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The Rtt109 histone acetyltransferase facilitates error-free replication to prevent CAG/CTG repeat contractions

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Abstract

Lysine 56 is acetylated on newly synthesized histone H3 in yeast, *Drosophila* and mammalian cells. All of the proteins involved in histone H3 lysine 56 (H3K56) acetylation are important for maintaining genome integrity. These include Rtt109, a histone acetyltransferase, responsible for acetylating H3K56, Asf1, a histone H3/H4 chaperone, and Hst3 and Hst4, histone deacetylases which remove the acetyl group from H3K56. Here we demonstrate a new role for Rtt109 and H3K56 acetylation in maintaining repetitive DNA sequences in *Saccharomyces cerevisiae*. We found that cells lacking *RTT109* had a high level of CAG/CTG repeat contractions and a two-fold increase in breakage at CAG/CTG repeats. In addition, repeat contractions were significantly increased in cells lacking *ASF1* and in an *hst3Δhst4Δ* double mutant. Because the Rtt107/Rtt101 complex was previously shown to be recruited to stalled replication forks in an Rtt109-dependent manner, we tested whether this complex was involved. However, contractions in *rtt109Δ* cells were not due to an inability to recruit the Rtt107/Rtt101 complex to repeats, as absence of these proteins had no effect on repeat stability. On the other hand, Dnl4 and Rad51-dependent pathways did play a role in creating some of the repeat contractions in *rtt109Δ* cells. Our results show that H3K56 acetylation by Rtt109 is important for stabilizing DNA repeats, likely by facilitating proper nucleosome assembly at the replication fork to prevent DNA structure formation and subsequent slippage events or fork breakage.

Keywords

trinucleotide repeat; Rtt109; H3K56 acetylation; replication; nucleosome assembly; genome instability

1. Introduction

Microsatellites are DNA sequences of 1-6 nucleotides that are repeated multiple times and are abundant in most genomes [1]. About 3% of the human genome is composed of microsatellites, which are present in both coding and non-coding regions [2]. Nearly 30 human neurological disorders are caused by expansions of these simple DNA repeats, many of which are trinucleotide repeat (TNR) diseases, including Huntington's disease, fragile X syndrome and

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myotonic dystrophy type 1 [3]. Previous studies have established that the occurrence of these diseases is repeat length-dependent, and longer repeats are associated with increased disease severity and earlier age of onset [3].

In order to understand the molecular mechanism of triplet repeat instability, studies have been done in *E. coli*, yeast, mouse, and mammalian cells containing repetitive sequences [1,3]. Due to the unusual secondary structures that TNRs can form, expansions and contractions occur during processes in which single-stranded DNA is produced, such as DNA replication, repair, and transcription [3]. CAG/CTG repeats are natural replication pause sites on plasmids in yeast or bacterial cells and are sites of replication fork reversal on a yeast chromosome and *in vitro*, indicating that they pose a challenge for replication fork progression [4-7]. Indeed, disruption of replication through CAG/CTG repeats can cause repeat instability [8,9]. TNR sequences can also interfere with DNA repair: base excision repair, nucleotide excision repair and mismatch repair can all contribute to CAG/CTG repeat expansions [10]. Studies in yeast and *E. coli* indicate that mistakes during double-strand break (DSB) repair are another mechanism of generating repeat instability [11]. For example, both contractions and expansions were observed when CAG/CTG repeats were used as a template to repair an induced DSB [12]. Also, a Rad52-dependent process has been shown to be responsible for repeat expansions and contractions that occur in yeast cells lacking either the Mre11-Rad50-Xrs2 (MRX) complex or the Srs2 helicase [6,13].

