RTEL1 Inhibits Trinucleotide Repeat Expansions and Fragility

Aisling Frizzell1,5, Jennifer H.G. Nguyen3,5, Mark I.R. Petalcorin4, Katherine D. Turner1,2, Simon J. Boulton4, Catherine H. Freudenreich3, and Robert S. Lahue1,2,*

1Centre for Chromosome Biology, School of Natural Sciences, National University of Ireland Galway, Newcastle Road, Galway, Ireland 2NCBES Galway Neuroscience Centre, National University of Ireland Galway, Newcastle Road, Galway, Ireland 3Department of Biology, Tufts University, Medford, MA 02155, USA 4DNA Damage Response Laboratory, London Research Institute, Cancer Research UK, Clare Hall, South Mimms EN6 3LD, UK

SUMMARY

Human RTEL1 is an essential, multifunctional helicase that maintains telomeres, regulates homologous recombination, and helps prevent bone marrow failure. Here, we show that RTEL1 also blocks trinucleotide repeat expansions, the causal mutation for 17 neurological diseases. Increased expansion frequencies of (CTG·CAG) repeats occurred in human cells following knockdown of RTEL1, but not the alternative helicase Fbh1, and purified RTEL1 efficiently unwound triplet repeat hairpins in vitro. The expansion-blocking activity of RTEL1 also required Rad18 and HLTF, homologs of yeast Rad18 and Rad5. These findings are reminiscent of budding yeast Srs2, which inhibits expansions, unwinds hairpins, and prevents triplet-repeat-induced chromosome fragility. Accordingly, we found expansions and fragility were suppressed in yeast srs2 mutants expressing RTEL1, but not Fbh1. We propose that RTEL1 serves as a human analog of Srs2 to inhibit (CTG·CAG) repeat expansions and fragility, likely by unwinding problematic hairpins.

INTRODUCTION

Expansions of trinucleotide repeats (TNRs) are the causative mutations in 17 human neurological disorders, including Huntington’s disease and fragile X syndrome (López Castel et al., 2010; McMurray, 2010; Mirkin, 2007). Although complex molecular mechanisms drive expansions (López Castel et al., 2010; McMurray, 2010; Mirkin, 2007), it is clear that two categories of protein factors are crucial in determining whether an expansion occurs. Promutagenic factors favor the expansion process, relentlessly driving TNRs toward longer allele lengths. Antimutagenic proteins inhibit the expansion process.
and are important in helping prevent the initiating expansions that trigger instability. Both categories of proteins likely act on aberrant DNA secondary structures, such as hairpins, that are widely thought to be intermediates in the expansion process (Liu et al., 2010; López Castel et al., 2010; McMurray, 2010; Mirkin, 2007). One of the most pressing issues is to identify human factors in both categories and determine how they regulate the enigmatic expansion process. In this study, we sought human proteins that inhibit expansions by examining model organisms for inhibitory factors that have human counterparts.

Studies in budding yeast identified the helicase Srs2 as a key protein for inhibiting TNR expansions. The likelihood of an expansion is increased up to 40-fold in srs2 mutants (Bhattacharyya and Lahue, 2004; Daee et al., 2007; Kerrest et al., 2009). In contrast, mutation of a related helicase gene, SGS1, did not recapitulate the expansion defects of srs2 mutants (Bhattacharyya and Lahue, 2004; Kerrest et al., 2009). In one study, Srs2 was shown to function with the error-free branch of postreplication repair (PRR) (Daee et al., 2007). Mutants in PRR genes RAD5 or RAD18 also showed higher rates of expansion, and double-mutant analysis was consistent with an epistatic relationship between SRS2 and PRR genes. In vitro, purified Srs2 enzyme was particularly effective at unwinding a number of TNR hairpin structures, which are thought to be key intermediates in the expansion process (Anand et al., 2012; Bhattacharyya and Lahue, 2005; Dhar and Lahue, 2008). In vivo, ATPase-defective alleles of srs2 were also deficient at blocking expansions (Bhattacharyya and Lahue, 2004) and in facilitating replication through CGG repeats (Anand et al., 2012). Thus, Srs2 most likely inhibits expansions by unwinding TNR hairpins, precluding their further processing into the full expansion mutation. This antiexpansion activity of Srs2 is in addition to its better-known role as a negative regulator of homologous recombination (HR) (Karpenshif and Bernstein, 2012; Marini and Krejci, 2010). During HR, Srs2 disassembles Rad51-single-stranded DNA filaments to prevent toxic recombination (Krejci et al., 2003; Veaute et al., 2003). Mutants of SRS2 are synthetically lethal when combined with SGS1 defects due to uncontrolled levels of recombination (Gangloff et al., 2000). The antirecombinase activity of Srs2 also plays a role in preventing TNR instability for long disease-length alleles, which are prone to breakage. Srs2 achieves this role by controlling sister-chromatid recombination initiated by repeat-associated fork blockage or breaks (Anand et al., 2012; Kerrest et al., 2009). Thus, Srs2 makes a major contribution to genomic stability by inhibiting TNR expansions both via hairpin unwinding and by blocking excessive HR.

