Published in final edited form as:

DNA Repair (Amst). 2017 August ; 56: 156–165. doi:10.1016/j.dnarep.2017.06.018.

Role of recombination and replication fork restart in repeat instability

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Abstract

Eukaryotic genomes contain many repetitive DNA sequences that exhibit size instability. Some repeat elements have the added complication of being able to form secondary structures, such as hairpin loops, slipped DNA, triplex DNA or G-quadruplexes. Especially when repeat sequences are long, these DNA structures can form a significant impediment to DNA replication and repair, leading to DNA nicks, gaps, and breaks. In turn, repair or replication fork restart attempts within the repeat DNA can lead to addition or removal of repeat elements, which can sometimes lead to disease. One important DNA repair mechanism to maintain genomic integrity is recombination. Though early studies dismissed recombination as a mechanism driving repeat expansion and instability, recent results indicate that mitotic recombination is a key pathway operating within repetitive DNA. The action is two-fold: first, it is an important mechanism to repair nicks, gaps, breaks, or stalled forks to prevent chromosome fragility and protect cell health; second, recombination can cause repeat expansions or contractions, which can be deleterious. In this review, we summarize recent developments that illuminate the role of recombination in maintaining genome stability at DNA repeats.

Keywords

trinucleotide repeat expansion; recombination; replication fork restart; chromosome fragility; DNA structure

INTRODUCTION

Expanded tracts of repetitive DNA sequences are the cause of over 30 genetic diseases and can consist of trinucleotide or larger repetitive units (1–5). The expandable repeats form stable non-B-form DNA structures which impede normal cellular processes like DNA

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The authors have no conflicts of interest to declare.

For DNA Repair Special Issue **DNARR17**, Edited by Phillip Hanawalt "Cutting-edge Perspectives in Genomic Maintenance IV"

replication and repair. Expanded trinucleotide repeats (TNRs) and other structure-forming repeats break at a greater frequency than non-repetitive DNA; types of DNA breaks that occur include nicks, gaps and double-stranded breaks (DSBs). These lesions must then be repaired in the context of the repetitive DNA. Much of the time the cell will succeed in repairing DNA damage at structure-forming repeats with fidelity, i.e. with no loss or gain of genetic material, thus preserving genome integrity. However, due to both the repetitive nature of the tract as well as the structure-forming potential, mistakes that lead to repeat expansions or contractions are relatively frequent.

There are multiple pathways that repair DNA damage that occurs within TNRs and other repetitive sequences. For example, nicks and gaps can be repaired by base excision repair (BER), or by transcription-coupled repair (TCR) within transcribed regions, both of which can generate TNR expansions (for recent reviews see (2, 5) and the review by Polyzos and McMurray in this issue). Damage that results in DSBs can be repaired by various types of end-joining, by annealing of processed ends, or by recombination-based mechanisms using either a sister chromatid or homolog as the template. In addition, recombination is a primary mechanism used in restarting stalled or collapsed replication forks and in repairing gaps left behind the replication fork. This review will summarize the current knowledge about the role of mitotic recombination in generating genomic changes within repetitive DNA. We will focus on structure-forming triplet repeats, but with comparisons to results found at other biologically relevant repeats and DNA structures.

DNA damage at expanded trinucleotide repeats is repaired by recombination

Deletion of genes required for recombination results in increased breakage of expanded TNRs, suggesting that recombination is normally required for healing these DNA breaks (6, 7). In replicating yeast cells, homologous recombination (HR) and ligase 4-dependent end joining (EJ) both contribute to the repair of breaks at CAG repeats (6). Genome-wide studies to identify novel genes preventing DSBs at GAA and Alu repeats identified several recombinational repair proteins as important, among them the nuclease Mre11, whose absence increased fragility of both repeats (7, 8). Additionally, dividing cells deficient in replication proteins exhibit cell cycle arrest and gross chromosomal rearrangements at Alu repeats because recombination intermediates cannot be resolved, which results in DSBs (8). Failure to heal breaks at expanded TNR repeats can have dire consequences for cells. Yeast cells that lack Rad52 or Ligase 4 and have expanded CAG repeat tracts undergo frequent cell cycle arrest and cell death (9).

