal-Bąkowska et al. 2011). It was further proposed that Uls1 drives Srs2-PCNA binding and inhibits HR-mediated repair at stalled forks by resolving Rad5- and Rad51-generated HR intermediates; this is supported by Srs2's anti-recombinase activity (Kramarz et al. 2017, Nguyen et al. 2017).

Supporting the hypothesis that Uls1 promotes error-prone repair pathways, Glineburg et al., (2019) found that *sgs1* Δ *uls1* Δ mutants caused a switch to an error-free DNA repair pathway. They further proposed that Uls1 decreases replication fork stability and potentially promotes error-prone repair pathways such as BIR (Glineburg et al. 2019). Sgs1, like Srs2, is a helicase required for DNA replication and repair; it is also synthetically lethal as a double mutant with *srs2* Δ (Lee et al. 1999). These data show that Uls1 is involved in several error-prone repair processes and could give insight into the roles of the Rad5 family proteins in different repair pathways.. Notably, Uls1's roles in DNA repair are dependent on its STUbL activity and interactions with other repair proteins to affect repair pathway choice.

V. Systems to study repair

Yeast Artificial Chromosome systems

Yeast artificial chromosomes (YACs) are constructs modeled off of an endogenous yeast chromosome, containing all essential features for its propagation through the strain, including a centromere, origin of replication, telomeres, and genes or sequences of interest. The

Freudenreich lab has produced several YAC systems to study TNR-related repair mechanisms, of which this paper will focus on CAG repeat fragility and instability. The output of the YAC fragility assay is FOA resistance (FOA^R); 5-Fluoroorotic acid is a nontoxic substance that is converted to its toxic form, 5-fluorouracil, in yeast that are Ura⁺. Therefore breakage at the repeat and subsequent loss of the right end of the YAC (resulting in Ura⁻ cells) will allow growth on 5-FOA plates. On the other hand, the output of the YAC instability assay is the presence of both arms of the YAC, with the CAG repeat tract being of the same tract length, expanded, or contracted.

It is also important to distinguish between fragility and instability; the fragility assay is a fluctuation protocol designed to characterize breakage and end-loss of the YAC. The instability protocol is a frequency of repeat tract length changes due to errors in replication and repair of daughter colonies. Although increased fragility often correlates with increased instability, they offer different insights into gene characterization and will be discussed further. Other conserved elements of the YAC systems include a G₄T₄ sequence allowing for *de novo* telomeric addition after a break at the CAG/CTG tract, and selection for presence of the unbroken YAC with YC-Leu-Ura selective plates (Polleys and Freudenreich 2018).

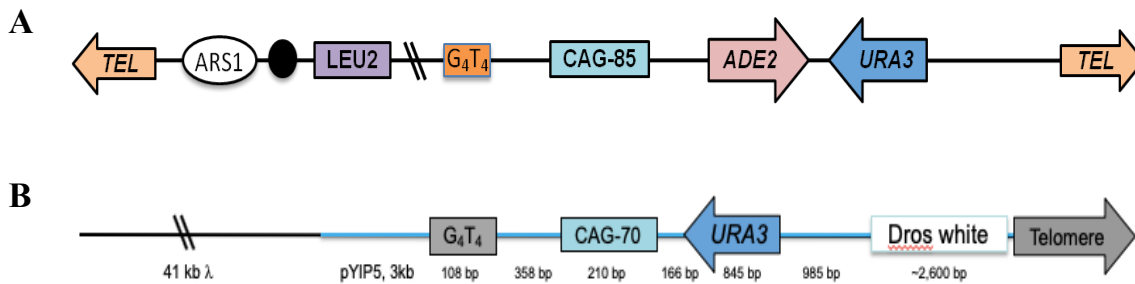


Figure 5. (A) ADE2 URA3 CAG₈₅ YAC construct, in the VPS105 background. **(B)** URA3 CAG₇₀ YAC construct, in the BY4705 background. Both constructs contain a G₄T₄ sequence that allows for *de novo* telomere addition upon breakage. Breakage event will also result in 5-FOA resistance. (Polleys and Freudenreich 2018)

A. ADE2 URA3 YAC – CAG-85 (Figure 5A) (EP senior thesis):

The ADE2 URA3 CAG-85 YAC is a low-breakage YAC in the VPS105 background that was used to first characterize the fragility phenotype of *irc20Δ* and other related genes.

Breakage rates are to the scale of 10^{-8} , and fragility is calculated using method of the median (MoM).

B. URA3 YAC – CAG-70 (Figure 5B):

The URA3 CAG-70 YAC is in the BY4705 background and has higher breakage rates (10^{-6}).

Fragility is calculated using the method of maximum likelihood (MLL).

Symington system to study SCR

Stalled replication forks during DNA synthesis have been shown to promote SCR as the primary repair pathway, since using the sister chromatid as the repair template is more likely to result in error-free repair (Kadyk and Hartwell 1992, Polleys et al. 2017). Furthermore, CAG repeats have been shown to induce spontaneous unequal SCR in *S. cerevisiae* and especially in the context of a directed DSB (Kadyk and Hartwell 1992, Nag et al. 2004). The Symington lab designed a substrate for a SCR assay. To identify successful repair by SCR, one of the *ade2*

alleles (*ade2-n* and *ade2-i*) must spontaneously break and recombine to produce ADE⁺ TRP⁺ cells via unequal sister chromatid gene conversion (Figure 6). Selective media plating can therefore identify the desired recombinants and repair by other pathways (Mozlin et al. 2007).

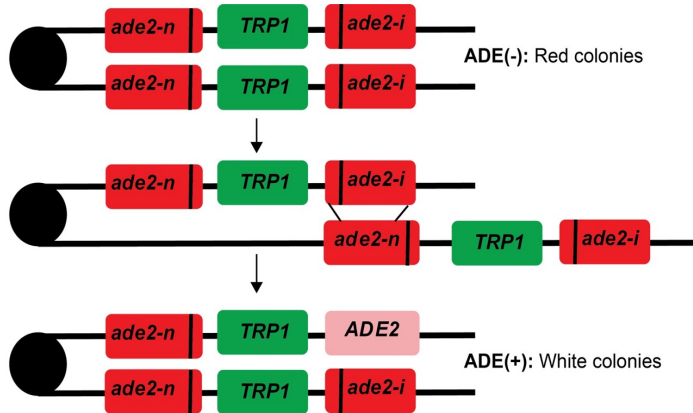


Figure 6. The SCR study system showing a possible repair mechanism. Unequal sister chromatid recombination where the invasion of *ade2-i* into *ade2-n* produces Ade⁺ Trp⁺ colonies. (Mozlin et al. 2008)

VI. Summary

We seek to understand the role of the Rad5 family of proteins in respect to CAG repeat fragility and instability, telomere maintenance, and SCR in this study. We suspect Irc20 could be involved in the template switch-mediated PRR pathway with Rad5, based on previous work by the Freudenreich lab and other studies. Since they are part of a larger family of proteins, by studying Irc20, Rad5, Rad16, and Uls1 together with respect to CAG repeats, we hope to identify possible characteristics and interactions that contribute to CAG repeat stability. We hypothesize that Irc20 may also have some compensatory functions or interactions with the Rad5 family proteins in the context of CAG repeat maintenance and repair.