Despite the absence of sequence homologs of yeast Srs2 protein in metazoan organisms, several helicases have functions analogous to Srs2 (Karpenshif and Bernstein, 2012; Uringa et al., 2011). Some functions of the essential helicase RTEL1 parallel those of Srs2 (Uringa et al., 2011). RTEL1 negatively regulates HR (Barber et al., 2008; Uringa et al., 2012), and RTEL1 is a helicase capable of unwinding unusual DNA structures in vitro (Vannier et al., 2012). Furthermore, rtel1 mutants in C. elegans are synthetically lethal when combined with mutation of HIM-6, the invertebrate homolog of yeast SGS1 (Barber et al., 2008). Inherited mutations in RTEL1 cause Hoyeraal-Hreidarsson syndrome, a severe form of dyskeratosis congenita that leads to early bone marrow failure (Ballew et al., 2013; Le Guen et al., 2013; Walne et al., 2013). A second protein with functions analogous to Srs2 is Fbh1, a human helicase/F-box protein (Kim et al., 2002). Fission yeast Fbh1 regulates recombination
Human Fbh1 expressed in budding yeast suppressed recombination defects of srs2 mutants and rescued the methylmethanesulfonate (MMS) sensitivity of rad18 or rad5 mutants in the presence of srs2 (Chiolo et al., 2007). A third analog, PARI, inhibits recombination and stabilizes the genome in a manner requiring proliferating cell nuclear antigen interaction (Moldovan et al., 2012). Unlike Srs2, PARI does not appear to be an active helicase and little is known about its in vivo function.

The goal of this study was to determine whether RTEL1 or Fbh1 inhibits TNR expansions and fragility. Our evidence supports RTEL1, not Fbh1, as an inhibitor of expansions in human cells and repeat-mediated chromosome fragility in budding yeast. Furthermore, the data suggest RTEL1 acts as a helicase to unwind TNR hairpins and thereby preclude the expansion process.

RESULTS

RTEL1 Inhibits Expansions and Unwinds TNR Hairpins

If either RTEL1 or Fbh1 inhibits expansions in human cells, then knockdown of the relevant factor should cause higher expansion frequencies. To assess RTEL1 and Fbh1 activity toward expansions, we combined small interfering RNA (siRNA) treatment with a previously described TNR expansion assay (Experimental Procedures; Figure S1). A human astrocytic cell line, SVG-A, was used because these cells support expansions in culture (Debacker et al., 2012; Gannon et al., 2012) and because in vivo expansions occur in glia (Shelbourne et al., 2007). Knockdown of Fbh1 in SVG-A cells produced virtually no change (<8%) in expansion frequencies (Figure 1A). In contrast, knockdown of RTEL1 increased expansions by 5- to 7-fold. This finding held for pooled siRNA and two unique interfering RNAs (Figure 1A). Knockdown efficiencies, measured by quantitative RT-PCR, showed Fbh1 was reduced by 68% (±1%) and RTEL1 was reduced by 55% (±4%) by pooled siRNA and 69% (±13%) by individual RNA #9 (Figure 1B). Although we cannot rule out that residual Fbh1 levels might inhibit expansions, it is clear that reducing RTEL1 mRNA levels by 55%–70% causes a pronounced effect on expansions. From these assays, we conclude that RTEL1 predominates at inhibiting expansions in this system.

Yeast Srs2 removes most TNR hairpins rather than acting selectively on a subset of hairpin sizes, based on the finding that mutation of SRS2 increases the rate of expansions without detectably changing their size distribution (Bhattacharyya and Lahue, 2004; Daee et al., 2007). To assess the expansion-blocking activity of human RTEL1, we examined expansion sizes (Figure S1D). There was virtually no difference in expansion sizes between scrambled control and siRNA against RTEL1. The range of expansions (+4 to +20 repeats), weighted average (+10.9 repeats), and median (+10 to +11 repeats) were extremely similar. Final allele sizes up to 42 CTG·CAG repeats were observed; thus, a subset of expansions that are inhibited by RTEL1 would cross the disease threshold of 30–40 repeats seen in some human neurological disorders.

Both yeast Srs2 and human RTEL1 are DNA helicases. Several lines of evidence suggest that the helicase function of Srs2 is critical for inhibiting expansions. An ATPase mutant of Srs2, K41A, is defective in blocking expansions in yeast (Bhattacharyya and Lahue, 2004),
and a similar mutant, K41R, is defective in facilitating replication through a CGG hairpin (Anand et al., 2012). Purified Srs2 efficiently unwinds TNR hairpins of different sizes and sequences (Bhattacharyya and Lahue, 2005; Dhar and Lahue, 2008). Similarly, the K48R ATPase mutant of RTEL1 fails to disassemble T-loops in cell-free extracts (Vannier et al., 2012). We extended this analysis by performing helicase assays on a TNR hairpin substrate with wild-type or K48R RTEL1. A duplex molecule containing a (CTG)_{11} hairpin was chosen to mimic a presumptive intermediate in the expansion process and because +11 repeat expansions lie in the midrange of RTEL1 activity based on the size spectra described above. In vitro, the wild-type enzyme, but not the mutant protein, efficiently unwound the (CTG)_{11} hairpin (Figures 1C and S2A). Unwinding by wild-type RTEL1 occurred efficiently in the presence of MgCl\_2 and was inhibited by CaCl\_2, suggesting that helicase activity requires Mg\^{2+} as a cofactor (Figure S2B). RTEL1 was also effective on a substrate with extrahelical CAG repeats. A (CAG)_{11} substrate was efficiently unwound by wild-type RTEL1, but not the K48R variant (Figure 1D). In summary, the helicase assays are consistent with the notion that RTEL1 inhibits expansions by unwinding TNR hairpins.