H3K56, which is located in the globular domain of histone H3, was found to be acetylated by mass spectrometry, and this modification has been shown to exist in yeast (both *S. cerevisiae* and *S. pombe*), *Drosophila*, and human cells [14,15]. Acetylation of H3K56 is catalyzed by the histone acetyltransferase (HAT) Rtt109 in budding yeast and CBP/p300 in *Drosophila* and human cells [16-19]. In addition to Rtt109, binding of histone chaperone Asf1 to histones H3 and H4 promotes efficient acetylation of H3K56 [20]. Deacetylation of H3K56 depends on two histone deacetylases (HDACs): Hst3 and Hst4 in yeast, and SIRT1 and SIRT2 in mammalian cells [15,21]. Interestingly, cells lacking an acetyltable lysine at H3K56 show hypersensitivity to a variety of DNA-damaging agents, such as methyl methanesulfonate (MMS), hydroxyurea (HU), and camptothecin (CPT), which suggests a critical role for H3K56 acetylation in maintenance of genome stability [21].

Several lines of evidence indicate that H3K56 acetylation plays an important role in DNA replication. Acetylation of H3K56 is a mark of newly synthesized histone H3 and the acetylation level increases during S phase [22]. Additionally, histone H3 acetylated on K56 is incorporated into replicating DNA by chromatin assembly factor 1 (CAF-1) and the histone chaperone Rtt106 [23]. This modification increased the binding affinity of CAF-1 and Rtt106 for H3 *in vitro* and *in vivo*, and enhanced replication-coupled nucleosome assembly in cell extract experiments [23]. Finally, both Rtt109 and Asf1 are required for the association of replication proteins Rfc3, PCNA, and Pole on a stalled replication fork, and this process is H3K56 acetylation-dependent [24,25]. Taken together, these data show that H3K56 acetylation maintains genome stability during replication by enhancing nucleosome assembly on newly replicated DNA and by stabilizing the replisome on stalled replication forks. Acetylation of H3K56 is also required for chromatin reassembly during repair of an HO-induced DSB [26]. Strains lacking Asf1 or Rtt109 no longer reassemble their chromatin at the DSB site even after proper DNA repair, and this defect can be rescued by using the acetylated mimic H3K56Q [26].

Notably, repetitive sequences can affect nucleosome assembly, and CAG/CTG repeat sequences are one of the strongest known natural nucleosome positioning elements [27]. Therefore, nucleosome repositioning or histone modification during DNA processes such as replication or repair could be particularly important at long CAG/CTG repeat tracts. At several

loci, highly expanded TNRs are associated with a silent chromatin state that affects transcription of nearby genes and contributes to the disease phenotype [27]. However, the role of histone modifications on the stability of repetitive DNA sequences has not been directly investigated.

Here, *S. cerevisiae* strains containing 85 CAG/CTG repeats on a yeast artificial chromosome (YAC) were used to examine the role of H3K56 acetylation in maintaining repeat stability. We found that contractions of CAG/CTG repeats were significantly increased in strains lacking *RTT109* and other genes involved in H3K56 acetylation and deacetylation. Our data support a model whereby defects in replisome integrity and inefficient chromatin assembly on newly replicated DNA allows DNA structure formation on the template strand, leading to repeat contractions.

2. Materials and Methods

2.1 Yeast strains and plasmids

Yeast strains used in this study (Table S2) were made by one-step gene replacement [28] in a BY4705 yeast strain background containing YAC CF1 with either no CAG repeat (CAG-0) or 85 CAG repeats (CAG-85) [8,29]. *rtt101Δ* and *rtt107Δ* strains were from the Stanford deletion set in the isogenic BY4742 background [30] and the YAC CF1 was introduced by cytoduction as described in Callahan et al. 2003 [8].

To test the effect of mutating the H3K56 residue, the *HHT2-HHF2* locus (one of two H3-H4 gene loci in yeast) was first replaced by the *KanMX6* gene [29] in yeast cells containing YAC CF1 with CAG-85. Plasmids with wild-type or mutated *HHT2* (H3 copy 2 gene) and wild-type *HHF2* (H4 copy 2 gene) provided by the Kirchmaier lab [31] were then transformed into the *hht2Δ-hhf2Δ* cells [32]. Lastly, the other H3-H4 gene locus (*HHT1* and *HHF1*) on yeast chromosome II was replaced by *His3MX6* [29].