RESULTS

I. *irc20*Δ does not increase ADE2 URA3 CAG-85 YAC fragility as a single mutant

Though previous work in a genetic screen showed that *irc20*Δ mutants had increased CAG repeat fragility, subsequent analysis was not able to confirm increased fragility in an *irc20*Δ single mutant. However, a synergistic increase in fragility was noted in a double mutant strain with *rad5*Δ (OTN thesis) (Figure 7C). Since the single mutants do not affect CAG-85 repeat fragility (Figure 7B), this suggested that Irc20 and Rad5 may have compensatory roles at a CAG repeat. As Rad5 and Irc20 are part of a larger family of proteins, we thought it would be interesting to expand our analysis to all of the Rad5 family of proteins, which includes Rad5, Irc20, Rad16, and Uls1 (Unk et al. 2010, Richardson et al. 2013). Although individual loss of Rad5 and Irc20 were insufficient to increase CAG repeat fragility, individual loss of Rad16 and Uls1 each produced significant increases in fragility (Figure 7B, see Appendix for statistics). Uls1's roles in telomere maintenance and replication stress response makes its high fragility interesting but perhaps unsurprising. Types of repair other than NER have also been shown to cause CAG repeat fragility, but the dramatic increase in fragility of a *rad16*Δ mutant was somewhat unexpected. Deletion of the TC-NER protein Rad26 was shown to significantly impact CAG-70 repeat breakage (2.4-fold over WT), and deletion of the Rad14 protein required for both TC-NER and GG-NER showed a similar increase in fragility (3.5-fold over WT, $p = 0.08$), suggesting that TC-NER is the more predominate pathway for preventing CAG repeat fragility (Su and Freudenreich 2017). My data suggests that GG-NER must also be considered as an important pathway needed to prevent CAG fragility.

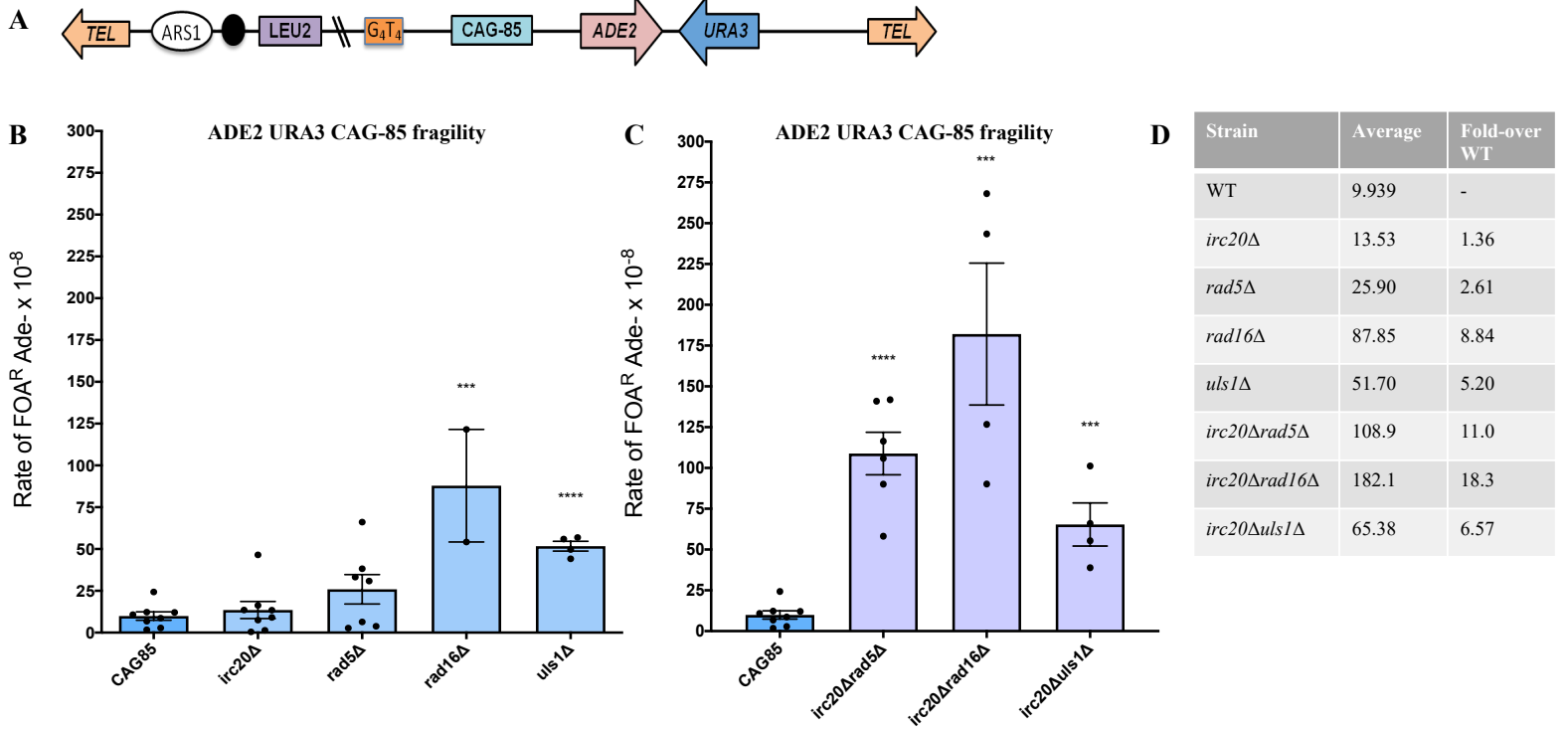


Figure 7. (A) ADE2 URA3 CAG-85 YAC construct. (B) Fragility rates of *RAD5* family single mutants. (C) Fragility rates of *irc20* mutants in combination with other Rad5 family members. (D) Average fragility rates (10^{-8}) and fold-over wildtype. Rates are calculated by method of the median (MoM). Combined data from EJP and OTN. See appendix for statistics.

AXH contributions: *rad16Δ*, *uls1Δ*, *irc20Δrad5Δ*, *irc20Δrad16Δ*

To address the question of whether the *rad16Δ* fragility is associated with general repair (GG-NER) or has CAG repeat specificity, we referred back to previous data on *rad16Δ*-induced fragility in a shorter CAG repeat (CAG-10). The lack of change in fragility between the single mutants (*irc20Δ* and *rad16Δ*) and double mutant (*irc20Δrad16Δ*) compared to wildtype in the CAG-10 tract confirms that Rad16 has a role specific to an expanded CAG tract (Figure 8).

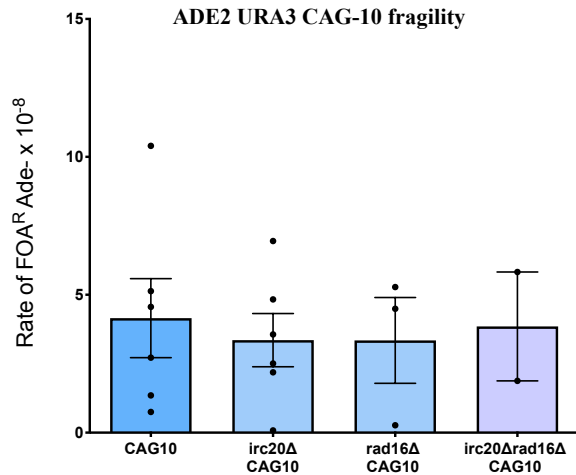


Figure 8. *IRC20* and *RAD16* fragility in the ADE2 URA3 CAG-10 YAC. No change in CAG-10 fragility across mutants suggests that *RAD16* has a CAG repeat-specific role in repair. Data by OF, TEP, MD, EJP.