Traditionally, DNA repair using recombination has been considered to be an error-free form of repair. However, in actuality, recombination can be highly mutagenic and a source of genomic instability (10–13). Though they are required for repair and cell health, both HR and EJ can be mutagenic when they occur within repetitive DNA, resulting in a loss (contraction) or gain (expansion) of repeat units (14). This is largely due to the challenges of replicating or aligning DNA across a repetitive region, especially one that has formed DNA secondary structures. These DNA structures are varied and include DNA hairpins (common in CAG/CTG and CGG/GCC repeats or inverted repeats), triplexes (formed by purine-rich repeats such as GAA/TTC) and G quadruplexes (for reviews see (5, 15–17)). Though the

structures are different, the common theme is that they impede DNA transactions so that replication and repair cannot proceed with fidelity within the repetitive sequence. This inaccurate repair can lead to the incorporation of errors that can range from the aberrant insertion/deletion of DNA bases, as seen in TNR repeat genetic diseases, to genomic rearrangements and loss of heterozygosity, which are commonly seen in cancers. Historically, misalignment of alleles during meiotic crossover was shown to be a mechanism for (GCN)_n repeat expansions that code for polyalanine tracts (18), but discounted as a mechanism for length changes of other TNRs, such as (CAG)_n repeat tracts encoding polyglutamine. However, these early studies focused on meiotic recombination and did not explore mitotic recombination as a potential mechanism for repairing DNA damage at TNRs and causing repeat instability. The following sections will delve into the various roles of recombination during DNA repair, how each contributes to genomic maintenance of repeat sequences, and the current knowledge of how recombination pathways result in repeat instability.

RECOMBINATION DURING REPLICATION RESULTS IN REPEAT INSTABILITY

Homology-dependent recombinational repair of forks stalled by DNA structures

Addition of repeat units by definition involves DNA synthesis. Incorporation of additional bases might arise as a result of strand slippage either during replication (19) or during fork restart (3). DNA structures formed by repetitive DNA sequences are impediments for DNA synthesis and can cause fork stalling, or gaps behind the replication fork if bypassed. GAA/TCC triplexes and GGC/CCG repeats strongly interfere with replication progression, acting as site-specific barriers (20–22). CAG/CTG repeats are much weaker barriers (23–26) but their replication generates joint molecules that likely represent both reversed fork and sister chromatid recombination intermediates (27, 28). Single stranded gaps occur when leading and lagging strand synthesis becomes uncoupled (reviewed in (29)), and pre-existing DNA nicks or gaps can become DSBs if replicated (5, 30, 31).

After a replication fork stalls at a DNA repeat structure, several types of fork restart can be envisioned (see (32, 33) for reviews on fork restart). First, unwinding of the DNA structure by a helicase may allow replication to continue without replisome dissociation, which would not lead to repeat instability unless slippage occurred (Figure 1C). Second, a fork reversal or template switch mechanism could be used to replicate through the DNA structure (Figure 1A, 1B). The outcome in terms of repeat contraction or expansion will vary depending on where the un-excised hairpin forms (template or nascent strand) and which hairpins are resolved. There are several possibilities for hairpin formation or mis-alignments during the fork restart process, which would likely involve the HR machinery (Figure 1A). Third, a break in the DNA could lead to an HR-dependent strand invasion, either on the same DNA template (broken fork repair (BFR), similar to what is drawn in Figure 1A but initiated from a break) or on a different template (ectopic break-induced replication (BIR; Figure 2). BIR is known to be a mutagenic process (10, 34, 35). Finally, repeat expansions are also known to occur due to hairpin impairment of Okazaki flap processing by the FEN1 endonuclease (Figure 1D; (36)).

Recently it was shown that expanded CAG repeats, which are natural replication fork barriers, result in the transient localization of chromosomes to the nuclear pore during S-phase (37). This relocation was dependent on replication, occurred in late S phase and was resolved by G2, and prevented repeat fragility. Yeast chromosomes exposed to both the alkylating agent MMS and the fork stalling drug hydroxyurea similarly relocate to the nuclear periphery (38). Interestingly, failure to relocate to the pore led to increased Rad52-dependent CAG repeat expansions and contractions. Taken together, these results suggest that relocation to the nuclear pore facilitates fork restart, and this may protect against DSBs and mutagenic Rad52-dependent repair (37, 39). Posttranslational modification of key repair proteins by sumoylation may be important in the re-localization and fork restart process, as deletion of the Slx5/8 SUMO-dependent ubiquitin ligase resulted in an increase in repeat instability and a decrease in nuclear localization of the expanded CAG repeat.