2.2 CAG stability assay

For each yeast strain, four individual colonies with a starting tract length of CAG-85 were inoculated separately in YC-Leu liquid cultures. After 16-hour incubation at 30°C, a dilution of each culture was plated onto YC-Leu-Ura for single colonies. Approximately 20 daughter colonies from each plate, at least 80 daughter colonies total, were tested for repeat tract length by colony PCR. CAG repeat sequences were amplified with primers newCAGforprimer (CCTCAGCCTGGCCGAAAGAAAGAAA) and newCAGrevprimer (CAGTCACGACGTTGT AAAACGACGG) using Taq polymerase (Phoenix Lab, Tufts Medical Center). Amplified products were separated on a 2% Metaphor gel (Lonza) for 80 minutes at 80 V, and repeat size was estimated by comparison with Hyladder 1 kb marker (Denville Scientific INC.). This method can detect tract length changes of 3 repeats (9 bp) or greater.

2.3 CAG fragility assay

A single starting colony from each strain background with the correct tract length as determined by colony PCR was suspended in 1ml YC-Leu media and used to inoculate 5 or 10 separate cultures. After growth in liquid media for 6-7 doublings at 30°C, cultures were plated onto FOA-Leu and YC-Leu plates as previously described [13]. The rate of FOA resistance was calculated by the method of the maximum-likelihood using SALVADOR software [33]. Assays were repeated 3-6 times for each tract length in each strain background and statistical significance was determined by a pooled variance *t* test using Systat software.

3. Results

3.1 CAG/CTG repeat contractions are significantly increased in cells with abnormal H3K56 acetylation

Since long CAG/CTG repeats interfere with DNA replication, and Rtt109 and Asf1-dependent H3K56 acetylation is important for replisome integrity at a stalled replication fork [24,25], we tested whether proteins involved in H3K56 acetylation and deacetylation help to protect CAG/CTG repeats from tract length changes. An expanded CAG/CTG repeat originally from a myotonic dystrophy type 1 patient was cloned into a YAC, orientated such that the CAG repeat tract is on the lagging strand template during replication [8]. The repeat is less contraction-prone and more expansion-prone in this orientation compared to when the CTG repeat tract is on the lagging strand template, although contractions still predominate in yeast [11]. Cells containing 85 CAG/CTG repeats on the YAC (abbreviated as CAG) were used to study repeat instability in mutants that affect H3K56 acetylation.

The frequency of CAG repeat contractions was significantly increased in cells lacking *RTT109* or *ASF1*, where no acetylation of H3K56 is detectable [16-18]. The *rtt109Δ* strain showed 36% contractions compared to 11% for a CAG-85 tract in the wild type (WT) background, a 3.3-fold increase ($p=2.8 \times 10^{-8}$; Fig. 1A). CAG contractions were also increased about two-fold in *asf1Δ* cells. Expansions were slightly increased in both mutant backgrounds, but the frequency was not significantly different from the WT strain (Table S1). To test whether contractions were occurring through the same pathway in *rtt109Δ* and *asf1Δ* mutants, a *rtt109Δasf1Δ* double mutant was made. Repeat contractions were significantly increased in the *rtt109Δasf1Δ* double mutant compared to the WT strain, but not different from either of the single mutants by statistical analysis (Fig. 1A). These results indicate that a shared function of Rtt109 and Asf1, such as H3K56 acetylation, is important for keeping CAG repeats stable. However, they do not rule out the possibility that Rtt109 also has an additional Asf1-independent role, since the difference between the contraction frequencies for these two mutants was significant ($p=0.02$). To gain further insight into the contraction phenotype, the pattern of repeat deletions in both *rtt109Δ* and *asf1Δ* mutants was compared to that in wild-type strains. As in wild-type, contractions ranged from deletion of 3 repeats (the limit of the assay) to deletion of almost the entire repeat tract (Figure 2). Large deletions were common in the mutants: the average deletion size was 51 and 52 repeats for *rtt109Δ* and *asf1Δ* mutants respectively, compared to 44 repeats for WT. These data support the interpretation that deletions are occurring by the same pathway in the two mutants.