II. *irc20*Δ increases ADE2 URA3 CAG-85 YAC fragility with *RAD5* family genes

To address the possibility of overlapping roles within the Rad5 family proteins, double mutants were produced and tested for fragility (Figure 7C). Previous work from Oliver Takacs-Nagy showed that *irc20*Δ*rad5*Δ produces an apparent synergistic increase, which supports Krijger et al., (2011)'s hypothesis that Irc20 is compensatory for Rad5 in one of the PRR pathways. An additive increase in fragility was observed in the *irc20*Δ*uls1*Δ double mutant, suggesting that Irc20 and Uls1 function in different pathways in respect to DNA repair specific to a CAG repeat.

The *rad16*Δ single mutant produced a significant increase in fragility compared to wildtype (Figure 7B). The *irc20*Δ*rad16*Δ fragility phenotype is about 2-fold higher than the *rad16*Δ single mutant, potentially suggesting some synergy between Irc20 and Rad16. However, it is also possible they have an additive relationship and therefore function in different pathways, as the broad range in fragility may be misleading. Rad16 has not been previously described in CAG repeat-specific maintenance, though previous work in the Freudenreich lab showed that a deletion increases CAG-85 repeat instability by 4.3-fold ($p = 0.014$) (Koch et al. 2018).

III. *RAD16* and *ULS1* are involved in telomere maintenance

Telomeres are another classic example of repeat tracts with structure-forming abilities; they are G-rich sequences predicted to form G-quadruplex structures. Current models of telomere replication show that fork stalling is prevalent and therefore can trigger template switch PRR pathways. Indeed, Fallet et al., (2014) showed that Rad5 plays a role in telomere length regulation in the absence of telomerase; it assists in HR repair with the sister chromatid as the repair template to elongate very short telomeres that cannot undergo more resection in danger of eliminating the telomeric repeat entirely. In order to determine whether members of the Rad5 family play a role in telomere length maintenance, we looked at telomeric Southern blots for changes in bulk telomere length. It was found that Rad5 did not significantly impact telomere length, and loss of *Irc20* showed no telomere length phenotype (Figure 9). This is consistent with previous reports on *Irc20* (OTN thesis, Askree et al. 2004, Gatbonton et al. 2006). Importantly, we see no change in telomere length in the *irc20Δrad5Δ* double mutant, which suggests that *Irc20* and Rad5 are not compensatory, at least in respect to telomeric repeat sequences.

It has been previously reported that *Uls1* plays a distinct role in NHEJ events at telomeres and loss of *Uls1* results in telomeric breakage-fusion-breakage cycles (Lescasse et al. 2013). Supportive of this, a diffuse telomeric band can be seen in all *uls1Δ* single and double mutants (Figure 9, rightmost bar). There is no difference observed between the *uls1Δ* and *irc20Δuls1Δ* mutants, further supporting that *Irc20* and *Uls1* are in unrelated DNA repair pathways.

A telomeric phenotype can also be observed for *rad16Δ* and *irc20Δrad16Δ* strains in a shift toward longer telomere lengths (Figure 9, leftmost bar). Notably, there is again no difference between the single *rad16Δ* mutant and the *irc20Δrad16Δ* double mutant, suggesting *Irc20* and Rad16 function in different pathways in respect to telomere maintenance. *IRC20* also was not

identified as a gene involved in telomere maintenance in the genome-wide screen by Askree et al., (2004). The *rad16* Δ telomere phenotype was unexpected, however; although Almeida and Ferreira, (2013) found that chromosome end fusions result from a Rad16-dependent SSA/MMEJ pathway in *S. pombe* and observed different telomere lengths in the absence of Rad16, there has been little research in *S. cerevisiae* to suggest some role of NER at telomeres.

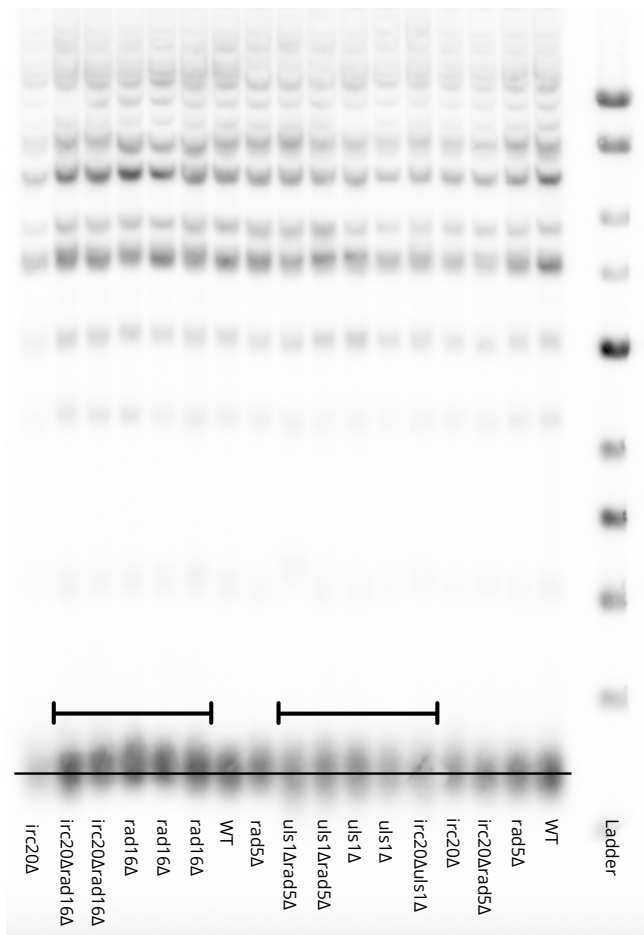


Figure 9. Telomeric Southern blot of *RAD5* family genes. Bulk telomeres shown at the bottom; all other bands are interstitial telomeric sequences and unique telomeric sequences. Bars represent *rad16* and *uls1* deletion sets from right to left.

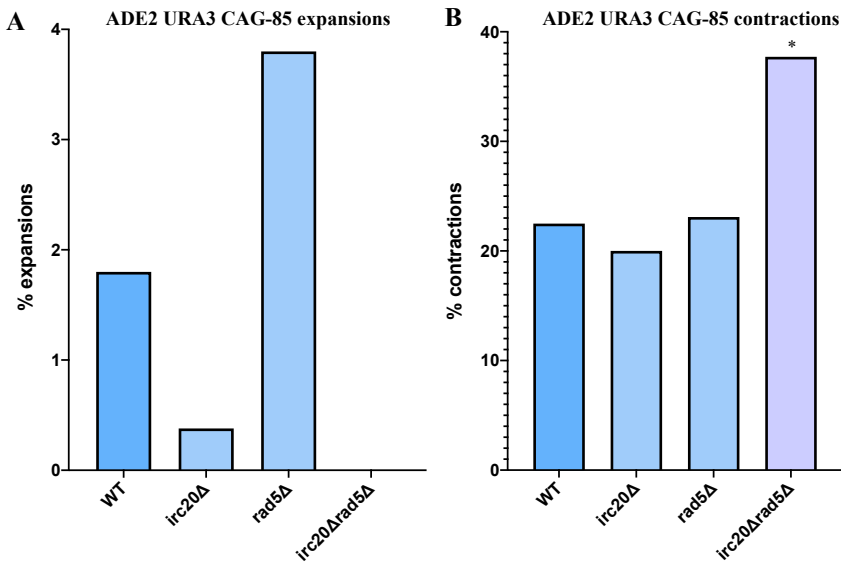
Data by AXH.

