Expanded CAG/CTG Repeat DNA Induces a Checkpoint Response That Impacts Cell Proliferation in *Saccharomyces cerevisiae*

**Rangapriya Sundararajan, Catherine H. Freudenreich**

Department of Biology, Tufts University, Medford, Massachusetts, United States of America

**Abstract**

Repetitive DNA elements are mutational hotspots in the genome, and their instability is linked to various neurological disorders and cancers. Although it is known that expanded trinucleotide repeats can interfere with DNA replication and repair, the cellular response to these events has not been characterized. Here, we demonstrate that an expanded CAG/CTG repeat elicits a DNA damage checkpoint response in budding yeast. Using microcolony and single cell pedigree analysis, we found that cells carrying an expanded CAG repeat frequently experience protracted cell division cycles, persistent arrests, and morphological abnormalities. These phenotypes were further exacerbated by mutations in DSB repair pathways, including homologous recombination and end joining, implicating a DNA damage response. Cell cycle analysis confirmed repeat-dependent S phase delays and G2/M arrests. Furthermore, we demonstrate that the above phenotypes are due to the activation of the DNA damage checkpoint, since expanded CAG repeats induced the phosphorylation of the Rad53 checkpoint kinase in a *rad52Δ* recombination deficient mutant. Interestingly, cells mutated for the MRX complex (*Mre11-Rad50-Xrs2*), a central component of DSB repair which is required to repair breaks at CAG repeats, failed to elicit repeat-specific arrests, morphological defects, or Rad53 phosphorylation. We therefore conclude that damage at expanded CAG/CTG repeats is likely sensed by the MRX complex, leading to a checkpoint response. Finally, we show that repeat expansions preferentially occur in cells experiencing growth delays. Activation of DNA damage checkpoints in repeat-containing cells could contribute to the tissue degeneration observed in trinucleotide repeat expansion diseases.

**Introduction**

Repetitive DNA is found dispersed throughout eukaryotic genomes, and in some cases is central to key biological processes such as chromosome segregation and chromosome end protection [1]. Repeat tracts are usually sites of variation among individuals, with some classes of repeats expanding to sizes that cause pathology. For example, expansion of CAG/CTG trinucleotide repeats (abbreviated CAG) have been observed to occur at several different genomic loci, causing diseases that include Huntington’s disease, myotonic dystrophy, and multiple subtypes of spinal cerebellar ataxia [2-3].

CAG trinucleotide repeats are among a class of repeats that are unique in that they form hairpin secondary structures which interfere with DNA replication and DNA repair [1,4]. The repeats exhibit a threshold length beyond which expansions become increasingly likely. For CAG repeats in humans, the expansion threshold is 35–38 repeats, 100–115 bp. In addition to the instability threshold, a disease-causing threshold also exists for trinucleotide repeats, which is at or above the expansion threshold, and is dependent on the locus and disease process. For Huntington’s disease the disease-causing threshold is 38–40 repeats, and is governed by the length at which the polyglutamine tract (coded for by CAG) within the Huntingtin gene becomes toxic. At the myotonic dystrophy locus, the disease threshold is closer to 200 repeats, the size at which the CUG RNA exerts toxic effects on muscle cells [2,4].

It is well established that in mammalian cells, proteins with an abnormally long polyglutamine tract due to a CAG expansion cause toxic effects that ultimately result in cell death [2-3]. In addition, RNA containing a long CUG tract can also cause toxicity and cell death by sequestering RNA binding proteins, as happens in patients with myotonic dystrophy where the expanded CTG repeat is transcribed but not translated [2-3]. However, less is known about whether the expanded repeat DNA itself is toxic to cells. CAG repeats of 55–80 repeats have been shown to block replication fork progression in plasmids, cause fork reversal on a eukaryotic chromosome [5-7], and interfere with ligation of 5’ DNA flaps that occur during Okazaki fragment maturation or gap repair [8-9]. Structure-forming trinucleotide repeats also cause double-strand breaks (DSBs) in a length-dependent manner resulting in chromosome fragility [10-11]. Thus multiple types of DNA damage occur at an expanded trinucleotide repeat tract, including stalled or reversed forks, single-strand breaks or gaps, and double-strand breaks (DSBs). Our recent results have shown...
Author Summary

Expansion of a CAG/CTG trinucleotide repeat is the causative mutation for multiple neurodegenerative diseases, including Huntington’s disease, myotonic dystrophy, and multiple types of spinocerebellar ataxias. Two reasons for the cell death that occurs in these diseases are toxicity of the repeat-containing RNA and of the polyglutamine-containing protein product. Although the expanded repeat can interfere with DNA replication and repair, it was not known whether the presence of the repeat within the DNA causes any additional cellular toxicity. In this study, we show that an expanded CAG/CTG tract placed within the chromosome of the model eukaryote, budding yeast, elicits a cellular response that interferes with cell growth and division. The effect is enhanced when DNA repair pathways, particularly double-strand break repair, are compromised. Moreover, cells experiencing an arrest were more likely to have undergone further repeat expansions. We show that the conserved MRX protein complex locates to the expanded repeat and is required to sense the damage and activate the DNA damage response. Our results suggest that DNA damage at expanded CAG/CTG repeats could contribute to both tissue degeneration and further repeat instability in affected individuals.