Repeat stability was also tested in cells with H3K56 hyperacetylation. Both Hst3 and Hst4 are HDACs responsible for removing the acetyl group from H3K56 and function redundantly in yeast cells [34]. Celic et al (2006) showed that H3K56 acetylation is slightly increased in *hst3Δ* strains but no increase was detected in *hst4Δ* cells [34]. However in the *hst3Δhst4Δ* double mutant, almost all of the histone H3K56 in the cell is acetylated and cells are quite sensitive to MMS and HU [34]. Therefore, Hst3 and Hst4 have a compensatory function in yeast, with Hst3 being slightly more important for H3K56 deacetylation. For CAG repeat stability, an *hst4Δ* strain did not show increased CAG contractions when compared with the WT strain, and there was a subtle increase of contractions detected in an *hst3Δ* strain but not to the level of statistical significance (Fig. 1A). However, in the *hst3Δhst4Δ* double mutant, CAG repeat contractions were significantly increased to 23%, a 2.1-fold increase over the WT frequency (Fig. 1A). These data reveal that a proper level of H3K56 acetylation is important to prevent CAG repeat contractions, as the frequency of CAG repeat contractions corresponded to the level of the H3K56 deacetylation defect. Notably, either hyper or hypoacetylation on H3K56 resulted in increased repeat instability.

To directly test if CAG repeat contractions were caused by altered H3K56 acetylation, CAG repeat stability was analyzed in strains expressing histone H3 with lysine 56 replaced with either arginine (H3K56R), which cannot be acetylated, or glutamine (H3K56Q), which mimics permanent acetylation. The CAG-85 containing YAC was introduced into strains in which both genomic loci encoding histone H3 were deleted and either WT H3 or mutant H3 (H3K56R or H3K56Q), was expressed from a plasmid. The strain with WT H3 expressed from the plasmid showed a higher basal level of CAG repeat contractions (15.9%) than strains with WT H3 expressed from the chromosome (11%) (Fig. 1A and 1B). H3K56Q, the acetylated lysine mimic mutant, did not show any increase in repeat contractions, but exhibited somewhat increased repeat expansions (Fig. 1B; Table S1). Cells with the unacetylated mimic, H3K56R, had 21.3% contractions, the same level as an *asf1Δ* mutant (Fig. 1A and B). However, because of the higher basal level of contractions in the plasmid-bearing strain, this represents a 1.3-fold increase in contractions over the WT H3 strain, which is not as high as was found in *rtt109Δ* (3.3-fold) or *asf1Δ* (1.9-fold) strains (Fig. 1A and B). Previous studies showed that cells expressing H3K56R were less sensitive to damaging agents than *asf1Δ* cells on low levels of MMS or CPT, which indicates that the K56R mutant might not function identically to unacetylated H3K56 [35]. Therefore, a phenotype equal to or slightly less severe than an *asf1Δ* strain, which is what we observed, is a reasonable expectation for the H3K56R mutant. Overall, these results are consistent with the conclusion that regulation of H3K56 acetylation plays a role in preventing CAG repeat contractions, but may not account for the full effect of deleting the *RTT109* gene.

3.2 CAG repeat contractions are not Rtt107 or Rtt101 dependent

Rtt107 and Rtt101 are found in a complex and have been shown to be involved in the same genetic pathway as *RTT109* and *ASF1* by a genetic interaction map (E-MAP) created by comparing growth rates of single and double mutants [36]. Both Rtt107 and Rtt101 are also recruited to chromatin in response to replication fork stalling by MMS, HU or CPT, and this recruitment depends on Rtt109 and Asf1 proteins [37]. One possible explanation for the increased CAG repeat contractions in *rtt109Δ* cells could be a failure to recruit the Rtt107/Rtt101 complex to the repeats during replication. To determine if this was the case, the stability assay was performed in *rtt107Δ* and *rtt101Δ* cells. Interestingly, neither showed increased CAG repeat instability (Fig. 1C), which indicates that the contractions found in the *rtt109Δ* strain are not due to the absence of Rtt107/Rtt101 recruitment to replication forks.