IV. *IRC20* phenotypes in instability and SCR

Instability is characterized as contraction and expansion of the repeat tract, which can arise from spontaneous breakage and repair or via replication slippage. Mutants that influence fragility often also increase repeat instability, although these phenotypes measure different aspects of

CAG repeat breakage and maintenance. There is also a relationship between SCR repair; House et al., (2018) found a correlation between a SCR defect and increased Rad5-dependent CAG expansions in H4 acetylation mutants. In addition, CAG instability was dependent on the availability of HR repair pathways, in which a mutation in proteins Rad5, Rad51, and Rad52 greatly increased instability in a *hta1*Δ mutant, which also has defects in SCR. This relationship is likely caused by the resolution of a stalled replication fork or breakage repair, in which both events could be funneled into the HR-mediated TS pathway. As mentioned in “Introduction”, repair by SCR is preferred due to the proximity of the sister chromatid and higher likelihood of error-free repair (Kadyk and Hartwell 1992).

The *irc20*Δ mutant shows approximately a twofold decrease in expansions but is not statistically significant ($p = 0.19$) (Figure 10A, C). This data could suggest that Irc20 actually functions in some mutagenic pathway, so that an *irc20*Δ mutant actually confers more accurate repair, but our data currently is not significant from wildtype. On the other hand, since Rad5 is essential for both PRR pathways, a *rad5*Δ mutation results in an accumulation of DSBs from replication fork collapse and has been shown to exhibit a two-fold increase in expansion rate (Gao et al. 2017). We see a similar frequency of *rad5*Δ mutant instability (about 1.5-fold higher than wildtype), but it is again not statistically significant ($p = 0.13$). Although we note a suppression in expansions in the *irc20*Δ*rad5*Δ mutant, only the increase in contractions is statistically significant compared to wildtype contractions ($p = 0.021$, see Appendix for full statistics) (Figure 10B).



C

Strain	Expansions (%)	Contractions (%)
WT	1.8	22.5
<i>irc20Δ</i>	0.38	20.0
<i>rad5Δ</i>	3.0	23.1
<i>irc20Δrad5Δ</i>	0.00	37.7 ($p = 0.02$)

Figure 10. ADE2 URA3 CAG-85 YAC instability determined via PCR and fragment analyzer. **(A)** Expansion frequency. **(B)** Contraction frequency. **(C)** Table of all instabilities. WT ($n = 226$) and *irc20Δ* ($n = 260$) are complete data sets by OTN and EJP. *rad5Δ* is $n = 52$ and *irc20Δrad5Δ* is $n = 69$. Significance determined by Fisher's exact test. See appendix for statistics.

AXH contributions: *rad5Δ*, *irc20Δrad5Δ*

To determine the role of the Rad5 family members on SCR, we utilized an unequal SCR system designed by the Symington lab (see “Introduction”), which was designed so that the successful repair of a broken allele via SCR results in ADE⁺ TRP⁺ cells. It was previously found that *irc20Δ* decreases SCR repair rates by approximately 2.5-fold (OTN). Miura et al., (2012) also reported a fivefold reduction in SDSA repair in an Irc20 knockout and proposed that Irc20 is involved in non-crossover recombination with Srs2, a major yeast helicase and translocase that promotes SCR (Friedl et al. 2001). However, reproduction of this data was unsuccessful; *irc20Δ*'s SCR rate is comparable to wildtype, and *irc20Δrad5Δ* has no significant decrease in SCR (Figure 11). If the relationship between increased instability and an SCR defect holds true, then we should have seen an SCR defect in the *irc20Δrad5Δ* strain. Previously published data

has shown that *rad5Δ* has a severe SCR defect as a result of its central role in HR and is mirrored in our data (House et al. 2014). Taken together, it is now unclear as to how *Irc20* interacts with *Rad5*, since their instability and SCR phenotypes do not construct one coherent hypothesis nor follow expected trends for the initial finding that they were acting in compensating pathways protecting against CAG-85 fragility.

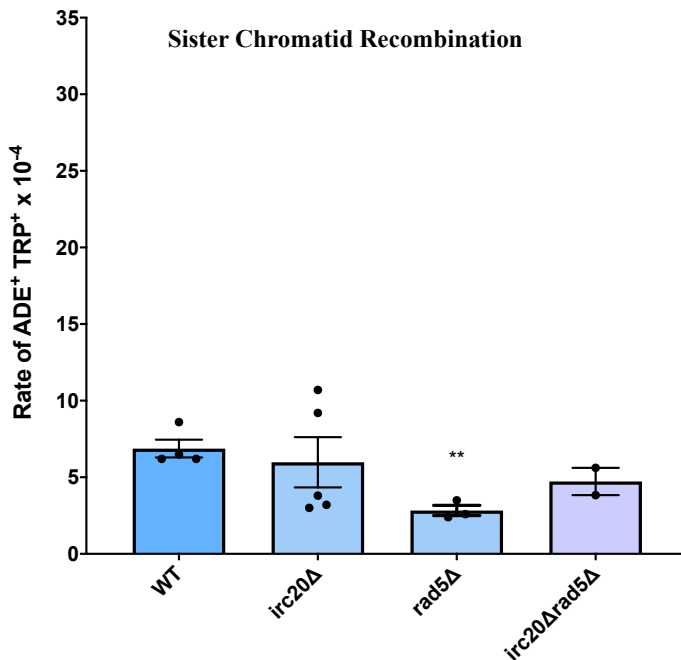


Figure 11. Refer to “Introduction” for SCR system schematic. SCR repair shows a defect in *rad5* mutants only. *irc20Δ* has a bimodal distribution but is insignificant compared to wildtype. *irc20Δrad5Δ* data is incomplete ($n = 2$). Combined data from OTN and EJP.