Recombination-mediated repeat instability at the replication fork is not unique to TNR repeats. In *S. pombe*, Swi1 promotes replication fork progression through telomeric repeats and prevents telomeric instability and aberrant recombination at telomeres (40). Additionally, in human cells, impaired replication of telomeric repeats results in fragile telomeres (41) and efficient replication requires the telomeric binding protein TRF1 and the helicases BLM and WRN to unwind G4 structures that can impede replication machineries (41, 42). Interestingly, replication in the context of HR repair or HR-dependent fork restart proceeds with less fidelity and more mutations than normal replication, even without the complication of copying DNA repeats (43–45). Recently, GAA repeats have been shown to induce mutagenesis up to 8 kb away from the repeat site in yeast, presumably through an HR-mediated repair event (22, 46–48). The authors hypothesize that a barrier to replication caused by a GAA secondary structure recruits the low-fidelity Pol ζ polymerase. DSB formation or fork stalling at the repeat leads to strand invasion of the homolog, where synthesis with Pol ζ leads to mutagenesis (46, 48). This repeat-induced mutagenesis (RIM) has also been observed for H-DNA and Z-DNA forming sequences introduced into mammalian cells (49–51). Taken together, expanded TNRs and other structure-forming repeat sequences are sites of replication fork collapses that are repaired by HR, and this repair may result in an increase in the mutation rate.

The role of helicases in replication of structure-forming repeats

Helicases have been shown to be important in preventing replication-associated repeat instability. One important helicase that helps to resolve repeat-induced replication fork stalls in yeast is the helicase Srs2 (Figure 1C). Using direct visualization of fork stalling *in vivo* by 2D gel electrophoresis, Srs2 has been shown to facilitate replication past a (CGG)₄₅ repeat that causes a barrier to replication via hairpin formation (52). Srs2 had no activity on replication barriers due to G-quadruplex structures or protein binding, thus it is specific to DNA hairpins. Srs2 function at stalled forks was unique among the helicases tested (Sgs1, Pif1, Rrm3), and was dependent on its helicase activity and its ability to interact with PCNA, but not on its Rad51 displacement motif. Srs2 can also unwind CAG hairpins *in vitro* and prevent expansions that occur during template switch (53, 54) and during sister-chromatid recombination (27). Recently, separation of function alleles were used to determine that Srs2 requires its helicase and PCNA interaction domains to protect against chromosome fragility,

for example by hairpin unwinding at the replication fork (Figure 1C), whereas its anti-recombinase function prevents repeat instability (Figure 1B) (28). These results further underscore the ability of replication-associated recombination events to generate repeat expansions.

In humans, unwinding of hairpins can be performed by the RTEL1 helicase as knockdown resulted in an increase in CAG expansion frequency to a similar level as knockdown of Rad18 and HLTF, homologs of yeast Rad18 and Rad5 (55). Strikingly, RTEL1 could substitute for Srs2 in yeast cells to prevent both CAG repeat fragility and instability (55). Though RTEL1 and Srs2 lack protein sequence homology and have opposite DNA unwinding polarities, these results indicate a strong functional conservation between the two enzymes with respect to CAG repeat replication. Both helicases are able to unwind CAG and CTG hairpin structures *in vitro*, though RTEL1 additionally acts at G4 DNA and is important in telomere maintenance (55–57). In *S. cerevisiae*, replication through G4 DNA is facilitated by the Pif1 helicase (Pfh1 in *S. pombe*) (58, 59). In a system used to detect gross chromosomal rearrangements due to G4 DNA Pif1 was shown to be important in preventing genomic instability, suggesting that Pif1-mediated unwinding of G4 DNA prevents error-prone replication or repair (60). Another helicase involved in TNR instability in mammalian cells is the 5′-3′ helicase FANCI, a member of the Fanconi Anemia pathway, which can unwind G4 DNA and CAG/CTG hairpins during replication to prevent repeat instability (61, 62). Unwinding non-B form DNA structures to prevent instability is a unique role for FANCI as other members of the Fanconi Anemia (FA) pathway did not exhibit similar roles (62). Similarly, yeast Sgs1 can unwind hairpins and G4 DNA *in vitro*, as can its human homologs WRN and BLM (52, 63–65). Sgs1 and WRN interact with Polδ, and thus are well positioned to unwind structures on the lagging strand template (Figure 1C). Indeed, deletion of Sgs1 led to a large increase in repeat contractions, consistent with such a role (27), and WRN was identified in a screen in HeLa nuclear extracts for proteins that were able to stimulate repair of a CTG hairpin on the template strand (66). The WRN helicase efficiently unwound CTG hairpins in this system to promote Polδ-catalyzed DNA synthesis across the gap and prevent deletions (66). Thus Srs2/RTEL1 and Sgs1/WRN may work together to resolve hairpins on different strands (Figure 1C). This mechanism could also be relevant during gap repair in non-dividing cells.