3.3 Contractions in the *rtt109Δ* strain are partially dependent on Rad51- and Dnl4-dependent pathways

In the absence of *RTT109*, replisome integrity is significantly affected, which might lead to DSBs [25]. We envisioned that repeat contractions could either occur due to strand slippage or other events at the replication fork, or that CAG repeats could break more in the absence of Rtt109 and the repeat contractions could occur during DSB repair. Cells have two major pathways to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ) [38]. In yeast, Rad52 is essential for DSB repair via HR [38]. Therefore, if contractions in *rtt109Δ* cells occurred during Rad52-dependent HR, deleting *RAD52* in *rtt109Δ* cells should decrease or eliminate CAG repeat contractions. However, the *rtt109Δrad52Δ* double mutant still showed significantly increased CAG repeat contractions compared with the WT strain (27% vs. 11%, Fig. 1D) or a *rad52Δ* single mutant (15% [13]). Although the double mutant did show fewer contractions when compared with the *rtt109Δ* mutant, it was not statistically significant. We conclude that Rad52-dependent HR is not a significant source of the repeat contractions that occur in the absence of *RTT109*.

Rad51 is a member of the Rad52 epistasis group, which binds to DNA and facilitates strand exchange between single-stranded DNA and homologous double-stranded DNA [39]. Even

though Rad52 is required for efficient mitotic recombination, Rad52-independent repair pathways do exist in yeast cells, such as single strand annealing (SSA) when homologies flanking a DSB are more than 1 kb, Rad51-dependent UV-induced gene conversion, and spontaneous HR initiated by non-DSB damage (e.g. nicks, gaps or reversed forks) [38,40, 41]. Cells lacking both *Rtt109* and *Rad51* still had increased CAG repeat contractions compared to WT cells (19.4% vs. 11%, $p=0.02$, Fig. 1D; *rad51Δ* frequency is 14% [13]). However, when compared with the *rtt109Δ* strain, contractions of CAG repeats in *rtt109Δrad51Δ* cells were significantly decreased ($p=0.002$). Therefore, approximately one-half of the contractions in *rtt109Δ* cells were caused by a Rad51-dependent pathway.

Since inhibiting HR repair by deleting *RAD52* did not eliminate repeat contractions in cells lacking *RTT109*, we next asked whether NHEJ contributed to any CAG repeat contractions in the *rtt109Δ* strain. To address this issue, repeat stability was analyzed in cells lacking both *RTT109* and *DNL4*, a DNA ligase required for NHEJ [38]. Repeat contractions were still significantly elevated in the *rtt109Δdnl4Δ* mutant compared with the WT strain, but somewhat reduced compared to *rtt109Δ* cells (22.7% vs. 36.5%, $p=0.02$, Fig. 1D; *dnl4Δ* frequency is 20% [13]). Altogether, CAG repeat contractions in *rtt109Δ* were partially (about one-half) due to a Rad51 dependent pathway, with a smaller contribution due to a Dnl4-dependent pathway.

3.4 The rate of CAG repeat breakage is increased in the *rtt109Δ* strain

To further investigate the mechanism of contractions in the *rtt109Δ* mutant, we assayed directly for whether CAG repeat breakage was elevated in this background. A relative measure of CAG repeat fragility can be determined by a previously described assay [8]. If breakage occurs near or within the CAG repeat tract and is not efficiently repaired, the broken chromosome can be rescued by telomere addition at a telomeric seed sequence proximal to the repeats, resulting in loss of the *URA3* gene distal to the repeats. The resulting cells will then become resistant to 5-fluoroorotic acid (FOA) and the rate of FOA resistance is proportional to the breakage rate of CAG repeats [8]. The level of CAG-85 repeat breakage was elevated two-fold in the *rtt109Δ* strain, an increase that is statistically significant ($p=0.018$, Fig. 3). These results are consistent with the genetic results that showed that a portion of contractions in *rtt109Δ* mutants occurred during a pathway dependent on DSB repair proteins.