**RTEL1 Functions with HLTF and Rad18 to Inhibit Expansions**

We also addressed whether additional factors work in conjunction with RTEL1 to inhibit expansions. One previous study of yeast Srs2 (Daee et al., 2007) indicates that the enzyme blocks expansions in concert with Rad5 and Rad18, proteins of the error-free branch of PRR (Daee et al., 2007). We evaluated the human homologs of these factors following depletion by siRNA. Of the two reported orthologs of Rad5 (Lin et al., 2011; Motegi et al., 2006; Unk et al., 2006), knockdown of HLTF, but not SHPRH, raised expansion frequencies by 3- to 4-fold (Figures 2A and 2B). Similarly, expansions were 4- to 5-fold more common upon siRNA treatment against human Rad18 (Figures 2A and 2B). Thus, single knockdown of RTEL1, HLTF, or Rad18 all conferred increases in expansion frequencies to similar extents, consistent with a concerted mode of action. Epistasis between RTEL1 and HLTF and between RTEL1 and Rad18 was evaluated by double-knockdown assays. In each case, expansions occurred about six times more frequently than control levels (Figure 2B), although the magnitude of this effect was not statistically different from the knockdown of any single component alone (Figures 2B and S1E). In summary, expansion assays in human cells suggests that RTEL1, not Fbh1, is the functional equivalent of Srs2 in vertebrates and that RTEL1 may function with HLTF and Rad18 to inhibit expansions.

Efficacious knockdown occurred for HLTF (87% ± 5%), SHPRH (75% ± 5%), and Rad18 (86% ± 9%) (Figures 2 and S3A). Other studies reported that knockdown of SHPRH to similar levels as seen here showed several phenotypes (Lin et al., 2011; Motegi et al., 2006). Thus, the lack of an expansion phenotype in our assays upon SHPRH knockdown is unlikely due to residual levels of the protein. Similar knockdown efficiency and specificity was seen upon double knockdowns of HLTF (84% ± 3%), Rad18 (81% ± 3%), and RTEL1 (59%–71%; Figures 2, S3B, and S3C).

Likewise, single knockdowns of HLTF or Rad18 yielded nearly identical expansion size results to control or RTEL1 knockdowns (Figure S1D), again consistent with coordination between RTEL1 and PRR enzymes. Expansion sizes were also similar in double...
knockdowns of RTEL1+HLTF or RTEL1+Rad18, although the size range was somewhat narrower and the weighted average and median values were slightly lower than comparable single knockdowns. Overall, we conclude that RTEL1, HLTF, and Rad18 may function coordinately to block most TNR expansions in this system and thereby control the incidence of expansions, not their sizes.

**RTEL1 Suppresses MMS Sensitivity and Triplet Repeat-Mediated Chromosome Fragility and Expansions of Yeast srs2 Mutants**

If RTEL1 or Fbh1 is an analog of Srs2, then expressing these human helicases in budding yeast might reverse srs2 phenotypes. The human *RTEL1* and *FBH1* genes were integrated into the *SRS2* locus, thereby inactivating the yeast gene and permitting expression of the human genes from the endogenous *SRS2* promoter (Figure S4A). Cells lacking Srs2 are moderately sensitive to the cytotoxic effects of MMS (Chiolo et al., 2007). The MMS sensitivity of the srs2Δ mutant was strongly suppressed by expression of RTEL1 but only weakly suppressed by Fbh1 (Figure 3A), suggesting that RTEL1 is the better substitute for Srs2. The genetic interaction of the helicases with Rad5 was also examined because Rad5 is needed for the error-free branch of PRR (Smirnova and Klein, 2003). rad5Δ mutants are exquisitely sensitive to MMS, in part because Srs2 restraints recombination. This hypersensitivity can be partly overcome in an srs2Δ rad5Δ double mutant, because deletion of Srs2 is thought to promote HR and therefore bypass the requirement for Rad5 (Schiestl et al., 1990; Ulrich, 2001). This leads to the prediction that MMS hypersensitivity should be restored in an srs2Δ rad5Δ double mutant upon expression of a human analog of Srs2. We found that expression of RTEL1 conferred extreme MMS sensitivity to the double mutant, whereas Fbh1 did not (Figure 3B). Thus, RTEL1, but not Fbh1, can substitute for the role of Srs2 in the Rad5 branch of the PRR pathway in yeast.