Template switch is a mechanism for trinucleotide repeat instability

Template switch repair is used to fill-in ssDNA gaps that result when the replication fork bypasses a fork-blocking lesion (Figure 1B) (reviewed by (67)). Given the ability of TNRs to form secondary structures that impair fork progression, it's not surprising that proteins required for template switch have been shown to be involved in the stability of repetitive sequences. Repair of post-replication gaps is dependent on ubiquitination of PCNA, and can be subdivided into two categories: translesion synthesis (TLS) and error-free post-replication repair (PRR) or template switching (reviewed by (68, 69)). The TLS branch is dependent on the PCNA ubiquitin ligases, Rad6 and Rad18, which together monoubiquitinate Lys164 of PCNA. Monoubiquitinated PCNA recruits translesion polymerases (e.g. Polζ or Polη) that synthesize across the lesion. Interestingly, mutations in the TLS polymerases had no effect on CAG repeat instability (70, 71) or GAA repeat stability (22) in wild-type budding yeast,

indicating that the TLS pathway is not a significant source of expansions. However recent data suggests that when replicative polymerases are compromised, some GAA repeat expansions do occur by a Pol ζ -dependent mechanism (22), as do short duplications initiated by small hairpins (72).

The error-free branch of PRR, template switch, requires additional ubiquitination action by Ubc13-Mms2-Rad5 E2–E3 ubiquitin ligases (mammalian HLTF/SHPRH), leading to a poly-ubiquitinated PCNA molecule (Figure 1B) (68, 69). Template switch further requires the action of the HR proteins Rad51, Rad52, Rad57 and Rad54 (73–75) (Figure 1B). The requirement for HR proteins is consistent with the use of the undamaged sister chromatid as a template for synthesis, though the precise mechanism is poorly understood (68, 69, 76, 77). In *S. cerevisiae*, spontaneous sister chromatid recombination (SCR) is proposed to occur because of gaps formed behind the replication fork (78), and is induced by both CAG repeats and inverted repeats (79). It is the propensity of these sequences to form secondary structures that is thought to impede replication and induce sister chromatid exchange, which was dependent on the presence of Rad52 (79).

Rad5, Rad18, HTLF, and PCNA ubiquitination have all been shown to inhibit (CTG)₁₃ or (CAG)₂₅ expansions in yeast and human cells (53, 55). At a longer (CAG)₈₅ repeat, deletion of *RAD5* also increased expansions 3-fold over wild-type (77), though at this repeat size, template switch can also *cause* repeat expansions (see chromatin section below). The role of Rad5 at short CAG repeats (e.g. less than 35 repeats) was epistatic to a deletion of *SRS2*, implicating a role for the Srs2 helicase, which was hypothesized to unwind 3' hairpins occurring during the template switch (53, 54). Altogether, these data indicate that CAG tracts induce both SCR and template switch events, and that in yeast, the Srs2 helicase is important to prevent instability during this process. This template switch event must play an important role in repairing TNR-related gaps, because in its absence (e.g. in *rad5* or *rad18* strains) expansions occur, by an alternative unknown pathway.

In contrast to short (CAG)_{13–25} repeats, ATTCT and GAA repeat expansions are promoted by the presence of Rad5 in yeast (80, 81). ATTCT repeats, which expand to cause SCA10, do not form structured DNA, but instead are DNA unwinding elements (82). In addition, a *rad5* mutant displays decreased ATTCT fragility (80), suggesting that template switching events can lead to chromosomal fragility at these repeats. Rad5-dependent expansions of the GAA repeat were proposed to occur by a template switching mechanism in which the GAA repeat expansions arise from dissociation of the leading strand from its normal template and aberrant copying from the newly synthesized Okazaki fragment (81). This model predicts that copying would not be dependent on DNA structure per se, but would be facilitated by pausing of the replication fork (80, 81).

How can one explain the different dependencies on Rad5 observed for different types and sizes of repeats? We previously proposed a model to account for the somewhat contradictory roles of proteins in the template switch pathway on repeat instability (5). For longer or more “slippery” repeats (GAA, ATTCT, longer CAGs), the fork stall could be strong enough to mediate a template switching event directly at the stalled fork, hypothesized to be facilitated by Rad5 (Figure 1A). There is experimental evidence for fork reversal at both CAG and

GAA repeats by direct visualization of replication intermediates by 2D gel electrophoresis and electron microscopy (27, 28, 83, 84). For CAG repeats, the size needed to produce a fork stall stable enough to be visualized on a 2D gel is approximately 90–100 CAGs (26, 28). After the stall there are two models for generating expansions: for hairpin-forming sequences, fold-back of the leading strand would allow DNA synthesis from the leading strand, resulting in a repeat expansion upon fork restart (Figure 1A, right pathway) (first proposed by (3)). For non-hairpin forming sequences, copying off of the lagging nascent strand provides the extra DNA synthesis, as proposed in (81) for large-scale GAA expansions (see (85) for review). On the other hand, a single hairpin is more likely to be bypassed, leading to a post-replicative template switch that initiates from a gap, and looks more like SCR (Figure 1B) (27, 77). This latter event may be more common for mid-length CAGs, above the expansion threshold of 35 repeats but still less than the size needed to produce a stable fork stall (e.g. ~45–85 repeats). For very short (CAG)_{13–25} tracts, post-replicative hairpin unwinding by the Srs2 protein could be sufficient most of the time, with less engagement of the full recombination pathway (86). This idea of length-dependent differences is supported by the fact that Tof1, a subunit of the replication-pausing complex, protects against instability for both GAA and ATTCT repeats (80, 81). Thus the replication pausing complex may act to limit the template switching events at stalled forks that can allow for repeat expansions.