Results

Microcolony assay to determine if a population of CAG repeat-containing cells has a growth disadvantage

Our earlier results indicated that checkpoint proteins are required in cells containing expanded CAG repeats in order to prevent increased fragility and instability of the repeats [26-27]. However, as these results were partly obtained using genetic assays that could detect rare events, it was not known if checkpoint activation in repeat-containing cells is a rare or common event, or whether it results in cell cycle delays. Therefore we sought to determine whether an expanded CAG repeat causes measurable effects on cell growth. We used a yeast strain containing a yeast artificial chromosome (YAC) with either no repeat (CAG-0) or an expanded CAG repeat originally cloned from a myotonic dystrophy patient [29]. The repeat was cloned into a region of the YAC not predicted to be transcribed or translated, and flanked by minimal human sequence, 50 bp and 150 bp on each side the repeat. In general, two allele sizes were studied, 70 and 155 repeats, which have been previously shown to exhibit both instability and fragility at this location [29]. Preliminary experiments in liquid culture revealed slight delays in exponential growth of wild-type yeast containing a CAG-195 repeat at this location (Figure S1). However, because bacterial cells with contracted repeats have a growth advantage in liquid culture over those with longer repeats [30], and since about 30% of CAG-155 repeats have a contraction event in wild-type (WT) yeast [29], we reasoned that the observed repeat-induced growth inhibition could be an underestimation. Therefore, a microcolony experiment, which measures population viability of cells on solid media, was performed to assess the extent of repeat-mediated growth inhibition. This experiment is similar in principle to that performed by Weinert and Hartwell [31], which originally described mutants that escaped the control of DNA damage checkpoints in S. cerevisiae.

Unbudded G1 cells were micromanipulated onto YC-Leu solid media that maintained selection for the repeat-containing chromosome, and their growth into microcolonies (small colonies, visible under the microscope) was monitored (Figure 1A). Since no essential genes required for cell survival are located in close proximity to the repeat, the growth differences observed between cells with or without the CAG tract can be attributed to a repeat-
mediated effect. A typical *S. cerevisiae* cell cycle time of 2 hours on synthetic media would result in about 15 doublings at 30 hours of growth. At 30 hours, we observed a bimodal distribution of microcolony growth in WT CAG-70 or CAG-155 strains. This distribution typically consisted of “survivors” (area ≥0.01 mm², more than 6 doublings, Figure 1A–1E) and “non-survivors” (area ≤0.005 mm², 6 or fewer doublings). The non-survivors likely represent terminally arrested lineages (frequencies, reported in [12], were ~10% for WT cells with or without a CAG repeat). Among survivors, WT CAG-70 or CAG-155 strains showed a significant 1.5 to 2-fold decrease in mean microcolony area compared to the CAG-0 control strain (Figure 1B). Therefore, in the WT strain, a large enough proportion of the cells containing an expanded CAG repeat experienced a growth delay that the overall rate of population growth was affected. We have seen the same effect in two other yeast strain backgrounds,
A. LaPorte, C. Weindel, and C.H. Freudenreich, data not shown.

We also assessed the survival efficiency of mutants lacking end-joining (dnl4Δ), homologous recombination (rad52Δ) or the MRX complex (mre11Δ) because we showed that all three of these pathways act to repair breaks at expanded CAG-70 or -155 repeats [12]. The dnl4Δ strain exhibited a pattern similar to WT, with CAG-70 and CAG-155 microcolonies attaining a mean area 3.3-fold and 1.2-fold below the CAG-0 control respectively (Figure 1C). The rad52Δ CAG-70 and CAG-155 microcolonies attained mean sizes 5.2 and 3.6-fold below CAG-0, revealing that the presence of expanded repeats significantly inhibited population growth in this background and more severely than in the WT strain (Figure 1D). Overall, the extent of repeat-induced growth inhibition depended on both the length of the repeat and the nature of the repair defect: Rad52 deficiency severely impacted the viability of both CAG-70 and CAG-155 survivors, while the Dnl4-Ej pathway deficiency impacted the viability of cells with 70 repeats more than those with 155 repeats, a trend that was present in all three backgrounds but was more pronounced in the dnl4Δ mutant.

Surprisingly, the repeat-induced growth defect phenotype observed in the rad52Δ and dnl4Δ repair mutants was absent among mre11Δ survivors (Figure 1E). Deletion of MRE11 caused a significant growth defect in all cells, with or without an expanded CAG repeat. The broader area distribution and relatively smaller colony sizes of both rad52Δ CAG-0 and mre11ΔA CAG-0 strains indicate that a wide spectrum of sporadically occurring damage in addition to CAG repeat damage require HR and MRX pathways for viability. However, the slow growth phenotype of the mre11Δ CAG-70 strain, rather than being enhanced, was somewhat relieved relative to CAG-0 or CAG-155 strains. This striking phenotype suggests that a proportion of damage that occurs at a CAG-70 repeat can escape detection by the cell cycle checkpoint machinery in the absence of the MRX complex. In addition to the effects graphed in Figure 1, a significant 3.5-fold increase in the frequency of non-survivors was observed in strains with CAG-70 or -155 repeats compared to the CAG-0 control in the rad52Δ and mre11Δ backgrounds, an effect not observed in wild-type or dnl4Δ strains [12]. Collectively, the above phenotypes indicate that repeat-containing cells have a proliferation defect frequent enough to result in reduced population viability, and that deficiency of DSB repair pathways exacerbates the defect.