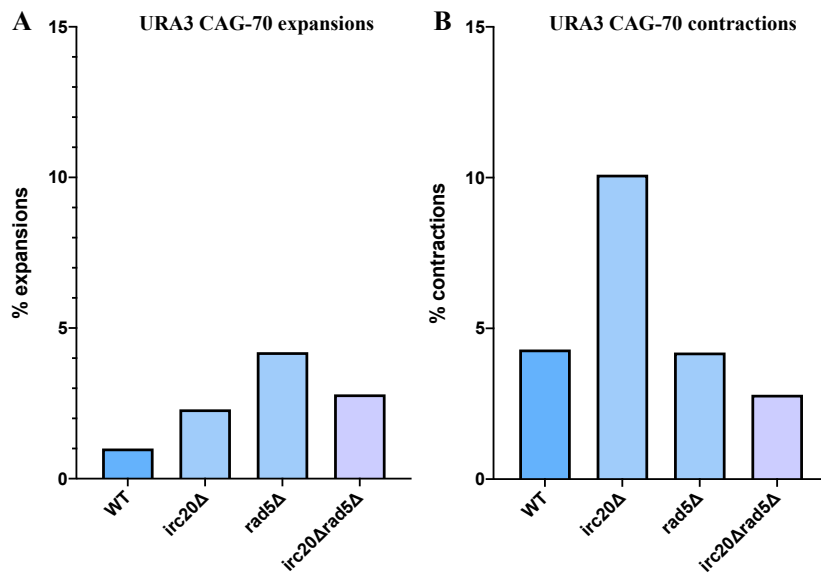
AXH contributions: *irc20Δ*, *irc20Δrad5Δ*

V. Phenotypes are not reflected in the URA3 CAG-70 YAC

Historical instability data has shown that the ADE2 URA3 CAG-85 YAC favors contractions and often has non-PCR amplification events not caused by YAC breakage that could skew data interpretation (EJP unpublished data). This means that the ADE2 URA3 CAG-85 YAC does not PCR amplify well, which could confer a lower frequency of expansions and contractions than is true. Therefore, studying the *Rad5* family proteins in a different YAC background could give more insight into some conflicting phenotypes across assays. We reasoned that a true positive

phenotype should carry over to the URA3 CAG-70 YAC system and show similar relative phenotypes even if this YAC system has higher breakage rates (10^{-6} compared to 10^{-8}) (Polleys and Freudenreich 2018).

Interestingly, in the URA3 CAG-70 YAC, the *irc20* Δ mutant has increased expansions (2.3%) and contractions (10.1%) compared to wildtype (1.0% and 4.3%, respectively; $p = 0.24$ and $p = 0.07$ for expansions and contractions, respectively). The *irc20* Δ *rad5* Δ double mutant (Figure 12A and B, purple bars) now has an increase in expansions but a decrease in contractions, differently from what was observed in the CAG-85 instability assay (Figure 12A and B, blue bars). However, it is important to note that this is an incomplete data set ($n = 89$) and changes in frequency could alter in additional assays performed (see Appendix for full statistics).



C

Strain	Expansions (%)	Contractions (%)
WT	1.0	4.3
<i>irc20</i> Δ	2.3	10.1
<i>rad5</i> Δ	4.2	4.2
<i>irc20</i> Δ <i>rad5</i> Δ	2.8	2.8

Figure 12. URA3 CAG-70 YAC instability determined via PCR and gel electrophoresis. **(A)** Expansion frequency. **(B)** Contraction frequency. **(C)** Table of all instabilities. WT ($n = 460$), *irc20* Δ ($n = 89$), and *rad5* Δ ($n = 96$) are data sets by the Freudenreich lab, EJP, and LG. *irc20* Δ *rad5* Δ is $n = 69$. Significance determined by Fisher's exact test. See appendix for statistics.

AXH contributions: *irc20* Δ *rad5* Δ

To revisit the fragility phenotype that first brought *Irc20* to our attention, *irc20Δrad5Δ* fragility is no longer synergistic in the URA3 CAG-70 YAC and is not statistically significant from wildtype ($p = 0.24$ and $p = 0.07$ for expansions and contractions, respectively) (Figure 13). Furthermore, in the process of creating all Rad5 family single and double knockouts, an *irc20Δuls1Δ* strain was created in the URA3 CAG-70 YAC. The fragility rate of the *irc20Δuls1Δ* strain based on two assays is similar to wildtype and *irc20Δ*, and there is not a *uls1Δ* mutant in this YAC system to compare the double mutant's fragility to. These data directly contradict previous data from the ADE2 URA3 CAG-85 YAC since there are no longer increases in fragility in these double mutants. It is unlikely that the slight difference in CAG tract length between the YAC systems is sufficient to explain these differences.

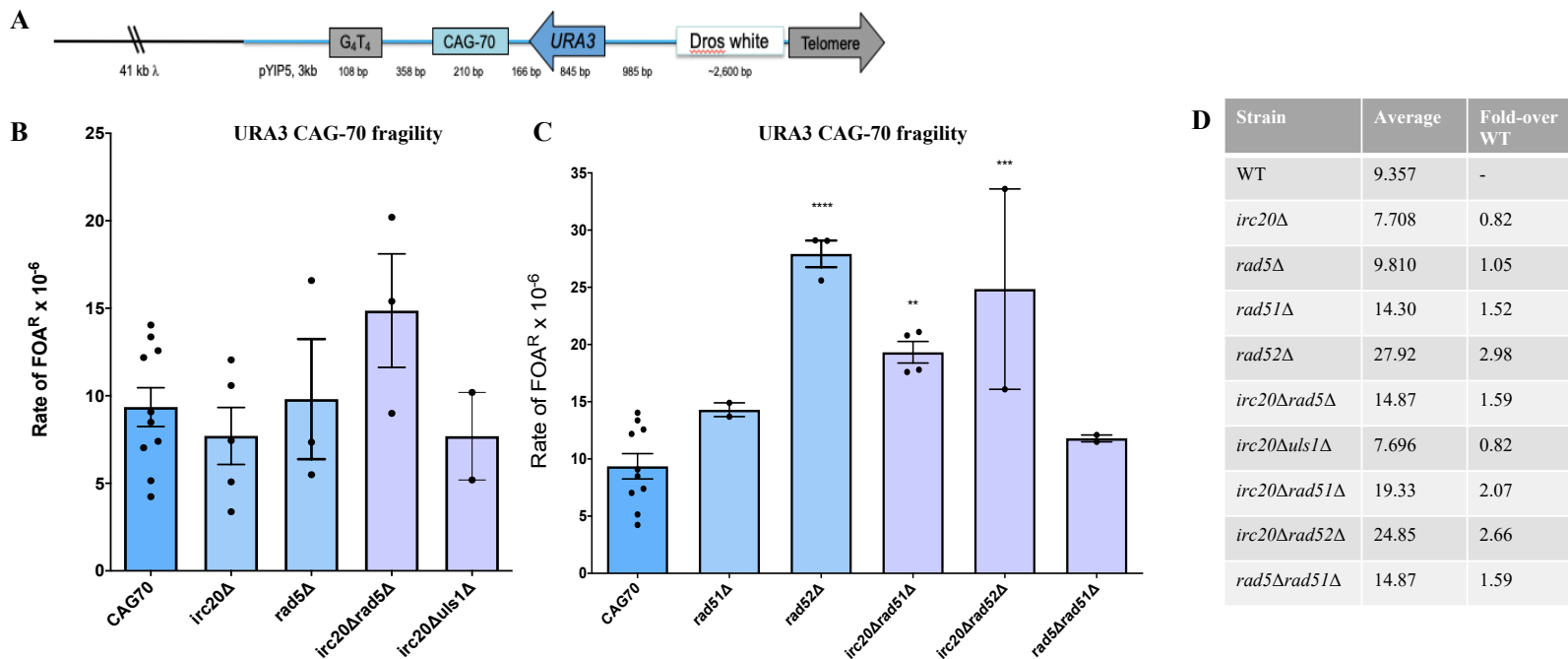


Figure 13. (A) URA3 CAG-70 YAC construct. (B) *irc20*, *rad5*, and *irc20rad5* mutant fragility rates. (C) Fragility rates of *rad51*, *rad52*, and combinations. (D) Average fragility rates (10^{-6}) and fold-over wildtype. Rates are calculated by maximum likelihood (MLL). Combined data from EJP and OTN. See appendix for statistics.