Chromatin modifications influence repair fidelity during template switch

The chromatin environment at gaps also contributes to efficient repair by error-free template switch (reviewed in (87, 88)). The absence of Anc1, a subunit of the chromatin modifying complexes INO80, SWI/SNF, and NuA3, leads to an increase in (CAG)₂₅ expansion frequency that is equivalent to the increase in *rad5* and *mms2* mutants (53, 89). Histone H4-K16 acetylation by the chromatin remodeling complex NuA4 is specifically enriched at expanded CAG repeats in yeast, and is required for high-fidelity template switch and (CAG)₈₅ repeat maintenance: in the absence of proper histone modification, Rad5 and Rad52-dependent expansions occur more frequently (Figure 1B) (77). Further, Rsc2, an acetyl-lysine binding subunit of the RSC chromatin remodeler, was recruited to the CAG repeat coincident with the peak in H4-K16 acetylation, suggesting a recruitment mechanism for this remodeler and a role in promoting template switch without expansions (Figure 1B) (77). Thus, although template switch is a protective pathway, it can be a source of repeat length changes if it occurs without accessory factors such as chromatin remodelers or modifiers (RSC, H4-K16ac, Anc1) that allow it to occur with fidelity.

RECOMBINATION DURING DSB REPAIR DRIVES REPEAT INSTABILITY

HR-dependent instability can cause large repeat expansions

Structure-forming DNA, including expanded CAG, CGG, GAA, and ATTCT repeats as well as palindrome-forming sequences, are natural fragile sites that cause chromosomal DSBs (reviewed in (5, 15, 90–92)). Consistently, expanded GAA/TTC repeats, Alu repeats, and internal telomeric repeats stimulate mitotic crossovers in yeast (93–95) and recombination in *E. coli* (96–98). In yeast, the effect of DSB repair by HR on CAG repeat stability was assessed directly by induction of a DSB and selection for repair events that used an

ectopically provided (CAG)₉₈ tract for repair (99). This experiment showed that repeat instability occurred during HR-mediated DSB repair, resulting in a much higher percentage of expansions (13%) and contractions (30%), than a control that did not undergo break induction (0% expansions, 10% contractions). Mre11, an endonuclease important in generating a single-stranded 3' end and initiating HR, was required for efficient repair, and the proportion of expansions increased in its absence (99, 100). In a second study without an induced break, (CAG)₇₀ expansions were also increased 11-fold in the absence of Mre11 with a bias to large expansions, pointing to a key role for this protein in preventing repeat instability (6). A nuclease-dead allele of Mre11 had a lesser effect, arguing that the structural function of the MRX complex was the most important factor in preventing expansions. These expansions were suppressed in the absence of Rad52, and therefore occurred through aberrant recombination (6). Mre11 has yet a different role at short (CTG)₂₀ repeats, as the presence (not absence) of the protein drives expansions during template switch, independent of both its nucleolytic function and its role in HR (101). At DSBs, one of the functions of Mre11 is to associate with Rad50 and Xrs2/Nbs1 to initiate DSB end processing (Figure 2A) and to activate the DNA damage checkpoint kinases Tel1/ATM and Mec1/ATR which are required for efficient DSB repair (102). At the expanded CGG repeat fragile site in a Fragile X mouse model, loss of one *Atm* allele increased the expansion frequency in both males and females (103), and similarly loss of one copy of the *Atr* gene was associated with an increased risk of expansion on maternal transmission (104). Additionally cutting by the CRISPR-Cas9 nickase at expanded CAG/CTG repeats in an ATR-deficient cell line resulted in increases in expansions and contractions (105). Although in these mammalian cells the mechanism creating expansions is not known, it is consistent with the data from yeast and bacteria that synthesis-dependent HR from a DSB that is lacking proper MRX/MRN scaffolding is a mechanism that can produce both repeat expansions and contractions (Figure 2E).