Single cell pedigree analysis reveals repeat-induced growth arrests with aberrant cell morphology

To better understand the basis of smaller microcolony sizes and reduced population viability of repeat-containing cells, a single cell pedigree analysis was performed. Single precursor cells were micromanipulated onto YC-Leu solid media, and successive divisions were followed for a span of ~18 hours, micromanipulating the daughter cells away at each division in order to follow a single lineage (Figure 2A). For each strain, about 30 lineages were analyzed for 5–7 divisions per lineage, for an average of 90 cell divisions per strain (Table S1). We first analyzed the division potential of each lineage, i.e. the ability of a progenitor cell with an expanded CAG repeat to sustain successive divisions in a lineage (Figure 2B). In the wild-type background, the CAG-70 and CAG-155 repeat-containing strains completed on average fewer divisions per lineage (3.6 and 4.1) than the CAG-0 no-tract control strain (4.6). This difference was even more striking in dnl4Δ and rad52Δ backgrounds, with fewer divisions completed compared to the no repeat control and some lineages failing to complete even one division (e.g. 60% for rad52Δ CAG-155, Figure 2B). Similar to the microcolony analysis, the profile was altered in the mre11Δ strain, with CAG-70 cells exhibiting a greater division potential than either CAG-0 or CAG-155 strains. This result supports the conclusion that damage at the CAG-70 tract is not being properly sensed or signaled in the absence of the MRX complex. This conclusion is further substantiated by a decreased frequency of cell-cycle arrests and decreased morphological defects in mre11Δ CAG-70 cells compared to mre11Δ CAG-0 and CAG-155 cells (see below, Figure 2C and Figure 3B).

To determine if reduced division potential was due to frequent cell cycle arrests, divisions were either categorized as normal divisions, lasting <3.0 hours, or arrest divisions, lasting >3.0 hours. While a majority of divisions in the WT CAG-70 and CAG-155 strains were normal divisions (cycling time of <3 hours), 55% and 38% of divisions, respectively, were >3 hour arrest divisions, an elevated frequency compared to the WT CAG-0 control (29%; Figure 2C). Both dnl4Δ and rad52Δ strains showed a further disparity between repeat-containing and no-tract control strains, with a 2.6-fold increase for CAG-70 and 3.3 to 3.5-fold increases for CAG-155 strains in the frequency of >3 hour divisions (Figure 2C). For the rad52Δ CAG-155 strain, about 60% of divisions took longer than 3 hours, compared to only 17% in the rad52Δ CAG-0 control. Thus the presence of an expanded CAG repeat had the potential to prolong cell division in a large proportion of cells, and arrests were 2 to 3-fold more frequent than in cells without an expanded repeat tract. The increased frequency of arrests explains the repeat-specific decrease in microcolony size and reduced division potential.

In addition to “normal” arrest times of 4–6 hours, we noted that a subset of arrests were very prolonged, >8 hours and up to 16 or more hours (the length of the experiment) (Figure 2C and Figure S2). Certain repair foci are known to last >8 hours in arrested cells, although yeast cells can also sometimes adapt after ~8 hours of arrest and re-enter the cell cycle even though the damage has not been repaired [21,32-33]. Adaptation coincides with release from arrest, re-entry into the cell cycle, and disappearance of Rad53 phosphorylated species [34]. WT CAG-70 and CAG-155 strains experienced >8 hour arrest divisions at a frequency 3 to 5-fold higher than the WT CAG-0 control (Figure 1C). Among the DSB repair-deficient mutants, 6.3% or 4.4% of dnl4Δ CAG-70 and CAG-155 strains experienced arrest divisions >8 hours, respectively, relative to 0% in the dnl4Δ CAG-0 control. The rad52Δ CAG-70 and CAG-155 strains experienced >8 hour arrests in 7.8% and 23% of divisions respectively, an 11- or 33-fold increase over the rad52Δ CAG-0 control. The higher frequency of >8 hour arrest divisions observed in repeat-containing cells suggests that expanded repeats are accumulating damage that is difficult to repair. Finally, among all comparisons, the dramatic increase in the frequency of >8 hour arrest divisions in the rad52Δ CAG-155 strain, which is 6.4-fold greater than the WT CAG-155 strain, indicates a requirement for Rad52-mediated HR in repairing damage at the CAG-155 repeat and ensuring timely cell cycle progression.

We further analyzed whether the arrests resulted in recovery, where the cell cycle resumed before 8 hours of arrest (usually indicating successful repair), adaptation, where the cell was arrested more than 8 hours but did eventually re-enter the cell cycle, or terminal arrests, which were not observed to re-enter the cell cycle. For wild-type cells, most arrest events were able to recover or adapt (96–100%), although the recovery frequency was lower for repeat-containing cells (Table 1). Cells lacking one of the main DSB repair proteins and containing a repeat tract were less likely to recover, and more likely to have an adaptation response or experience a terminal arrest. Notably, with the exception of the
mre11Δ mutant, terminal arrests were not observed in the CAG-0 control strain even in rad52Δ or dnl4Δ backgrounds, suggesting that the presence of an expanded repeat can lead to a more severe outcome than deficiency in a major DSB repair pathway. However the most striking difference between cells with and without an expanded repeat was the likelihood of experiencing a...
second arrest in the next cell division, as these recurring arrests appeared only in repeat-containing lineages (Table 1). This phenomena was repeat-length dependent, occurring more often in cells with a CAG-155 than CAG-70 repeat, and the second arrest was often longer than the first. Apparently, the damage at a CAG-155 strain had less severe swelling than the no-tract background and was highly significant compared to the CAG-0 control (Figure S4). Along with previous results, these data suggest that the CAG-155 repeat arrests result in a perturbation of replication that is severe enough to cause a slowing of S phase, while the damage at CAG-70, although it may also originate in S phase, does not always induce the S phase checkpoint but rather is induced by the CAG-155 strain, with the vast majority of arrests occurring in the G2 phase (71–93%); absolute percentages are 2.8 and 2.7-fold over CAG-0, Table S1). Furthermore, the increase in S phase arrests in cells with a CAG-155 repeat was evident in the rad52Δ background and was highly significant compared to rad52Δ CAG-0 or CAG-70 (26% vs. 7–8%, Figure 4A). Overall, these data support the idea that the CAG-155 repeat arrests result in a perturbation of replication that is severe enough to cause a slowing of S phase, while the damage at CAG-70, although it may also originate in S phase, does not always induce the S phase checkpoint but rather is more often resolved in G2. Furthermore, Rad52-dependent HR is a major reason for repairing repeat-induced DNA damage, a result consistent with the increased repeat tract fragility observed in a rad52Δ strain [12]. Shifts in arrest phase also occurred in repeat-containing dnl4Δ and mre11Δ cells; the majority of arrests in the absence of either protein were at the G2/M boundary, with mre11Δ cells additionally exhibiting a reduction in G1 arrest frequencies compared to the CAG-0 control (Figure S4).