4. Discussion

H3K56 acetylation, which is catalyzed by *Rtt109* and regulated by *Asf1*, *Hst3*, and *Hst4*, is conserved in yeast, *Drosophila*, and mammals, and appropriate acetylation of H3K56 is important for genome stability [19,42]. Mutants of genes responsible for H3K56 acetylation and deacetylation are sensitive to a subset of genotoxic agents, which is possibly due to the failure of proper nucleosome reassembly following DNA replication and repair [43]. We showed here that all of the known players involved in H3K56 acetylation and deacetylation are important for preventing contractions of CAG/CTG repeats. Since acetylated H3K56 occurs primarily during chromatin assembly after DNA synthesis, we conclude that defects in this process are most likely leading to repeat contractions.

One interesting question is why defects in two opposing modifications, hyperacetylation and hypoacetylation, showed a similar phenotype of increased repeat contractions. This result implies that a proper balance of H3K56 acetylation is important. Nucleosomes with acetylated H3K56 are deposited onto newly replicated strands during S phase and then subsequently deacetylated by *Hst3* and *Hst4* [23,35]. Thus, the defect in H3K56 acetylation in *rtt109Δ* and *asf1Δ* mutants should directly affect nucleosome assembly, whereas a defect in the deacetylation step may disrupt the next round of replication or a subsequent repair process. Interestingly, H3K56 hypoacetylation (*rtt109Δ* and *asf1Δ*) and hyperacetylation (*hst3Δhst4Δ*) mutants also share several other phenotypes including sensitivity to both MMS and HU and

defects in the DNA damage response [42,44]. Thaminy et al. (2007) have proposed that local H3K56 acetylation may play a role in recruiting DNA damage response proteins to damage sites [44]. Therefore in the absence of H3K56 deacetylation, when acetylated H3K56 is distributed throughout chromatin, cells may be unable to use this modification to properly direct damage response proteins to the repeat to facilitate replication or repair. Alternatively, the K56Ac mark has been shown to be necessary for deactivation of the checkpoint response [26], therefore an over-acetylated chromatin state as in the *hst3Δhst4Δ* mutants could prevent a proper checkpoint response to a replication problem at the repeats, leading to increased breakage and contractions during subsequent repair. Indeed, mutation of checkpoint proteins also led to increased CAG repeat fragility and contractions [45].

Fewer contractions were detected in H3K56R mutants than in *rtt109Δ* cells. One possible explanation is that the H3K56R mutant does not completely phenocopy the unacetylated histone H3 lysine 56. In support of this interpretation, it has been shown that the H3K56R mutant is less sensitive to DNA damaging agents than *asf1Δ* cells on low levels of MMS and CPT that induce DNA replication damage [35]. As CAG/CTG repeats cause a relatively weak block during replication [5,6], the effect of H3K56R on repeat stability might also be less than deletion of *ASF1* or *RTT109*. If this is the case, contractions in *rtt109Δ* or *asf1Δ* cells may be entirely due to hypoacetylation of H3K56. On the other hand, *rtt109Δ* and H3K56R mutants do show a similar level of reduction of replication protein association at a stalled replication fork, including Rfc3, PCNA and Pole, which suggests that H3K56R is able to mimic that aspect of the *rtt109Δ* mutant phenotype [25]. A second possibility for the higher level of contractions in the *rtt109Δ* strain compared to the H3K56 mutant could be that another target of Rtt109 HAT activity is also involved. Rtt107 and Rtt101 have been shown to be recruited to replication forks stalled by MMS-induced DNA damage in an Rtt109-dependent and H3K56 acetylation-independent pathway [37]. Despite this association, CAG tracts were stable in the absence of either *RTT107* or *RTT101*, indicating that the Rtt107/ Rtt101-dependent fork restart pathway is not important for stabilizing CAG/CTG repeats and is unlikely to play a role in fork restart at DNA structures. Another possible target of Rtt109 HAT activity is H3K9, which is acetylated on newly synthesized histone H3 by both Rtt109 and another HAT, Gcn5 [46]. However, the H3K9R mutant does not show sensitivity to DNA damaging agents in yeast [46] and no increase in repeat instability was detected in a *gcn5Δ* mutant (J. Yang, J. Moy, and C. H. Freudenreich, unpublished data). Thus, H3K9 acetylation does not appear to be the relevant Rtt109 target responsible for preventing increased contractions. Therefore, if there is another target of Rtt109 besides H3 lysine 56 important for stabilizing CAG/CTG repeats, it remains to be discovered.