The yeast strains expressing RTEL1 or Fbh1 were also tested for triplet-repeat-mediated chromosomal fragility and instability. Long TNR tracts, such as (CAG)$_{70}$, undergo chromosome fragility in yeast (Anand et al., 2012; Callahan et al., 2003; Freudenreich et al., 1998; Kerrest et al., 2009). DNA breaks are prevented by Srs2, based on the finding that srs2 mutants show increased levels of chromosome fragility (Anand et al., 2012; Kerrest et al., 2009). Protection against chromosome fragility was attributed to its function as a helicase that unwinds hairpins encountered during replication (Anand et al., 2012) and/or to a role in promoting fork reversal (Kerrest et al., 2009). We found that chromosome fragility in the control strain lacking a repeat tract was unchanged in the srs2Δ mutant and expression of Fbh1 or RTEL1 caused modest increases in breakage rates (Figures 3C and S4B). The presence of the (CAG)$_{70}$ tract resulted in higher levels of breakage in the wild-type strain, an effect that is exacerbated by the srs2Δ mutation, in agreement with previous studies (Anand et al., 2012; Kerrest et al., 2009). Expression of Fbh1 did not detectably alter fragility rates compared to the srs2Δ strain, whereas expression of RTEL1 conferred complete rescue (Figures 3C and S4B). The fragility data suggest that RTEL1 can substitute for Srs2 in facilitating replication through TNRs. We also examined expansion frequencies for a (CAG)$_{85}$ tract (Figures 3D and S4C). The srs2Δ strain showed 5-fold more expansions than the wild-type control, similar to published values (Kerrest et al., 2009). Expression of RTEL1 reduced expansion levels to levels similar to wild-type. In contrast, the expansion
frequency was still partly elevated following expression of Fbh1. The partial rescue of (CAG)\textsubscript{85} expansions by Fbh1 is consistent with its known role in preventing recombination (Chiolo et al., 2007), because expansions of this repeat size occur during a Rad51-dependent process in srs2\textDelta yeast (Kerrest et al., 2009). Similarly, both Fbh1 and RTEL1 were able to rescue the sensitivity of the srs2\textDelta strain to camptothecin (Figure S4D). These two rescue phenotypes of Fbh1 prove that the protein is expressed in yeast, thereby eliminating the possibility that failure of Fbh1 to rescue other srs2 phenotypes is due to insufficient expression. Thus, although both helicases can compensate for some Srs2 antirecombinase functions, RTEL1 is uniquely able to complement Srs2 roles in replication and postreplication repair, based on the RTEL1 rescue of srs2\textDelta MMS sensitivity, restoration of MMS hypersensitivity in a rad5\textDelta background, and rescue of CAG repeat-mediated fragility and instability in a srs2\textDelta mutant. We conclude that expression of RTEL1 in yeast provides similar biochemical functions as Srs2 in DNA repair and in inhibiting triplet-repeat-mediated chromosome fragility and expansions.

**DISCUSSION**

**Parallels between RTEL1 and Yeast Srs2 in Inhibiting TNR Expansions and Fragility**

This work identifies RTEL1 as a key human enzyme that inhibits TNR expansions and fragility. Knockdown of RTEL1 in SVG-A astrocytes caused a substantial increase in expansion frequencies, whereas targeting Fbh1 conferred little effect (Figure 1A). Purified RTEL1 readily unwound CTG and CAG hairpin structures in vitro, in a manner dependent on its ATPase activity (Figures 1C and 1D). Similar increases in expansions were also observed upon knockdown of Rad18 or the Rad5 ortholog HLTF, and double knockdowns of RTEL1 with Rad18 or HLTF are suggestive of a single pathway for inhibiting expansions (Figure 2). Our expansion results reveal a clear delineation between the Rad5 orthologs HLTF and SHPRH, consistent with the findings of Lin et al., who identified distinct functions for the two enzymes in suppressing damage-induced mutagenesis (Lin et al., 2011). These RTEL1 findings—inhibition of expansions, hairpin unwinding, and function with the Rad5 ortholog HLTF and Rad18—all closely resemble yeast Srs2, indicating the strong functional conservation between the two enzymes with respect to (CTG·CAG) repeat expansions, despite their lack of protein sequence homology. We note that mutations in yeast SRS2, RAD5, or RAD18 have different mutagenic effects depending on the type of microsatellite (Cheng et al., 2011; Daee et al., 2007; Shishkin et al., 2009), perhaps due to the different DNA structures they can adopt (Mirkin, 2007). We found expression of RTEL1 in yeast srs2 mutants efficiently suppressed MMS sensitivity and CAG-repeat-dependent chromosomal fragility and expansions, whereas expression of Fbh1 was substantially poorer in reversing these phenotypes (Figure 3). We conclude that RTEL1 is an important human factor that blocks triplet repeat expansions and repeat-mediated chromosome fragility and that its DNA helicase function is likely important in doing so.

In contrast, we found little or no evidence for a role in triplet repeat instability for the alternative Srs2 analog Fbh1. Fbh1 was originally identified as a human helicase/F-box protein (Kim et al., 2002) and in fission yeast as a regulator of recombination (Morishita et al., 2005; Osman et al., 2005). Fbh1 was subsequently shown to function at replication forks...
A previous study showed that human Fbh1 expressed in budding yeast suppressed recombination defects and MMS sensitivity of an srs2Δ mutant; however, Fbh1 did not restore MMS hypersensitivity to a rad5Δ srs2Δ double mutant (Chiolo et al., 2007), in agreement with our data (Figure 3B). Despite some parallels to Srs2, our experiments showed no detectable role for Fbh1 at triplet repeats in SVG-A cells (Figure 1A) and little evidence in yeast (Figure 3). Although we cannot rigorously rule out that Fbh1 imparts protection at TNRs in human cells, the current data do not support this hypothesis.