To determine whether repeat-specific S and G2 arrests were also visible at a population level, cell cycle distributions were analyzed by flow cytometry (FACS). In this method, all cells are analyzed, not just those undergoing an arrest. The S phase slowdown was more difficult to detect by FACS than single cell analysis, but the tendency of WT repeat-containing cells to accumulate with 2N DNA content (G2/M) was evident (Figure 4B). In contrast, the WT CAG-0 strain contained mostly

Damage at expanded CAG/CTG repeats elicits frequent S phase and post-replication G2/M delays

In order to determine at what point in the cell cycle delays were occurring, and thereby gain insight into the potential types of damage causing the delays, we determined the cell cycle distribution of growth arrests. The frequencies of G1, S and G2/M arrests were recorded in wild-type or mutant cells with or without an expanded repeat tract (Figure 4A). Analysis of individual divisions from the pedigree experiment revealed that wild-type repeat-containing cells showed a bias towards arresting in the S and G2/M cell cycle phases relative to a CAG-0 control (Figure 4A). Specifically, the CAG-70 strain exhibited a modest but significant increase in S phase arrests (to 33% vs. 28% for CAG-0) and a greater tendency to arrest in G2 (51% vs. 40%), whereas the CAG-155 strain exhibited a further increase in frequency of arrests that began in S phase (38%). The profile was shifted dramatically in a rad52Δ strain, with the vast majority of the arrests occurring in the G2 phase (71–93%); absolute percentages are 2.8 and 2.7-fold over CAG-0, Table S1). Furthermore, the increase in S phase arrests in cells with a CAG-155 repeat was evident in the rad52Δ background and was highly significant compared to rad52Δ CAG-0 or CAG-70 (26% vs. 7–8%, Figure 4A). Altogether, these data suggest that the CAG-155 repeat arrests result in a perturbation of replication that is severe enough to cause a slowing of S phase, while the damage at CAG-70, although it may also originate in S phase, does not always induce the S phase checkpoint but rather is more often resolved in G2. Furthermore, Rad52-dependent HR is a major reason for repairing repeat-induced DNA damage, a result consistent with the increased repeat tract fragility observed in a rad52Δ strain [12]. Shifts in arrest phase also occurred in repeat-containing dnl4Δ and mre11Δ cells; the majority of arrests in the absence of either protein were at the G2/M boundary, with mre11Δ cells additionally exhibiting a reduction in G1 arrest frequencies compared to the CAG-0 control (Figure S4).
cells with 1N DNA content (G1/G0) near stationary phase. In the rad52Δ background, the proportion of cells that accumulated with 2N DNA content was increased even in the CAG-0 control but was further increased in the CAG-155 strain, confirming the importance of this protein for repair of repeat-induced damage in addition to spontaneous damage occurring within non-repetitive regions.

Based on the above phenotypes, we conclude that expanded CAG repeats have the potential to induce both the intra-S and G2/M DNA damage checkpoints. Our results show that specific S and G2/M phase arrests, as opposed to a general overall slowed progression through the cell cycle, contribute to longer cell division times in repeat-containing cells. Rad52-dependent homologous recombination is particularly crucial for prevention of S phase delays and release from the G2/M block to allow resumption of cell proliferation.

### CAG expansion events are frequently associated with cell cycle delays

Since the above results indicated that checkpoint-mediated cell cycle arrests are associated with a repair or fork restart event, we wished to determine the status of the CAG repeat locus in cells that had experienced an arrest. Due to technical difficulty in amplifying the repeat from a single arrested cell, two approaches were taken. First, we isolated swollen cells (one indicator of arrest, Figure 3B), allowed them to divide to form colonies, and then assessed repeat length in the resulting colonies. To obtain a large enough sample size the analysis was done in the rad52Δ strain background that formed normal-size colonies, there were 14% contractions (Table 2), frequencies that are almost identical to CAG-70 tract stability in wild-type cells exposed to a sub-lethal dose of hydroxyurea (0.1 M). Treatment with hydroxyurea resulted in a 7-fold increase in expansions (to 5.6%), relative to the untreated control (0.8%, p < 0.01, Table 2). Contractions remained similar to the untreated wild-type control. We conclude that these HU-dependent expansions, which occurred independently of Rad52- and Dnl4-dependent DSB mis-repair events, result from impaired replication across the repeats. These data suggest that the expansions that arose in the rad52Δ poorly growing colonies could be due to slippage events at slowed or restarting replication forks. We showed previously that Rad52- and Dnl4-dependent DSB mis-repair events are other mechanisms for generation of expansions [7,12].

An expanded CAG tract induces Rad53 phosphorylation and Mre11p localization

The above results show that cell cycle checkpoints are activated in response to damage or interference with fork progression at an expanded CAG repeat. If the level of repeat-induced damage is sufficient or present in a large-enough proportion of cells, phosphorylation of the Rad53 checkpoint kinase should occur. Therefore, we directly tested the phosphorylation status of Rad53 in CAG repeat-containing cells.