AXH contributions: *irc20Δ*, *irc20Δrad5Δ*, *irc20Δuls1Δ*, *rad5Δrad51Δ*

Since Rad51 is required for template switch-mediated repair, double knockouts of *irc20Δrad51Δ* and *rad5Δrad51Δ* could help determine if Irc20 functions in the TS pathway of PRR as hypothesized (Krijger et al. 2011, Symington 2002). These assays were done in the URA3 CAG-70 YAC because the Freudenreich lab has traditionally used this YAC system to characterize repair proteins, and so at the time this experiment was performed, Rad51 and Rad52 were better characterized in this YAC system; it was also done before we found the loss of the *irc20Δrad5Δ* fragility phenotype in this YAC. It was previously shown that *irc20Δrad51Δ* has a borderline additive fragility compared to their single mutants (OTN thesis, Figure 13C). On the other hand, *rad5Δrad51Δ* produces a similar fragility to wildtype as expected, since Rad5 and Rad51 are in the same pathway according to the current PRR model (Figure 13C, see Appendix for statistics).

Comparing the *irc20Δrad51Δ* and *rad5Δrad51Δ* fragility data, Irc20 and Rad51 do not seem to interact in the same way as Rad5 and Rad51 interact. Rad5 is important in PRR pathway choice, while Rad51 promotes D-loop formation in the TS pathway, so it seems Irc20 at least does not affect PRR pathway switching to the same degree as Rad5 (Polleys et al. 2017). A triple mutant (*irc20Δrad5Δrad51Δ*) would help clarify the relationship between Irc20 and Rad51, but it is highly likely that Irc20 is not participating in template-switch mediated repair with Rad5 as we initially hypothesized. Irc20 may participate in a Rad52-dependent pathway, since the *irc20Δrad52Δ* mutant showed the same level of CAG-70 fragility as the *rad52Δ* single mutant, though since the *irc20Δ* single did not show a phenotype, this is not a strong conclusion.

DISCUSSION

The goal of this study was to characterize the role of the Rad5 family proteins (Irc20, Rad5, Rad16, and Uls) in CAG repeat repair and maintenance as well as identify any possible interactions that could help explain the synergistic increase in CAG repeat fragility in an *irc20Δrad5Δ* double mutant in the ADE2 URA3 CAG-85 YAC (Figure 7C). Rad5, Rad16, and Uls1 are well characterized in repair and produce expected phenotypes in our assays that are in line with their respective roles in repair. We also hypothesized that Irc20 has some redundant role to Rad5 in the template switch PRR pathway based on the initial results measuring CAG-85 fragility, but our new data does not support that hypothesis. Still, there are some differing phenotypes between the YAC systems that need to be further explored.

A *uls1Δ* mutant produced consistently high fragility rates in the ADE2 URA3 CAG-85 YAC and was unaffected by the knockout of *irc20Δ*. Uls1's role in replicative stress responses and PRR predicts this fragility phenotype in that the loss of Uls1 likely causes a buildup of DNA lesions since it is associated with slowed S-phase progression (Cal-Bąkowska et al. 2011). From the unchanged fragility between the *uls1Δ* single mutant and the *irc20Δuls1Δ* double mutant, we expect no interactions between Irc20 and Uls1 in CAG-specific maintenance and DNA repair. Interestingly, an *irc20Δuls1Δ* double mutant in the URA3 CAG-70 YAC did not show an increase in fragility despite what is seen in the ADE2 URA3 CAG-85 YAC. There is no *uls1Δ* mutant in the URA3 CAG-70 YAC for comparison, but the *irc20Δuls1Δ* data suggests that *uls1Δ* may not show an increase in fragility in the URA3 CAG-70 YAC despite its roles in DNA repair. This fragility phenotype in the URA3 CAG-70 YAC is in agreement that Irc20 and Uls1 do not seem to interact with each other in DNA repair.

Uls1 is also hypothesized to take part in PRR pathway choice by physically interacting with PCNA and targeting SUMO-modified Srs2 (Kramarz et al. 2017). This could lead to some consideration about interactions between the Rad5 family proteins and ubiquitinated substrates, since they contain RING finger domains. Brühl et al., (2019) studied SHPRH, a Rad5 mammalian homolog, and suggested that the Rad5 family proteins direct DNA repair via modification of nucleosome ubiquitination in different situations; this supports that the Rad5 family proteins are not directly interacting in DNA repair pathways.

Rad16 does have some involvement in histone acetylation during GG-NER, but that mechanism is not well understood (Teng et al. 2008). On the other hand, Rad16 has no established role in CAG repeat fragility although it has been shown to affect CAG repeat instability (Koch et al. 2018). Thus, the large increase in CAG repeat fragility in a *rad16Δ* mutant is exciting (Figure 7B). On the other hand, an *irc20Δrad16Δ* double mutant produced an either additive or greater than additive fragility compared to the single mutants, depending on whether the upper two or lower two data points are considered (Figure 7C). If *irc20Δ* and *rad16Δ* are additive, then Irc20 and Rad16 function in different, unrelated pathways. If they are synergistic, then Irc20 and Rad16 function in compensating pathways important for repairing breaks in an extended CAG repeat or preventing breaks from occurring. As previously mentioned, there have not been studies showing a role for GG-NER in CAG repeat maintenance.

A deletion of Irc20 was not statistically significant compared to wildtype in fragility and instability in both YAC systems, in telomeric Southern blotting, and in SCR as a single mutant. As a double mutant with *rad5Δ*, it shows a synergistic increase in fragility in the ADE2 URA3 CAG-85 YAC and has a statistically significant increase in contraction frequency in the URA3 CAG-70 YAC. Otherwise, *irc20Δrad5Δ* produces negative phenotypes in CAG-85 instability on

the ADE2 URA3 YAC, URA3 CAG-70 YAC fragility, the telomeric Southern blot, and SCR. Therefore, our new data do not support an important role for Irc20 in maintaining expanded CAG repeats as we first envisioned.

It is also important to note at this point that the ADE2 URA3 CAG-85 YAC construct consistently produces low fragility rates across the board and utilizes two selective markers, making it useful in the detection of genes that dramatically increase fragility (Freudenreich lab data). We chose to utilize the well-characterized URA3 CAG-70 YAC, which has a slightly shorter repeat tract, to complement and reassess data from the ADE2 URA3 CAG-85 YAC. However the *irc20Δrad5Δ* synergy in fragility was not observed in the URA3 CAG-70 YAC system. Steps were also taken to complete the Rad5 family double mutant combinations in both YAC systems, but work is incomplete, and only the *irc20Δuls1Δ* strain was made and assayed.

It would be interesting to see if the *rad16Δ* and *irc20Δrad16Δ* fragility phenotypes are reflected in the URA3 CAG-70 YAC or if they return to wildtype like *irc20Δ* and *irc20Δrad5Δ*. It was also suggested that a quadruple mutant (*irc20Δrad5Δrad16Δuls1Δ*) would serve to determine if these mutants, combined, could inform any redundancy between proteins and repair pathways. It may also be worthwhile to follow up on the fragility in the *uls1Δ* strain and test whether Uls1 and Rad16 are acting in the same or different pathways, given the potential telomere phenotype observed in both mutants.