**Potential Mechanism of TNR Stabilization by RTEL1**

Srs2 has been proposed to inhibit expansions through its helicase activity on TNR hairpins (Bhattacharyya and Lahue, 2004, 2005; Daee et al., 2007; Dhar and Lahue, 2008) and/or by unwinding TNR structures to facilitate passage of replication forks (Anand et al., 2012; Kerrest et al., 2009). Purified RTEL1 is capable of efficiently unwinding (CTG)\(_{11}\) and (CAG)\(_{11}\) hairpins (Figures 1C and 1D), analogous to Srs2 (Bhattacharyya and Lahue, 2005; Dhar and Lahue, 2008). The ATPase function of both enzymes is important for unwinding TNRs (Figure S2) (Anand et al., 2012; Bhattacharyya and Lahue, 2004), consistent with a requirement for helicase activity. These results support the possibility that RTEL1 unwinds TNR hairpins before they can be converted into full expansion mutations. Our genetic analysis suggests that RTEL1 may work in the same pathway as HTLF and Rad18, based on the requirement for Rad18 and HLTF in expansion assays (Figures 3A and 3B), the double-knockdown data of RTEL1+Rad18 and RTEL1+HLTF (Figure 2B), the similar profiles of expansion sizes in all the knockdowns (Figure S2), and the genetic complementation of Srs2’s role in the PRR pathway (Figure 3). Thus, RTEL1 function at TNRs could act during postreplication repair or a template-switching event such as fork reversal (Figure 4).

In the PRR model, RTEL1 could unwind hairpins that initiate PRR at gaps left behind the replication fork or hairpins that occur during error-free PRR repair synthesis (Figure 4A). This model is strengthened by the similarity of the D-loop structure (Figure 4A, lower panel) to substrates that RTEL1 has been proposed to unwind during recombination and at T-loops (Uringa et al., 2011; Vannier et al., 2012). In this mode of action, RTEL1 could antagonize error-prone recombination that leads to expansions of long TNRs as seen in yeast (Kerrest et al., 2009; Sundararajan et al., 2010). We postulate that RTEL1 is recruited to the TNR by postreplication repair factors Rad18 and/or HLTF or that these factors modify another target that in turn attracts RTEL1 (Figure 4A). The activity of all three proteins would remove the hairpin and prevent expansions, whereas lack of RTEL1, HLTF, or Rad18 would result in higher expansion frequencies.

Another potential mode of action is through HLTF-mediated, error-free resolution of TNR replication stress (Figure 4B). HLTF, like Rad5, mediates reversal of model stalled replication forks by unwinding of the leading and lagging strands and then annealing of the nascent and parental strands to form a chicken-foot structure (Blastyák et al., 2010). This raises the possibility that replication fork reversal by HLTF could provide a means for stabilizing TNR tracts. In this case, RTEL1 would unwind hairpins at a stalled or reversed fork to allow restart without expansions or fragility. A hairpin on the reversed fork end was previously proposed as a potential expansion intermediate (Mirkin, 2007) and provides a
single-stranded DNA/double-stranded DNA junction, proposed to be a good substrate for RTEL1 loading and 5′ to 3′ unwinding (Uringa et al., 2011; Youds et al., 2010). Similarly, a template switch at the fork, mediated by HTLF and Rad18, could also require RTEL1 to unwind aberrant structured intermediates that might occur (Kim and Mirkin, 2013; Mirkin, 2007). This unwinding activity of RTEL1 is supported by the facts that Srs2 excels at unwinding G-rich structured DNA encountered during replication (Anand et al., 2012) and RTEL1 also appears to have in vivo roles in unwinding G-rich or structured regions at telomeres and recombination hot spots (Uringa et al., 2011; Vannier et al., 2012). The fork restart model could also explain how the lack of RTEL1, HLTF, or Rad18 would lead to excess expansions.

There is emerging evidence that RTEL1 and HLTF help prevent human disease. Polymorphisms in the RTEL1 gene are susceptibility loci for glioma risk (Shete et al., 2009; Wrensch et al., 2009), and inherited mutations in RTEL1 cause a severe form of dyskeratosis congenita (Ballew et al., 2013; Le Guen et al., 2013; Walne et al., 2013). For HLTF, silencing of its promoter by methylation is associated with colon, gastric, and uterine cancers (Debauve et al., 2008; Moinova et al., 2002). Based on our findings in this paper, we suggest that downregulation or loss of RTEL1 or HLTF might also lead to genetic predisposition toward TNR expansions.

**EXPERIMENTAL PROCEDURES**

**Expansion Assays, siRNA Treatment, and Expression Analysis in SVG-A Cells**

SVG-A astrocytes were cultured and assayed for expansions as described previously (Gannon et al., 2012) and as summarized in Figure S1. Briefly, cells were seeded on day 0 and transfected on day 1 with siRNA. On day 3, cells were transfected with triplet repeat shuttle vector and also retransfected with siRNA. After another 2 days, expansion frequencies were measured (Gannon et al., 2012), in parallel with analysis of expression levels from whole-cell lysates. The Supplemental Experimental Procedures provides details regarding siRNA, PCR primers, and antibodies. Subsequent PCR analysis of individual yeast colonies confirmed the number and size of TNR expansions. Statistical details are given in Figure S1E.