The wild-type strain harboring a CAG-155 repeat failed to show a detectable shift in mobility of the Rad53 protein, similar to the CAG-0 control (compare lanes 2 and 3, Figure 5A). Because CAG fragility is proportional to increasing repeat length [29,38], CAG-195 and CAG-240 repeat lengths were also tested, however a phosphorylated Rad53 species was still not detectable (Figure 5A). In contrast, control samples treated with 0.05% MMS or 0.1 M HU exhibited Rad53 phosphorylation, although the intensity and size of the shifted species was considerably less with HU relative to MMS treatment. Since the wild-type strain has functional repair pathways, the lack of detectable Rad53

### Table 1. Modes of exit from cell cycle arrest.

| Strain Background | % Recovery response >3<8 hours | % Adaptation response >8<14 hours | % Terminal arrests >14 hours | % Recurring arrests >3 hours |
|-------------------|-------------------------------|----------------------------------|-----------------------------|-----------------------------|
| WT CAG-0          | 96                            | 3.8                              | 0                           | 0                           |
| WT CAG-70         | 90 *                          | 5.8                              | 3.9                         | 11 *                        |
| WT CAG-155        | 90 *                          | 10                               | 0                           | 13 *                        |
| dnl4Δ CAG-0       | 100                           | 0                                | 0                           | 0                           |
| dnl4Δ CAG-70      | 84 *                          | 9.4                              | 6.3                         | 0                           |
| dnl4Δ CAG-155     | 92 *                          | 6.0                              | 2.0                         | 17 *                        |
| rad52Δ CAG-0      | 96 *                          | 4                                | 0                           | 0                           |
| rad52Δ CAG-70     | 83 *                          | 14 *                             | 3.4                         | 13 *                        |
| rad52Δ CAG-155    | 62 *                          | 31 *                             | 7.7                         | 36 *                        |
| mre11Δ CAG-0      | 64                            | 25                               | 11                          | 0                           |
| mre11Δ CAG-70     | 77 *                          | 15 *                             | 7.7                         | 29 *                        |
| mre11Δ CAG-155    | 62                            | 29                               | 8.8                         | 31 *                        |

Percentages are out of total number of ≥3 hr arrests. * p < 0.05. Statistical significance was determined by logistic regression analysis (columns 1–3) or Fisher’s exact test (column 4).

doi:10.1371/journal.pgen.1001339.t001
phosphorylation could be due to either successful repair of CAG-associated damage or a level of phosphorylated species too low to be visible as a mobility shift on the gel. Indeed, only 3.6% of WT CAG-155 cells exhibited arrests >8 hrs (Figure 2C). Therefore, we monitored the Rad53 phosphorylation status in rad52Δ cells that showed a greater frequency of long arrests (7.8% and 23% for CAG-70 and -155 respectively), and elevated CAG fragility [12]. Indeed, rad52Δ CAG-70 or CAG-155 strains but not the rad52Δ CAG-0 control showed a discernible Rad53 phosphorylation response (Figure 5B), indicating that the DNA damage checkpoint is activated in a repeat-dependent manner in this mutant background. The level of Rad53 phosphorylation was repeat-length dependent with the longer CAG-155 repeat eliciting a more robust checkpoint response than the intermediate CAG-70 repeat length as determined by densitometric quantification (Figure 5B, right). We conclude that in the absence of Rad52-dependent repair, the types of damage that occur at an expanded CAG repeat induce a signaling cascade that results in Rad53 phosphorylation and associated downstream checkpoint events. Based on the data from the microcolony and pedigree experiments, it is likely that the same events happen in a wild-type background, but the damage is at a lower level or repaired more quickly so that phosphorylated Rad53 does not accumulate to a level detectable by Western blotting.

To determine whether CAG repeat damage was capable of eliciting a cell cycle checkpoint response in the absence of the MRX complex, we determined the Rad53 phosphorylation status in mre11Δ cells. The results revealed two surprising observations. First, Rad53 phosphorylation was observed in mre11Δ CAG-70 and CAG-155 strains (Figure 5C), even though CAG-70 cells, and to a lesser degree CAG-155 cells, appeared to escape cell cycle arrests in this background (Figure 1, Figure 2, Figure 3). Second, Rad53 phosphorylation was also observed in mre11Δ CAG-0 cells, a pattern unlike the WT and rad52Δ CAG-0 controls. Based on these results, we infer that the local checkpoint signaling in response to CAG damage is compromised in mre11Δ cells, while global checkpoint signaling in response to non-repeat damage in the genome is intact. A local Mre11-dependent response at the repeat is further supported by physical detection of the Mre11 protein at the repeat tract, which is enhanced 12-fold compared to a non-repeat reference locus by chromatin immunoprecipitation (ChIP) (Figure 5C, right). Intriguingly, Mre11 localization to the repeat peaks in S phase, suggesting that the relevant structure sensed by Mre11 is formed during DNA replication.

In conclusion, an expanded CAG repeat at a single genomic locus can induce a myriad of cellular arrest responses that depend on signaling via the MRX complex, and culminating in a detectable Rad53 phosphorylation response if the damage is not promptly or efficiently repaired by Rad52-dependent recombination.