Several other lines of data support the idea that Irc20 and Rad5 actually act in different pathways. For example, we mentioned in “Introduction” that Srs2 is a major yeast helicase and translocase that interacts with Irc20, Rad5, and Uls1, and is required for PRR and HR (Friedl et al. 2001, Kramarz et al. 2017). The model by Miura et al., (2012) proposed that Irc20 functions in the same SDSA pathway involving Srs2 and acts prior to D-loop formation while Srs2 directs

D-loop processing to prevent double Holliday junction formation. Srs2 was also shown to interact with Rad5 since inactivation of Srs2 suppressed UV sensitivity of *rad5Δ* mutants and overall reduced SCR repair (Friedl et al. 2011). However, since an *irc20Δ* single mutant does not increase fragility in our data, but *srs2Δ* single mutants have significantly higher fragility compared to wildtype. It would seem that Irc20 and Srs2 are not involved in the same DNA repair pathway nor functionally analogous; they should produce similar fragility phenotypes if they are in the same pathway (Miura et al. 2012, Nguyen et al. 2017).

Furthermore, *irc20Δ* had no viability defect in our SSA assay system, which is a repair mechanism that requires extensive resection, a major step prior to D-loop formation (OTN thesis). This means that Irc20 would have to be involved in the repair pathway choice between SDSA and DSBR or in homology search. Rad5 and Rad51 act in the same TS pathway based on our URA3 YAC fragility data and previous studies. Rad51 is required for SDSA and SCR, both of which also involve Rad5 (Polleys et al. 2017). However, the *irc20Δrad51Δ* double mutant is approximately additive, and the *rad5Δrad51Δ* fragility is similar to wildtype in the same YAC (Figure 13B, C). If Irc20 is involved with Rad5 in template switch-mediated repair, then the fragility phenotype of the *irc20Δrad51Δ* strain and the *rad5Δrad15Δ* strain should be comparable. It is therefore unlikely that Irc20 acts in the template switch-mediated pathway of PRR with Rad5.

The unsuccessful reproduction of the high *irc20Δrad5Δ* fragility in the URA3 YAC also lead us to reassess our data in the ADE2 URA3 CAG-85 YAC. We initially hypothesized that the high *irc20Δrad5Δ* fragility was caused by a point mutation elsewhere in the genome, but that might be incorrect since a newly made *irc20Δrad5Δ* strain containing the ADE2 URA3 CAG-85 YAC) produced a similarly high fragility rate (Figure 7C, $\mu = 141.8 \times 10^{-8}$). The new

irc20Δrad5Δ strain was created by mating to a wildtype strain, sporulating, and isolating a haploid to refresh the strain background, hopefully eliminating any accumulated mutations. This does not exclude the possibility that a mutation elsewhere still remains, as there is a 50/50 chance it separated into the same spore, and especially since only one *irc20Δrad5Δ* spore was found during random spore analysis. However, current data supports that there is a possibility that the *irc20Δrad5Δ* fragility phenotype in the ADE2 URA3 CAG-85 YAC is a strain-specific phenomenon. In particular, the *irc20Δuls1Δ* strain in the URA3 CAG-70 YAC produced a fragility rate comparable to wildtype ($p = 0.55$); although we previously discussed that Irc20 and Uls1 are not likely interacting, we expected to see an increase in fragility caused by the lack of Uls1. However, the wildtype-level fragility of the *irc20Δuls1Δ* strain insinuates that a *uls1Δ* single mutant would also not affect fragility in the URA3 CAG-70 YAC. This in turn supports the hypothesis that there is strain specificity to the high fragility rates seen in the ADE2 URA3 CAG-85 YAC, because Uls1 has very distinct roles in DNA repair and a clear fragility phenotype in the ADE2 URA3 CAG-85 YAC strain.

While addressing the possible strain specificity of *irc20Δrad5Δ* fragility, whole genome sequencing analysis of the VPS105 and BY4705 strain backgrounds of the two YAC systems (ADE2 URA3 YAC and URA3 YAC, respectively) led to interesting findings related to ubiquitination (Alex Ferrazzoli, Brandeis; Alexandra Khristich, Mirkin lab). In the VPS105 background of the ADE2 URA3 CAG-85 YAC, there are tandem repeat deletions in *POL4* and *GRR1*, which are predicted to be deleterious. *POL4* codes for DNA polymerase IV, which mediates NHEJ (Leem et al. 1994). Sundararajan et al., (2010) showed that knocking out the NHEJ pathway (via a Dnl4 mutant) increased CAG-70 tract's fragility (3.2-fold over WT, $p < 0.05$) and instability (20% contractions, $p < 0.01$). A broad hypothesis could suggest that

inability to ubiquitinate Pol4p in the absence of one of the Rad5 family proteins could lead to ineffective NHEJ repair, thus conferring the high fragility rate of *irc20Δrad5Δ* in the ADE2 URA3 CAG-85 YAC but not in the URA3 CAG-70 YAC. Next steps could include deleting Pol4 and Grr1 in the URA3 CAG-70 YAC and looking for changes in fragility rate of the Rad5 family mutants.

GRR1, on the other hand, is an F-box protein component of the SCF complex, which is a subfamily of E3 ubiquitin ligases (Hermand 2006). This recalls some function of each of the Rad5 family proteins. Irc20 and Rad5 are characterized as E3 ubiquitin ligases; Uls1 is a STUbL; Rad16 is a subunit of the ECS ligase complex (Cherry et al. 2012). Another broad hypothesis could involve the SCF complex and characterize Irc20, Rad5, Uls1, and Rad16 as proteins that bind to the SCF complex and help guide ubiquitination to target repair proteins for degradation. More work is needed to clarify the relationships between these proteins, although it is highly likely that there are several unknown factors at play.

Although several phenotypes in our data are just borderline significant from wildtype, the outputs of these assays support that the Rad5 family of proteins likely act in unrelated DNA repair pathways that collectively contribute to CAG repeat maintenance and repair. Determining the phases of the cell cycle during which Irc20 is active could help identify DNA repair pathways it is involved in, since it does not seem to interact with Rad51, Uls1, and possibly Rad5 or Rad16 based on our data. Another major factor to consider is the Rad5 family proteins' roles as E3 ubiquitin ligases and their interactions with ubiquitinated substrates, which include PCNA and histones, and could provide better insight into their respective roles.

METHODS AND MATERIALS

I. Assay systems

Fragility

A total of 11-12 2 mL YC-Leu cultures were set up. Of those, 2 cultures were used as OD controls and inoculated to an OD₆₀₀ of 0.02-0.05. The other 9-10 cultures were inoculated by eye from single colonies of correct tract length (CAG-70 or CAG-85). Cultures were grown for 6-7 divisions in a 30°C rotating incubator. Final ODs were taken of the OD control cultures (Polleys and Freudenreich 2018).

For URA3 CAG-70 YAC: Cells were plated onto 5-fluoroorotic acid (5-FOA) -Leu plates without dilution. Total cell count (TCC) was measured by combining 100 µL of each culture and plating 10⁻⁵ dilutions on YC-Leu plates in duplicate.