One subpathway of HR is BIR, a highly mutagenic form of HR where a DSB end invades a region of homology and replicates for several kilobases, potentially to the telomere end (10, 34, 35). Pol32, a non-essential subunit of Polδ, as well as the helicase Pif1 are essential for BIR mediated repair in yeast (106, 107). Replication during BIR is highly mutagenic as it proceeds via a migrating bubble of conservative DNA synthesis (108, 109). Recent work has shown that large-scale CAG/CTG expansions (defined as addition of more than 20 repeats) of a (CAG)₁₄₀ repeat tract in a yeast model utilizes traditional HR machinery as well as Pol32 and Pif1, implicating BIR as a mechanism for generation of large-scale repeat expansions (Figure 2F) (110). These large-scale expansions depended on replication, therefore they could also be generated though a mechanism similar to broken fork repair (111) or HR-dependent fork restart that occurs at protein barriers to replication (33) (Figure 1A). Intriguingly, such large-scale CAG/CTG expansions occur in replicating cells of the pre-meiotic male germline in humans with pre-mutation Huntington's disease alleles and in a DM1 mouse model (112, 113). Expansions that occur in non-dividing cells could also arise by BIR, as BIR readily occurs in non-dividing cells; in this case DSBs could be generated during mismatch repair or transcription rather than by replication (7, 44). At critically short telomeres, BIR may serve a similar function in extending difficult to replicate sequences, as BIR has recently been shown to be a mechanism for alternative lengthening of telomeres

(114, 115). It was hypothesized that PCNA can load at alternative structures with recessed 3' ends to recruit the Polδ polymerase for telomere synthesis (114).

Despite the strong evidence for HR-induced repeat instability in yeast, in the above cited DM1 mouse model, loss of the Rad54 gene product did not significantly suppress CTG instability, and the absence of the mouse Rad52 gene decreased the size of expansions, but did not eliminate them (116). These results have been interpreted to mean that HR has no effect on CAG/CTG instability in mammalian cells. However, because elimination of HR in mammalian cells is lethal, the Rad52^{-/-} and Rad54^{-/-} lines used in the DM1 mouse studies do not eliminate HR: Rad52 knockouts exhibit only a slightly reduced HR frequency and are not hypersensitive to DSB-inducing agents; Rad54 is not an essential HR protein, but serves to facilitate chromatin remodeling during HR (117, 118). The fact that the mean size of expansions was significantly decreased in the Rad52^{-/-} DM1 mouse model supports the idea that rare large expansions could actually be arising during HR in these mice. Due to the technical difficulties of eliminating HR in mouse models, the role of HR in repeat instability in mammals remains to be fully assessed. Study of HR in mammalian cell culture models where repeat expansions can be detected would be one way to bridge this gap.

Repair of DSBs by single-strand annealing (SSA) and end-joining mechanisms (NHEJ, MMEJ) can cause repeat instability, predominantly contractions

In addition to HR, DSBs occurring at fragile repeat sequences can be repaired by various types of end joining pathways. At an expanded CAG repeat in yeast, deletion of both Rad52 and Dnl4 (Ligase 4) led to an additive level of fragility (6). Also, breaks at Z- and H-DNA forming sequences are repaired by NHEJ in mammalian cells, causing deletions (49, 50, 90, 119)(Figure 2A). In the absence of Dnl4, CAG repeat contraction frequency is significantly increased in yeast and remains so in *dnl4 rad52* cells, indicating that these contractions are not occurring through HR (6). The absence of HR proteins Rad52, Rad51 and Rad54 also increased repeat contractions ~2.5-fold (6). Because broken DNA ends undergo greater resection in both *rad52* and *dnl4* backgrounds, end processing followed by SSA between repeats is an attractive mechanism to explain these contractions; another possibility is microhomology-mediated end joining (MMEJ), which uses short stretches of homology (5–25 bp) to align the broken DNA ends and promote ligation to resolve the lesion (120)(Figure 2B, C). Indeed, in a yeast system where breaks are induced within a CAG repeat, contraction via SSA is a prominent outcome (121), and induction of a DSB by a CAG/CTG-specific TALEN induced 100% contractions in a highly specific manner (122). The same mechanism appears to be operating in human cells, as a CAG-targeted DSB by a zinc finger nuclease also induced frequent contractions (123). Additionally, a nickase version of CRISPR-Cas9 directed toward CAG or CTG repeats resulted in increased large-scale contractions that depended on activation of the ATM branch of the DNA damage response to promote repair via SSA (105). This makes nuclease-directed cleavage of TNRs an attractive method for inducing contractions, which could potentially be used therapeutically (124).