**RTEL1 Purification and Helicase Assays**

Wild-type and K48R mutant RTEL1 were expressed as N-terminal fusions to glutathione-S-transferase (GST) and expressed in Sf-9 cells using the baculovirus expression system. Briefly, infected cells were lysed by sonication, and protein was precipitated with ammonium sulfate and fractionated by gel filtration. Fractions containing GST-tagged proteins were further purified with glutathione affinity chromatography, cleaved by protease digestion of the tag, and concentrated. Details are given in the Supplemental Experimental Procedures. The formation of labeled helicase substrates and the helicase assays were carried out as described by Dhar and Lahue (2008).
Yeast Genetic Assays for Chromosome Fragility and Triplet Repeat Instability

Strains (Figure S4A) were isogenic BY4705 background containing a yeast artificial chromosome with 0, 70, or 85 CAG·CTG repeats, with the CAG strand on the lagging-strand template (Callahan et al., 2003; Sundararajan et al., 2010). Mutants were created through PCR-mediated gene replacement to generate srs2::Fbh1 and srs2::RTEL1 constructs. CAG tract length was tested by PCR amplification and analyzed for expansions ±9 bp (three repeats). Fragility and instability assays were performed as previously described (Kerrest et al., 2009). Two independent transformants were tested for each strain. Spot assays were repeated twice independently with two different transformants to test reproducibility. See the Supplemental Experimental Procedures for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Science Foundation Ireland award 10/IN.1/B2973 (to R.S.L.), the Irish Research Council for Science, Engineering and Technology and the Thomas Crawford Hayes Fund (to A.F.), the Tufts University dean’s fund (to C.H.F.), and a Tufts GSRA award (to J.N.). S.J.B. is funded by Cancer Research UK and by an ERC Advanced Investigator Grant (RecMitMei) and is a recipient of a Royal Society Wolfson Research Merit Award.

References

Anand RP, Shah KA, Niu H, Sung P, Mirkin SM, Freudenreich CH. Overcoming natural replication barriers: differential helicase requirements. Nucleic Acids Res. 2012; 40:1091–1105. [PubMed: 21984413]

Ballew BJ, Yeager M, Jacobs K, Giri N, Boland J, Burdett L, Alter BP, Savage SA. Germline mutations of regulator of telomere elongation helicase 1, RTEL1, in Dyskeratosis congenita. Hum. Genet. 2013; 132:473–480. [PubMed: 23329068]

Barber LJ, Youds JL, Ward JD, McIlwraith MJ, O’Neil NJ, Petalcorin MI, Martin JS, Collis SJ, Cantor SB, Auclair M, et al. RTEL1 maintains genomic stability by suppressing homologous recombination. Cell. 2008; 135:261–271. [PubMed: 18957201]

Bhattacharyya S, Lahue RS. Yeast Srs2 DNA helicase selectively blocks expansions of trinucleotide repeats. Mol. Cell. Biol. 2004; 24:7324–7330. [PubMed: 15314145]

Bhattacharyya S, Lahue RS. Srs2 helicase of Saccharomyces cerevisiae selectively unwinds triplet repeat DNA. J. Biol. Chem. 2005; 280:33311–33317. [PubMed: 16085654]

Blastyák A, Hajdú I, Unk I, Haracska L. Role of double-stranded DNA translocase activity of human HLTF in replication of damaged DNA. Mol. Cell. Biol. 2010; 30:684–693. [PubMed: 19948885]

Callahan JL, Andrews KJ, Zakiai VA, Freudenreich CH. Mutations in yeast replication proteins that increase CAG/CTG expansions also increase repeat fragility. Mol. Cell. Biol. 2003; 23:7849–7860. [PubMed: 14560028]

Cherng N, Shishkin AA, Schlager LI, Tuck RH, Sloan L, Matera R, Sarkar PS, Ashizawa T, Freudenreich CH, Mirkin SM. Expansions, contractions, and fragility of the spinocerebellar ataxia type 1 pentanucleotide repeat in yeast. Proc. Natl. Acad. Sci. USA. 2011; 108:2843–2848. [PubMed: 21282659]

Chiolo I, Saponaro M, Baryshnikova A, Kim J-H, Seo Y-S, Liberi G. The human F-Box DNA helicase FBH1 faces Saccharomyces cerevisiae Srs2 and postreplication repair pathway roles. Mol. Cell. Biol. 2007; 27:7439–7450. [PubMed: 17724085]

Daee DL, Mertz T, Lahue RS. Postreplication repair inhibits CAG·CTG repeat expansions in Saccharomyces cerevisiae. Mol. Cell. Biol. 2007; 27:102–110. [PubMed: 17060452]

Cell Rep. Author manuscript; available in PMC 2018 January 24.
Debacker K, Frizzell A, Gleeson O, Kirkham-McCarthy L, Mertz T, Lahue RS. Histone deacetylase complexes promote trinucleotide repeat expansions. PLoS Biol. 2012; 10:e1001257. [PubMed: 22363205]

Debaveu G, Capouillez A, Belayew A, Saussez S. The helicase-like transcription factor and its implication in cancer progression. Cell. Mol. Life Sci. 2008; 65:591–604. [PubMed: 18034322]

Dhar A, Lahue RS. Rapid unwinding of triplet repeat hairpins by Srs2 helicase of Saccharomyces cerevisiae. Nucleic Acids Res. 2008; 36:3366–3373. [PubMed: 18440969]

Freudenberg CH, Kantrow SM, Zakian VA. Expansion and length-dependent fragility of CTG repeats in yeast. Science. 1998; 279:853–856. [PubMed: 9452383]