### Table 2. CAG-70 repeat instability in colonies exhibiting poor growth due to frequent arrests or treated with 0.1 M hydroxyurea.

| Genotype / Treatment | Precursor cell and colony morphology | # colonies tested | % Instability | % Expansions | % Contractions |
|----------------------|-------------------------------------|------------------|--------------|-------------|---------------|
| rad52Δ a             | normal cell—normal colony           | 204              | 21           | 5.9         | 15            |
| rad52Δ b             | swollen cell—normal colony          | 21               | 19           | 4.8         | 14            |
| rad52Δ c             | normal cell—poorly growing colony   | 12               | 67 **        | 33 **       | 33            |
| WT (−HU)             | normal cell—normal colony           | 243              | 6.6          | 0.8         | 5.8           |
| WT (+HU)             | normal cell—normal colony           | 195              | 10           | 5.6 **      | 4.6           |

a data from Sundararajan et al (2010) [12] included for comparison. b swollen cells isolated by micromanipulation. c very small colonies after 3 days growth; repeat expansions were +10 to +20 repeats. Statistical significance determined by Fisher’s exact test, * p<0.05, **p<0.01, upon comparison of with normal untreated colonies of same genotype.

doi:10.1371/journal.pgen.1001339.t002
Discussion

Despite the knowledge that expanded CAG repeats interfere with replication, nick ligation, and are fragile sites, direct evidence on whether such damage is at a level or type sufficient to activate DNA damage checkpoints was lacking. This question is especially important since repeated or long checkpoint arrests can affect cell growth potential and lead to apoptosis in higher eukaryotes. It is also of interest to better understand the cellular response to structure-forming sequences, since there are many examples of these sequences in the human genome. Using yeast containing an expanded CAG repeat, we were able to follow the growth potential and fate of single live cells. The presence of a CAG-70 or CAG-155 repeat did not elicit visible Rad53 phosphorylation by Western blot in wild-type cells. We were nonetheless able to detect significant differences between cells with and without an expanded repeat in growth potential, number of arrests, duration of arrests, and morphological abnormalities. Thus even in a wild-type cell, the presence of a long repeat tract was a significant burden on the cell that resulted in measurable effects on growth and division potential. Notably, the arrests that occurred within repeat-containing cells were sometimes of a very long duration, greater than 8 hours and frequently accompanied by severe cell swelling, indicating that a type of damage had occurred that was particularly difficult to repair [21,32,34].

What is the origin of the damage inducing the checkpoint response? Recently, it was reported that convergent transcription through CAG repeats induces apoptosis in both dividing and non-dividing human cells [39]. Our CAG repeat is not within a gene or known transcriptional unit, however RT-PCR experiments did show low but equivalent levels of transcript in WT and rad52Δ cells, which could reflect read-through transcription from the neighboring URA3 gene (M. Koch, J. Yang, and C.H. Freudenreich, data not shown). Therefore, it is possible that some of the damage may initiate during transcription. However, the similar transcript levels in the two strains, together with the S-phase delays, the S-phase binding by Mre11p, and the importance of Rad52-dependent repair are all more consistent with the primary damage sensed by the checkpoint occurring during DNA replication.

Whatever the initiating event, our data indicate that Rad52-dependent recombination is a key mechanism for overcoming repeat-dependent damage in cells, and without it a strong checkpoint response is induced and cellular growth is severely compromised. For the CAG-155 repeat-containing cells lacking Rad52, a quarter of the divisions displayed arrests of greater than 8 hours and 36% had morphological abnormalities. In addition, most of those cells had a recurring arrest in the next cell division, indicating that the damage had persisted. The checkpoint responses were all highly repeat-length dependent in the rad52Δ background being significantly greater at CAG-155 compared to CAG-70, indicating that damage at the longer repeat requires rescue by recombination mechanisms more frequently. In addition, the cell cycle analysis indicated that cells with a CAG-155 tract had a greater tendency to show an S phase delay compared to CAG-70, a difference exacerbated by Rad52 deficiency. We conclude from this data that the longer repeat has a greater effect on replication, and that a Rad52-dependent process is likely involved in fork restart events for this tract length. This interpretation is supported by comparatively increased CAG-155 fragility observed in a rad52Δ strain [12]. The CAG-70 repeat likely also interferes with replication, as there was a slight increase in S-phase delays, and we showed previously that Srs2-dependent fork reversal occurs at a CTG-55 repeat [7]. Perhaps at this repeat length, fork restart can usually occur without recombination, consistent with genetic results. However if fork reversal or integrity is compromised, recombination may become the preferred pathway, since the CAG-70 expansions that occur in mre11Δ mutants are Rad52-dependent [7,12].

The Dnl4 ligase, needed to complete end-joining repair of DSBs, also played a role in preventing repeat-mediated cell cycle arrests and promoting normal growth and division potential. The requirement for end-joining was more subtle than that for HR, although deficiencies in either process led to a large percentage of cells arrested in G2 with 2N DNA content. While the Dnl4 protein strictly localizes to DSBs, the Mre11 and Rad52 proteins have also been found at stressed or collapsed replication forks and may aid in fork restart [40-41], potentially explaining the greater requirement for these proteins. Altogether, our data are consistent

Figure 5. Rad53 phosphorylation status and Mre11 localization in strains with expanded CAG/CTG repeats. Western blots of protein extract from (A) WT (B) rad52Δ, and (C) mre11Δ cells probed with Rad53 antibody. Extracts treated with 0.1 M HU (A, lane 1) or 0.05% MMS (A, lane 6) are included as positive controls. Quantified equal amounts (15 ng) of total protein prepared from asynchronous cultures were loaded per lane; the positions of unphosphorylated Rad53 (93 kDa) and hyperphosphorylated Rad53 species (Rad53-P) are indicated by arrows and square brackets, respectively. Rad53 hyperphosphorylated species were quantified and normalized to the Rad53 intact band; average of 3 experiments with standard error of the mean (SEM) is shown for rad52Δ (B, right) or mre11Δ (C, right) strains. Similar ratios were obtained when Rad53-P signal was normalized to an internal loading control (data not shown). (D) Mre11-TAP is recruited to the CAG repeat-containing DNA fragment. A strain with TAP-tagged Mre11 and a loading control (data not shown). (D) Mre11-TAP is recruited to the CAG repeat-containing DNA fragment. A strain with TAP-tagged Mre11 and a loading control (data not shown). (D) Mre11-TAP is recruited to the CAG repeat-containing DNA fragment. A strain with TAP-tagged Mre11 and a loading control (data not shown).
with the idea that the expanded CAG repeat causes multiple types of damage sensed by the checkpoint, including stalled or reversed forks in S phase needing Rad52-dependent restart, and DSBs in G2 that can be repaired by either Rad52-dependent HR or Dnl4-mediated end joining. Importantly, cells which showed an initial arrest response but were able to continue dividing for a limited time to form small, poorly growing colonies showed a significantly elevated frequency of repeat instability. Thus repeat instability may preferentially occur during inefficient or initially failed repair or fork restart events.