Taken together, our data suggest a model in which repetitive sequences in WT cells are replicated with relatively few changes, and newly synthesized histone H3 acetylated on lysine 56 is efficiently incorporated into nucleosomes on the newly replicated DNA. In contrast, cells lacking *RTT109* or *ASF1* will have defects in nucleosome assembly during replication [23]. Therefore, a region with partially assembled nucleosomes is likely present behind the replication fork in *rtt109Δ* or *asf1Δ* mutants (Fig. 4). We propose that under these conditions, CAG/CTG repeats are more likely to form secondary structures on the template strand. In addition, strains deleted for *RTT109* or *ASF1* or with an H3K56R mutation have an extended doubling time and defects in replisome assembly at stalled replication forks [16,24,25]. Therefore another possibility is that a slow replication rate or unstable replisome leads to longer-lived single-stranded DNA regions at the advancing fork, allowing hairpin formation on the template strand (Fig. 4). These two models are not mutually exclusive. Even though structure formation is generally more likely on the lagging strand template as it has a longer transient single-stranded region (as drawn in Fig. 4), it is possible that structures could also form on the leading strand template where the CTG repeat with higher hairpin stability is located.

The formation of secondary structure caused either by defective nucleosome assembly or by defects in replisome stability could impair replication fork progression. One possible way to continue DNA synthesis is that the polymerase may simply slip over the structure formed by the repeats, resulting in a repeat contraction [47]. This pathway would likely be independent of the repair proteins tested (Fig. 4, middle pathway). A replication slippage model for generating repeat contractions is supported by observations in replication mutants. For example, a point mutation in Pol δ , *pol3-t*, has a phenotype of slow lagging strand replication that could lead to more single stranded DNA exposed on the lagging strand template [8,48]. Similar to *rtt109 Δ* cells, contractions of an expanded CAG repeat tract were significantly increased in this mutant, and a small increase of breakage was detected (2-fold at CAG-45) [8]. Increased contractions were also detected in other Pol δ temperature-sensitive mutants and several *pol30* (PCNA) mutants [9,49,50]. Alternatively, if the hairpin blocks replication, fork reversal may occur to facilitate hairpin removal and replication restart [6]. However, if template hairpin removal is not complete, as could be the case if increased ssDNA in the *rtt109 Δ* mutant allows multiple hairpins to form, this process could still result in some level of contractions.

In addition to contractions that occur during the replication process in *rtt109 Δ* and *asf1 Δ* mutants, the reduced level of CAG/CTG repeat contractions in *rtt109 Δ dnl4 Δ* and *rtt109 Δ rad52 Δ* mutants indicates that DSB repair does contribute to the generation of some repeat contractions in *rtt109 Δ* cells. Our fragility results also indicate that DSBs within the CAG/CTG repeat are increased in *rtt109 Δ* cells, consistent with a role for DSB repair in creating a portion of the contractions that occur in *rtt109 Δ* cells. DSBs may occur because of breakdown or enzymatic cleavage of one of the fork structures depicted in Fig. 4. End processing during Dnl4- and/or Rad52-dependent repair could then create contractions of the repeat (Fig. 4, left pathway).