Although more rare, repeat expansions also appear to occur during some end joining events (Figure 2A, 2B). In the yeast study by Sundararajan et al. (6), more than half of the expansions in a *rad52* strain were eliminated in the *rad52 dnl4* background, indicating

that an end-joining pathway contributes to expansions in this system. In addition, CRISPR-Cas9 induced DSBs resulted in an increase in both CAG repeat expansions and contractions, further supporting the possibility that end-joining can result in repeat expansions (105). The observation of large expansions created during repair of DSBs by HR or end-joining repair is intriguing, as large expansions that occur during maternal transmission of the DM1 CTG and Fragile X syndrome CGG repeats appears to happen during oogenesis. A prominent stage of oogenesis is meiosis, where many breaks occur and are repaired. In yeast, breaks occur frequently at CAG repeats during meiosis and are repaired to give both expansions and contractions (125, 126). Similarly, while MMEJ favors contraction events, it can also result in templated insertions during Pol Θ -dependent fill-in in metazoans (120, 127). Using human proteins in a reconstituted *in vitro* system, it was revealed that Pol β - and Pol λ -dependent microhomology-mediated strand annealing promotes CAG expansions, and the frequency of these events is limited by the 9-1-1 (Rad9/Hus1/Rad1) DNA damage checkpoint complex (128).

Homologous recombination is a mechanism for genome rearrangement

Repetitive DNA sequences can present challenges to maintaining genomic stability. Genomic changes that result from breakage at DNA structures can range from small-scale insertions/deletions to loss of heterozygosity or other large-scale genomic rearrangement, which is a hallmark of cancer genomes (11, 129). Given that structure-forming repeats break at a higher frequency than non-repetitive DNA, they are ideal DNA substrates for the machineries that drive genomic rearrangement.

DNA hairpins formed by some TNRs and long inverted repeats are thought to be cleaved at the base to form DSBs that have hairpin-capped ends (130). These ends must then be processed by the MRX/MRN complex in concert with Sae2/CtIP in order to promote recombination and repair fidelity. In the absence of Sae2 or the MRX complex, recombination is inhibited at inverted Alu repeats, which leads to inverted duplications (94). Another factor that is key to preventing genomic rearrangements is replication protein A (RPA), which coats the 3' ssDNA that is revealed during end resection of DSBs (Figure 2C) and thus prevents DNA secondary structure formation by intramolecular base pairing at the 3' overhang. In the absence of RPA, the 3' DNA overhang can form hairpin-capped ends that recruit Pol32, suggesting that DNA synthesis is required to synthesize a foldback structure (131). The coordination between the MRX complex, Sae2 and RPA is important as it has been demonstrated that loss of this coordination results in palindromic gene amplification and gross chromosomal rearrangements of the type that are commonly seen in cancer cells (131, 132).

Replication barriers are also a driver of genome rearrangement by promoting template switch between ectopic sequences (133, 134). Repetitive sequences can serve as ideal homologies for template switches to occur. Indeed, simultaneous transcription and replication through a potential G4 DNA-forming sequence resulted in an increase in gross chromosomal rearrangements, where the initiating breakpoint was located within 4 kb of the G4 forming sequence (135). These rearrangements depended on topoisomerase I (Top1) to ease torsional stress associated with transcription through the G4 motifs (135). Taken

together, DNA breaks that occur at structure forming sequences must be processed in such a way that DSB repair proteins have access to the broken end, and impairment of end processing can result in large-scale genome rearrangements.

CONCLUSIONS

Many repetitive DNA sequences form secondary structures that serve as constant challenges to DNA replication and repair machineries, resulting in stalled forks, nicks, gaps, and DSBs. Recombination is an important pathway to repair these lesions, and serves as a powerful guardian of the genome. However, recombinational repair at repetitive DNA tracts can be tricky as it can also be a source of mutation, including repeat expansions and contractions. This dichotomy between recombination being a high fidelity form of repair and also a source of instability is important, as it has challenged the canonical idea that HR is error free. Through the study of repeat instability, the scientific community has learned how recombination proteins such as nucleases, helicases, and chromatin remodelers navigate secondary structures in an effort to preserve genomic integrity. A better understanding of how recombination impacts repeat instability could have far-reaching impacts for both inherited genetic disorders, where repeat length correlates to disease severity, and cancers, where structure-forming repeats may initiate genomic instability. Future research will be imperative in dissecting the steps of HR and the exacerbating cellular conditions that drive mutation at repetitive DNAs.

Acknowledgments

Research in CHF's laboratory is supported by the National Institutes of Health (award P01GM105473 and R01GM122880), National Science Foundation (MCB1330743), and Tufts University.