For ADE2 URA3 CAG-85 YAC: Cells were plated onto 5-FOA-Leu low Ade plates without dilution; only red colonies were counted. Total cell count was measured by combining 100 µL of each culture and plating 10⁻⁵ dilutions on YC-Leu plates in duplicate.

Instability

Single colonies with correct tract length (CAG-70 or CAG-85) were inoculated into 2 mL of YC-Leu to OD₆₀₀ of 0.02-0.05 in the “allow breakage” condition. Cultures were grown for 6-7 divisions in a 30°C rotating incubator. Cultures were then diluted to 10⁻⁴ and plated on YC-Leu-Ura to select for both arms of the YAC (Polleys and Freudenreich 2018).

For CAG-70 URA3 YAC: tract length PCR and a 2% gel electrophoresis (MetaPhor™ agarose) were used to determine tract length changes.

For CAG-85 ADE2 URA3 YAC: colony PCR was performed on 33 daughter colonies (per mother colony) and run on the fragment analyzer. CAG repeat expansions and contractions

thresholds were determined by counting daughter colonies with tract lengths outside of +/- 3 CAG repeats.

Sister chromatid recombination (SCR) assay

The SCR assay was adapted from Mozlin et al., (2008). Ten Trp⁺ Ade⁻ colonies of correct tract length were inoculated into 5 mL of YEPD and grown to saturation in a 30°C rotating incubator. Dilutions of 10⁻³ were plated onto selective media (YC-Ade-Trp). Total cell count was determined by combining 100 µL of each culture and plating 10⁻⁵ dilutions on YEPD plates in duplicate. Recombination rates were calculated with method of the median and statistical significance in recombination rate was determined using Student's t-tests. See Figure 4 for a SCR repair schematic.

Telomeric Southern blotting

Cells were grown to saturation in 10 mL of appropriate minimal media at 28°C. Genomic DNA was prepared by zirconia/silica bead disruption and a standard phenol extraction, then digested with XhoI. DNA was separated on a 0.8% agarose gel (35 V for approximately 24 hrs), then transferred to a Hybond XL membrane (Amersham) using an alkaline transfer. Gels were probed with a radiolabeled telomeric DNA fragment excised from plasmid Ap135 (Williams et al. 2014).

II. Yeast strain construction

Fragility and instability (Figure 7, 8, 10, 12, 13):

Strain number	Genotype	CAG tract length and YAC
1666, 1667	rad5::KAN	CAG 70 URA3
1672, 1673	rad5::KAN; rad51::HIS3	CAG 70 URA3
2137, 2138	rad51::TRP1	CAG 70 URA3
3911, 3912	irc20::KAN	CAG 85 ADE2 URA3
4064, 4065	irc20::KAN	CAG 70 URA3
4536	irc20::KAN; rad5::TRP1	CAG 85 ADE2 URA3
4606, 4607	irc20::KAN; rad16::TRP1	CAG 85 ADE2 URA3
4608, 4609	uls1::NAT	CAG 85 ADE2 URA3
4610	irc20::KAN; uls1::NAT	CAG 85 ADE2 URA3
4716, 4717, 4718	rad16::TRP1	CAG 10 ADE2 URA3
4719, 4720, 4721	irc20::KAN; rad16::TRP1	CAG 10 ADE2 URA3
4793, 2794	rad5::HPH	CAG 85 ADE2 URA3
4803, 4804	rad5::HPH; uls1::NAT	CAG 85 ADE2 URA3
*5152, 5153, 5154	irc20::KAN; rad5::KAN	CAG 70 URA3
*5155, 5156	rad5::KAN; rad51::KAN	CAG 70 URA3
5157	rad5::KAN	CAG 70 URA3
*5171, 5172	uls1::NAT	CAG 70 URA3
*5173, 5174	rad5::KAN; uls1::NAT	CAG 70 URA3
*5175, 5176	irc20::KAN; uls1::NAT	CAG 70 URA3
*5177, 5178	irc20::KAN; rad5::KAN uls1::NAT	CAG 70 URA3
*5179	irc20::KAN; rad5::TRP1	CAG 85 ADE2 URA3
*5180, 5181	rad16::TRP1	CAG 85 ADE2 URA3
*5182, 5183	irc20::KAN; rad16::NAT	CAG 85 ADE2 URA3

SCR assay (Figure 6, 11):

Strain number	Genotype
2867	WT SCR
2996, 2997	rad5::KAN
4155, 4156	irc20::KAN
*5104, 5105, 5106, 5107	WT SCR (refreshed)

*strains made by AXH; all other strains made by OF, OTN, TEA, MD, EJP

APPENDIX

I. ADE2 URA3 CAG-85 YAC: Figure 7, 10

ADE2 URA3 CAG-85 YAC fragility

Strain	<i>p</i> -value (to WT)	<i>p</i> -value (to <i>irc20Δ</i>)
<i>irc20Δ</i>	0.54	-
<i>rad5Δ</i>	0.087	-
<i>rad16Δ</i>	0.0006 ***	-
<i>uls1Δ</i>	< 0.0001 ****	-
<i>irc20Δrad5Δ</i>	< 0.0001 ****	< 0.0001 ****
<i>irc20Δrad16Δ</i>	0.0002 ***	0.0002 ***
<i>irc20Δuls1Δ</i>	0.0002 ***	0.0002 ***

ADE2 URA3 CAG-85 YAC instability

Strain	Expansions	Expansions <i>p</i> -value	Contractions	Contractions <i>p</i> -value	<i>n</i>
WT	4	-	50	-	226
<i>irc20Δ</i>	1	0.19	52	0.66	260
<i>rad5Δ</i>	3	0.13	13	0.72	52
<i>irc20Δrad5Δ</i>	2	0.63	29	0.021	69

II. URA3 CAG-70 YAC: Figure 12, 13

URA3 CAG-70 YAC fragility

Strain	<i>p</i> -value (to WT)	<i>p</i> -value (to <i>irc20Δ</i>)
<i>irc20Δ</i>	0.41	-
<i>rad5Δ</i>	0.87	-
<i>rad51Δ</i>	0.085	-
<i>rad52Δ</i>	< 0.0001 ****	-
<i>irc20Δrad5Δ</i>	0.059	0.0673
<i>irc20Δuls1Δ</i>	0.55	1.00
<i>irc20Δrad51Δ</i>	0.0002 ***	0.0007 ***
<i>irc20Δrad52Δ</i>	0.0030 **	0.0243 *
<i>rad5Δrad51Δ</i>	0.3653	-

URA3 CAG-70 YAC instability

Strain	Expansions	Expansions <i>p</i> -value	Contractions	Contractions <i>p</i> -value	<i>n</i>
WT	5	-	20	-	460
<i>irc20Δ</i>	2	0.32	9	0.070	89
<i>rad5Δ</i>	4	0.057	4	1.00	96
<i>irc20Δrad5Δ</i>	2	0.25	2	0.75	72

III. Whole genome sequencing analysis:

Chromosome	CDS	Change	Polymorphism type	Protein effect
chr03	POL4	(TTC)7 -> (TTC)6	Deletion (tandem repeat)	Deletion
chr10	GRR1	(TTGTTG)5 -> (TTGTTG)4	Deletion (tandem repeat)	Deletion

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