Gangloff S, Soustelle C, Fabre F. Homologous recombination is responsible for cell death in the absence of the Sgs1 and Srs2 helicases. Nat. Genet. 2000; 25:192–194. [PubMed: 10835635]

Gannon A-MM, Frizzell A, Healy E, Lahue RS. MutSβ and histone deacetylase complexes promote expansions of trinucleotide repeats in human cells. Nucleic Acids Res. 2012; 40:10324–10333. [PubMed: 22941650]

Karpenshif Y, Bernstein KA. From yeast to mammals: recent advances in genetic control of homologous recombination. DNA Repair (Amst.). 2012; 11:781–788. [PubMed: 22889934]

Kerrest A, Anand RP, Sundararajan R, Bermejo R, Liberi G, Dujon B, Freudenberg CH, Richard G-F. SRS2 and SGS1 prevent chromosomal breaks and stabilize triplet repeats by restraining recombination. Nat. Struct. Mol. Biol. 2009; 16:159–167. [PubMed: 19136956]

Kim JC, Mirkin SM. The balancing act of DNA repeat expansions. Curr. Opin. Genet. Dev. 2013; 23:280–288. [PubMed: 23725800]

Kim J, Kim J-H, Lee S-H, Kim D-H, Kang H-Y, Bae S-H, Pan Z-Q, Seo Y-S. The novel human DNA helicase hFBH1 is an F-box protein. J. Biol. Chem. 2002; 277:24530–24537. [PubMed: 11956208]

Krejci L, Van Komen S, Li Y, Villetmain J, Reddy MS, Klein H, Ellenberger T, Sung P. DNA helicase Srs2 disrupts the Rad51 presynaptic filament. Nature. 2003; 423:305–309. [PubMed: 12748644]

Le Guen T, Jullien L, Touzot F, Schertzer M, Gaillard L, Perderiset M, Carpentier W, Nitschke P, Picard C, Couillault G, et al. Human RTEL1 deficiency causes Hoyeraal-Hreidarsson syndrome with short telomeres and genome instability. Hum. Mol. Genet. 2013; 22:3239–3249. [PubMed: 23591994]

Lin JR, Zeman MK, Chen JY, Yee MC, Cinprich KA. SHPRH and HLTF act in a damage-specific manner to coordinate different forms of postreplication repair and prevent mutagenesis. Mol. Cell. 2011; 42:237–249. [PubMed: 21396873]

Liu G, Chen X, Bissler JJ, Sindon RR, Leffak M. Replication-dependent instability at (CTG) × (CAG) repeat hairpins in human cells. Nat. Chem. Biol. 2010; 6:652–659. [PubMed: 20676085]

López Castel A, Cleary JD, Pearson CE. Repeat instability as the basis for human diseases and as a potential target for therapy. Nat. Rev. Mol. Cell Biol. 2010; 11:165–170. [PubMed: 20177394]

Lorenz A, Osman F, Folkyte V, Sofueva S, Whitby MC. FbH1 limits Rad51-dependent recombination at blocked replication forks. Mol. Cell. Biol. 2009; 29:4742–4756. [PubMed: 19546232]

Marini V, Krejci L. Srs2: the “Odd-Job Man” in DNA repair. DNA Repair (Amst.). 2010; 9:268–275. [PubMed: 20096651]

McMurray CT. Mechanisms of trinucleotide repeat instability during human development. Nat. Rev. Genet. 2010; 11:786–799. [PubMed: 20953213]

Mirkin SM. Expandable DNA repeats and human disease. Nature. 2007; 447:932–940. [PubMed: 17518176]

Moinova HR, Chen WD, Shen L, Smiraglia D, Olechnowicz J, Ravi L, Kasturi L, Myeroff L, Plass C, Parsons R, et al. HLTF gene silencing in human colon cancer. Proc. Natl. Acad. Sci. USA. 2002; 99:4562–4567. [PubMed: 11904375]

Moldovan GL, Dejsuphong D, Petalcorin MI, Hofmann K, Takeda S, Boulton SJ, D’Andrea AD. Inhibition of homologous recombination by the PCNA-interacting protein PARI. Mol. Cell. 2012; 45:75–86. [PubMed: 22153967]

Morishita T, Furukawa F, Sakaguchi C, Toda T, Carr AM, Iwasaki H, Shinagawa H. Role of the Schizosaccharomyces pombe F-Box DNA helicase in processing recombination intermediates. Mol. Cell. Biol. 2005; 25:8074–8083. [PubMed: 16135799]
Motegi A, Sood R, Moinova H, Markowitz SD, Liu PP, Myung K. Human SHPRH suppresses genomic instability through proliferating cell nuclear antigen polyubiquitination. J. Cell Biol. 2006; 175:703–708. [PubMed: 17130289]

Osman F, Dixon J, Barr AR, Whitby MC. The F-Box DNA helicase Fbh1 prevents Rhp51-dependent recombination without mediator proteins. Mol. Cell. Biol. 2005; 25:8084–8096. [PubMed: 16135800]

Schiestl RH, Prakash S, Prakash L. The SRS2 suppressor of rad6 mutations of Saccharomyces cerevisiae acts by channeling DNA lesions into the RAD52 DNA repair pathway. Genetics. 1990; 124:817–831. [PubMed: 2182387]