Cell cycle arrest responses were dramatically altered in the absence of a functional MRX complex. In general, all mre11Δ cells, with or without a repeat tract, are quite compromised for growth with small microcolonies, a reduced division potential, a very high frequency of divisions arresting for >3 hrs (90% for CAG-0), and a third arresting for >8 hrs. Notably, although mre11Δ cells were often swallen due to the frequent and long arrests, they did not exhibit many morphological defects. Opposite to the situation in wild-type cells or other DSB repair mutants, the arrest and growth phenotypes were usually less severe in the mre11Δ repeat-containing cells, especially for the CAG-70 tract. Cells containing a CAG-70 tract and lacking the Mre11 protein had a relief of the microcolony growth defect, underwent significantly more divisions, fewer arrests and less cell swelling compared to CAG-0 cells. These results indicate that an intact MRX complex is required for efficient induction of the repeat-mediated checkpoint, and that the majority of CAG-70 damage and some of the CAG-155 damage likely escapes detection by the cell cycle checkpoint machinery in the absence of the MRX complex. This conclusion is further substantiated by the physical detection by ChIP of the Mre11 protein at the repeat tract. Therefore the MRX complex is likely acting as a sensor of damage at the repeat tract, interfacing with a signaling kinase such as Tel1 or Mec1. Our previous observation of increased CAG fragility in a mre11Δ sml1Δ strain of similar magnitude to that observed in mre11Δ, whereas a tel1Δ did not increase CAG fragility, suggests that Mec1 is a good candidate for signaling from Mre11 bound damage [12,27]. An alternative interpretation is that MRX is needed to create the checkpoint signal, for example by exposing ssDNA that can be coated by RPA. However, our previous results showed that mutation of the Mre11 nuclease activity or associated Exo1 or Sac2 nucleases did not fully recapitulate the mre11Δ phenotype [12], suggesting that MRX has a function in addition to processing. Interestingly, the overall checkpoint response is not compromised in mre11Δ cells, as constitutive Rad53 phosphorylation was detected, and the CAG-0 arrest phenotypes also indicate a robust and intact global checkpoint response. The S-phase localization of Mre11p to the repeat and the reduced recovery of S phase arrests in the mre11Δ CAG-155 strain (Figure S4) suggest that the primary repeat-induced damage sensed by the MRX complex may arise during replication. Since Mre11p has also been found at HU stalled forks [40], it may be recognizing the double-strand end at either a reversed or broken fork. The consequences of the absence of MRX sensing are a large increase in CAG fragility, expansions, and cytotoxicity [12].

An interesting and unexpected finding was that the two repeat lengths, CAG-70 and CAG-155, did not behave identically, suggesting that there are some differences in the DNA structures eliciting the checkpoint at each repeat. Based on the relief of growth and arrest defects by deletion of MRE11, Mre11 appears to be the primary sensor of damage at CAG-70. In contrast, cells with a CAG-155 tract still showed some arrest phenotypes in mre11Δ cells, but were particularly dependent on Rad52 for normal growth. Based on these results and previous data that a strain mutated for Mrc1 checkpoint function had a high rate of CAG-155 fragility, we speculate that the longer repeat is detected more efficiently by sensors of fork stalling, such as Mrc1, making it less dependent on signaling through the MRX complex. It may be that both repeats elicit fork reversal and occasional DSBs that are sensed by MRX, but that the CAG-155 repeat is also able to stall a replication fork long enough to elicit an Mrc1-dependent checkpoint signal. Intriguingly, despite a robust checkpoint response in CAG-155 cells as measured by Rad53 phosphorylation, they formed a slightly larger average microcolony size than cells with a CAG-70 tract. This could be either due to the greater tendency of the CAG-155 strain to adapt (Table 1), or due to the greater amount of cell swelling (Figure 3B), taking up more space in the microcolony. Perhaps the hypothesized better S-phase structure detection allows for a timelier repair process at the longer repeat.

Do other structure-forming sequences elicit similar checkpoint responses? Expanded CGG/CCG repeats, inverted repeats, and alternative DNA structures such as H-DNA and Z-DNA are hotspots of replication stalling, chromosome breakage and rearrangements, and thus might elicit a similar response [42]. Yet genetic data suggest that expanded CGG repeats may be less efficient at eliciting a checkpoint than CAG repeats, as fork arrest at a CGG repeat was not dependent on the checkpoint function of Mrc1 whereas suppression of CAG fragility and instability is [26,28,43]. On the other hand, mice with an expanded CGG repeat at the fragile X locus and heterozygous for ATR or ATM exhibit increased frequencies of repeat expansion during inter-generational transmission and in somatic cells [44-45], suggesting that there is some level of checkpoint response to expanded CGG repeats. Variables that could affect the response to different sequences include the nature of the initial damage, processing of the damage, the amount of exposed ssDNA, or the chromatin structure at the repeat. Paradoxically, the ability of CAG repeats to be efficiently recognized by the checkpoint machinery may be helpful in preventing some level of CAG fragility, which is recovered at a lower rate than fragility at expanded CGG/CCG repeats in yeast [46] and has not yet been detected at human disease loci. It will be informative to directly compare the checkpoint responses to CAG versus CGG repeats and other structure-forming sequences in the future.