Interestingly, Rad51 appears to have a Rad52-independent role in facilitating CAG repeat contractions in *rtt109 Δ* mutants. There is evidence that unreplicated gaps left behind an advancing fork, such as those caused by UV lesions [51], or in this case a bypassed hairpin DNA structure, can be repaired by a Rad51-dependent and Rad52-independent process [40, 41]. In addition, Kerrest et al. (2009) showed that Rad51 is responsible for the increased repeat expansions and contractions that occur in the absence of Srs2-mediated fork regression at CAG/CTG repeats [6]. We hypothesize that the Rad51 role could be in a pathway that is not associated with DSBs. For example, Rad51-mediated sister chromatid recombination could be employed to fill a gap opposite a hairpin to create a contraction (Fig. 4, right pathway). Alternatively, Rad51 could act directly to facilitate bypass of a template strand structure to allow fork restart as proposed for RecA [52]. Rad51 has been localized to HU stalled replication forks by chromatin immunoprecipitation, where it stabilizes Pol ϵ [53,54]. In summary, our data are most consistent with the idea that template strand structures form behind the fork in cells defective in H3K56 acetylation, and that contractions ensue when the hairpin is either replicated over, bypassed by sister chromatid recombination, or deleted during DSB repair events (Fig. 4).

Recently, HDAC inhibitors have been investigated as a potential therapy for polyglutamine repeat diseases through regulating expression of the affected genes [55]. For example, several trinucleotide repeat diseases are caused by down-regulation of one or more genes due to the triplet repeat expansion, either directly due to promoter inactivation (Fragile $\times$ syndrome) [56] or indirectly due to abnormal interactions of the mutant protein with HATs and other transcriptional regulators (Huntington's disease) [57]. Treatment with HDAC inhibitors can increase histone acetylation and thereby increase the transcription of genes which have been downregulated [55]. Compared with the extensive studies of the effect of histone modifications on transcriptional levels in the repeat expansion diseases, few studies have been done to understand the significance of histone modification on repeat stability at the DNA level. Our

results indicate that histone modification, specifically H3K56 acetylation, does affect repeat stability. Therefore, HDAC inhibitors targeted to histone H3 deacetylases could have the side benefit of inducing repeat contractions. Importantly, our data also reveals that improper chromatin assembly during DNA replication can be a cause of genome instability by allowing the template strand to fold into DNA structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Instability of a CAG-85 tract in mutants related to H3K56 acetylation. Percent of repeat contractions are shown for each mutant. *, $P < 0.05$; **, $P < 0.01$ by Fisher's exact test compared to corresponding WT strain. #, $P < 0.05$; ##, $P < 0.01$ by Fisher's exact test compared to *rtt109Δ* mutant.

Fig. 2.

Size distribution of repeat contractions and expansions. The percentage of repeat contraction and expansion events in each size range are shown. The dotted line represents an intact tract length (I) range of 80-90 repeats. Repeat lengths are binned into groups of ten repeats, which means that deletions of 3-5 repeats are contained within the intact (I) bin in this figure. The range of contractions in each strain were: WT: 3-77 repeats (ave. 44); *rtt109Δ*: 3-85 repeats (ave. 51); *asf1Δ*: 8-85 repeats (ave. 52).

Fig. 3. Fragility assay of YAC CF1 containing zero or 85 CAG repeats in wild type and *rtt109Δ* backgrounds. The average of 3-9 experiments with standard error is shown. The rates of FOA^R were calculated by the method of maximum likelihood. The asterisk represents the rates that were significantly different from those of the wild type by a pooled variance *t* test (*, $P < 0.05$).

Fig. 4. Model for CAG repeat contraction formation in mutants defective in H3K56 acetylation (*rtt109Δ*, *asf1Δ* and H3K56R). Red lines represent CAG/CTG repeats. Solid circles represent nucleosomes with unacetylated H3K56, and open dashed circles represent sites where newly deposited nucleosomes with acetylated H3K56 are missing.