Shelbourne PF, Keller-McGandy C, Bi WL, Yoon SR, Dubeau L, Veitch NJ, Vonsattel JP, Wexler NS, Arheim N, Augood SJ. US-Venezuela Collaborative Research Group. Triplet repeat mutation length gains correlate with cell-type specific vulnerability in Huntington disease brain. Hum. Mol. Genet. 2007; 16:1133–1142. [PubMed: 17409200]

Shete S, Hosking FJ, Robertson LB, Dobbins SE, Sanson M, Malmer B, Simon M, Marie Y, Boisselier B, Delattre JY, et al. Genome-wide association study identifies five susceptibility loci for glioma. Nat. Genet. 2009; 41:899–904. [PubMed: 19578367]

Smirnova M, Klein HL. Role of the error-free damage bypass postreplication repair pathway in the maintenance of genomic stability. Mutat. Res. 2003; 532:117–135. [PubMed: 14643433]

Sundararajan R, Gellon L, Zunder RM, Freudenreich CH. Double-strand break repair pathways protect against CAG/CTG repeat expansions, contractions and repeat-mediated chromosomal fragility in Saccharomyces cerevisiae. Genetics. 2010; 184:65–77. [PubMed: 19901069]

Ulrich HD. The srs2 suppressor of UV sensitivity acts specifically on the RAD5- and MMS2-dependent branch of the RAD6 pathway. Nucleic Acids Res. 2001; 29:3487–3494. [PubMed: 11522817]

Vannier JB, Pavicic-Kaltenbrunner V, Petalcorin MI, Ding H, Boulton SJ. RTEL1 dismantles T loops and counteracts telomeric G4-DNA to maintain telomere integrity. Cell. 2012; 149:795–806. [PubMed: 22579284]

Veauve X, Jeusset J, Soustelle C, Kowalczykowski SC, Le Cam E, Fabre F. The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments. Nature. 2003; 423:309–312. [PubMed: 12748645]

Walne AJ, Vulliamy T, Kirwan M, Plagnol V, Dokal I. Constitutional mutations in RTEL1 cause severe dyskeratosis congenita. Am. J. Hum. Genet. 2013; 92:448–453. [PubMed: 23453664]
Figure 1. RTEL1 Inhibits Expansions in Cells and Unwinds Hairpins In Vitro

(A) Expansion frequencies after siRNA treatment. “RTEL1” denotes pooled siRNA, whereas “RTEL1 #9” and “RTEL1 #7” designate unique siRNAs. Expansion frequencies are normalized to those observed with scrambled control siRNA. See Figure S1E for details.

(B) Knockdown efficiency based on mRNA levels, as measured by quantitative RT-PCR. For (A) and (B), “Scr” denotes scrambled control siRNA and error bars represent SEM. *p < 0.05 compared to scrambled control.

(C and D) Unwinding of (CTG)_{11} hairpin (C) or (CAG)_{11} hairpin (D) by purified wild-type RTEL1 or K48R mutant RTEL1.
Figure 2. Expansions in SVG-A Cells following Knockdowns of RTEL1, HLTF, SHPRH, and Rad18

(A) Expansion frequencies for single knockdowns of HLTF, SHPRH, or Rad18 and immunoblot analysis of protein levels.

(B) Expansion frequencies following single or double knockdowns of RTEL1, HLTF, or Rad18 and immunoblot results of protein levels.

*p < 0.05 compared to scrambled control, and error bars represent SEM. Additional expression results are in Figure S3. See Figure S1E for details.
Figure 3. Expression of RTEL1 in Yeast Suppresses srs2 Phenotypes

(A) Human RTEL1 (hRTEL1) can complement Srs2 and rescue srs2Δ sensitivity to MMS more effectively than hFbh1.

(B) hRTEL1, but not hFbh1, expression in rad5Δ mutants restores hypersensitivity to MMS.

(C) Chromosome fragility on a yeast artificial chromosome (YAC) carrying a (CAG)_{70} tract (Kerrest et al., 2009). Rate values indicated above the bars; error bars represent SEM. Significance was determined compared to wild-type (*) or srs2Δ (†). *p ≤ 0.05, **p ≤ 0.001. See Figure S4B for details.

(D) Expansions of a (CAG)_{85} tract integrated on a YAC. See Figure S4C for details.
Figure 4. Models for Inhibition of Expansions by the Concerted Activities of HLT, Rad18, and RTEL1

(A) A triplet repeat hairpin is formed by DNA replication (shown) or repair (not shown). HLT and Rad18 recruit RTEL1 directly to the hairpin or modify another factor that does so. Once at the hairpin, RTEL1 uses its helicase activity to unwind the hairpin to help avoid an expansion. Lower panel: recombinational repair attempts to resolve the hairpin but creates another hairpin in the process. RTEL1 acts to resolve the hairpin(s) and avoid expansion.

(B) A hairpin is formed during DNA replication in conjunction with fork stalling. The hairpin could be alleviated directly by RTEL1 (top panel), or HLT in conjunction with Rad18 acts to reverse the stalled fork, which brings RTEL1 to the hairpin for unwinding. Loss or reduced levels of HLT, Rad18, or RTEL1 preclude hairpin unwinding such that the hairpin is subsequently processed to a triplet repeat expansion.