Abbreviations

TNR	Trinucleotide repeat
HR	Homologous recombination
EJ	End joining
DSB	Double stranded break
SCR	Sister chromatid recombination
NHEJ	Non homologous end-joining
MMEJ	Microhomology-mediated end-joining
DM1	Myotonic Dystrophy type I
HD	Huntington's Disease
SCA	Spinocerebellar ataxia
BER	Base excision repair

TCR	Transcription-coupled repair
TLS	Translesion synthesis
PRR	Post-replication repair
SSA	Single strand annealing
G4 DNA	G-quadruplex forming DNA

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Figure 1. Structures formed by DNA repeats cause replication fork stalling and template switch
 During replication, leading or lagging strand hairpins may cause fork stalling. To recover from these fork stalls, there are two recombinational mechanisms that can be employed: first, by restarting the replication fork, which may involve fork reversal (A), and second, by structure bypass (B). For simplicity, fork reversal is only shown for a leading strand hairpin and structure bypass for a lagging strand hairpin, though either mechanism could be triggered by each barrier. Alternatively, helicase unwinding of the DNA structures could allow fork restart without recombination (C). **A.** 1) Fork reversal and template switch to the

lagging strand may occur without hairpin resolution; the hairpin could then be bypassed during fork restart resulting in a repeat contraction. 2) If fork reversal results in the fold-back of the leading strand (or excess synthesis from the lagging strand without hairpin formation), DNA synthesis would result in an expansion upon replication fork restart. Both mechanisms of fork restart are predicted to utilize recombination proteins such as Rad51 for strand invasion. **B.** If DNA structures are bypassed, ssDNA gaps must be filled in after replication by using the sister chromatid as a template. Repair of post-replication gaps is dependent on poly-ubiquitination of PCNA by Ubc13-Mms21-Rad5 to initiate the template switch, which is mediated by recombination proteins Rad51, Rad52, Rad55/57 and Rad54 for strand invasion. In addition, the histone H4 acetyltransferase NuA4 and the RSC chromatin remodeler are required for efficient sister chromatid recombination and accurate repair within repetitive DNA (77). Upon strand invasion into the sister chromatid, repeat instability can occur by two mechanisms. 1) strand invasion can result in bypass of the DNA structure which will result in a contraction. 2) Alternatively, D-loop extension during template switching may be prone to replication slippage leading to a repeat expansion. Repeat expansions and contractions could also occur by misalignment of repeat units during either (A) or (B) (not shown). **C.** Helicases can prevent repeat instability by unwinding DNA structures, which will allow replication to continue without replisome dissociation, or prevent hairpin formation during template switch (e.g. as in B.2). The helicase Srs2 is a 3'-5' helicase that can unwind hairpins to facilitate fork progression through hairpin structures. This function is dependent on PCNA interaction but independent of its role in inhibiting recombination. Sgs1/WRN is another 3'-5' helicase that can unwind hairpins as well as G4 DNA. Sgs1/WRN interacts with Polδ and is thought to unwind DNA structures on the lagging strand template during replication. RTEL1 and FANCI are 5'-3' helicases that can also unwind DNA structures like hairpins and G4 DNA sequences. **D.** During replication, structure formation on the lagging strand can impede the 5' flap endonuclease activity of FEN1/Rad27 which can lead to inefficient or "alternative" flap cleavage and repeat expansions.

Figure 2. Double stranded break repair can drive repeat instability

DSBs occurring within repetitive DNA may be repaired by multiple pathways. **A.** DSB ends are initially bound by the MRX/MRN complex which can mediate either end-joining or HR pathways. Post initial processing by MRX/N, repair via NHEJ may occur. NHEJ requires the Ku heterodimer and Ligase 4 to re-ligate two ends of a DSB that has undergone minimal end processing. NHEJ can result in TNR contractions or expansions **B.** Sae2 interacts with MRX to initiate end processing and resection; structure-forming repeats may have hairpin capped ends that require additional processing. After initial processing by the MRX/N complex and

Sae2/CtIP, one mechanism for repair of breaks at repeats is MMEJ which can result in TNR contractions, and perhaps expansions. **C.** After initial processing, long range resection occurs by the 5'-3' exonuclease Exo1 or the RecQ helicase Sgs1 (WRN/BLM) in concert with the nuclease Dna2. After extensive resection by exonucleases, repair can occur via SSA, which requires Rad52 and favors repeat contractions. **D.** To prevent secondary structure formation, the RPA complex binds to the resected ssDNA. Rad52 mediates the removal of RPA and the nucleation of Rad51 bound filaments; accessory proteins Rad55/57 form co-filaments with Rad51 which increases the stability of the filament (136). **E.** The Rad51 filaments initiate the homology search and invade homologous DNA which initiates D-loop formation; hairpin formation during D-loop extension provides an opportunity for slippage and repeat expansions. Upon completion of DNA synthesis, repair via SDSA results in re-ligation with the original chromosome end which can result in contractions or expansions depending on the location of hairpin formation and alignment of repeat units. **F.** For break induced replication, polymerase δ along with Pol32 and Pif1 binds to the D-loop and proceeds with long range conservative DNA replication, which can result in extensive polymerase slippage and large scale TNR expansions