What relevance might our results have for human repeat expansion diseases? It is known that the RNA and protein products of transcribed and translated CAG/CTG repeats can be toxic to cells. Now we provide the additional knowledge that the expanded DNA itself can be toxic through mechanisms involving DNA replication and DNA damage repair. Since sense or antisense transcription across repeats could contribute to structure formation [47], the baseline of repeat-induced cytotoxicity may be higher in instances where the CAG repeat locus is also heavily or convergently transcribed [39]. In a multi-cellular organism, many of the long and recurring arrests we observed would probably lead to apoptosis and cell death, the main cause of disease symptoms and morbidity. Indeed, checkpoint activation and cell cycle re-entry have been observed during apoptosis of aging brains of patients with Huntington’s as well as other neurodegenerative diseases [48]. A second finding with potential relevance for repeat expansion diseases is that repeat expansions are more frequent in cells undergoing a checkpoint response. Intriguingly, re-entry into the cell cycle after DNA damage can facilitate repair in postmitotic neurons [49], which could possibly contribute to further repeat expansions. Therefore, DNA repair occurring in the context of an activated checkpoint response may be a cause of repeat expansions in mammalian cells as well.
Materials and Methods

Yeast strains, YAC, and media

YAC CF1 harboring CAG repeats is described in [12,29]. In this YAC, the CAG repeat is oriented such that the CAG strand is the lagging strand template (the more stable and expansion-prone orientation). For all experiments, yeast strains harboring YAC-CF1 with CAG repeats (CAG-70, 155, or 195 repeats; Table S2) were plated onto YC-Ura-Leu solid media for single colonies and grown for 3 days at 30 °C. CAG repeat length from a portion of the colony was determined by colony PCR [29]. Starting colonies with intact tract lengths were chosen for experiments.

Microcolony and single cell pedigree analyses

20 µl of overnight culture (~7.0 doublings) was spread as a stripe onto yeast complete solid media lacking Leucine (YC-Leu). Single un budded, normal-sized G1 cells from the stripe were micromanipulated away to designated locations on the plate using a Nikon Eclipse E400 tetrad dissection scope. Precursor cells were allowed to divide for either 30 hours (micromanipulation experiment) or 18 hours (pedigree experiment) at 30 °C. For the micro colony experiment, the growth of precursor cells into microcolonies (small colonies) was recorded at 3-hour, 10-hour and 30-hour time intervals. An average of 40 cells (range 22–73) from two experiments were analyzed per strain. Pictures were taken at 10X magnification using an Olympus microscope, and microcolony area at 30 hours was measured using the National Institutes of Health (NIH) ImageJ software. Survivors (plotted) were defined as area>0.01 mm²; nonsurvivors (not shown) were defined as area≤0.005 mm² (cutoff values in [12] reported incorrectly and corrected here). The data set was subject to non-linear regression analysis and graphed using the Prizm curve-fitting software (GraphPad Software, San Diego, CA). Analysis of variance (ANOVA) was used to compare microcolony areas. Fisher’s LSD post-hoc test was used to quantify growth differences among CAG -0, -70 and -155 repeat-containing strains within each genotype.

For single cell pedigree analysis, individual divisions within and across pedigrees were monitored for a duration of ~18 hours (5–7 divisions) on YC-Leu media at 30 °C. This number of divisions was chosen to minimize any confounding effects of senescence and allow a fair comparison between WT and DSB repair mutants that have a compromised division potential (see Text S1 for further information). 15–41 lineages were monitored in parallel on five plates for a total of 31–183 cell divisions; see Table S1 for raw numbers. Mother cells were followed as they do not have a size-related growth delay; daughter cells were discarded. As a rule, precursor cells that failed to initiate growth (increase in cell volume) or initiate division were excluded from the experiment since they could have been damaged during micromanipulation. The duration of individual cell division, i.e. growth from a single un budded cell until separation into daughter cells was recorded; the duration of a normal yeast cell division was set at ≤53.0 hours to factor in delays in division introduced by mechanical stress due to micromanipulation, observed to be 30–45 minutes. A bud to mother ratio of <33% was deemed an S phase cell (small-medium bud), or >33% a G2/M phase cell (large budded) [30]. Cell cycle position at the G1, S or G2/M phases was recorded at least twice within a division cycle. Elongated buds were typed as S phase arrests since they occurred after S phase onset. Multibuds were classified as G2/M arrests since they arose after G2 phase onset. Cells that were small budded when a cell cycle delay occurred were categorized as S phase “arrests”, although the S phase checkpoint does not result in a true arrest, but a slowing of S phase and eventual entry into G2. Cell sizes greater than the size of a normal yeast cell (>8 µM diameter) were counted as swollen. Because of interdivisional variation in cell size, the G1 cell of each division was set as the normal size standard for that division, allowing for unbiased assessment of cell swelling among cell divisions. Pictures of swollen cells were taken using a Nikon D40 camera under 90X magnification. Cell area was measured by NIH ImageJ software. Results were graphed using MATLAB version 7.9 (R2009b) software (The Mathworks, Natick, MA).

CAG repeat tract PCR

CAG-70 tracts were chosen to analyze, as expansions are more reliably detected for this length. For the HU experiment, overnight WT CAG-70 cultures were grown for ~7.0 doublings in YC-Leu liquid media +/- 0.1 M HU, plated on YC-Leu solid media +/- 0.1 M HU, and allowed to form daughter colonies at 30 °C for 3 days. Alternatively, single cells from an overnight YC-Leu culture were micromanipulated on to YC-Leu solid media, and grown for ~3 days until they attained maximal sizes. A partial normal-sized colony or entire poorly growing colony (defined as growing to one-third or less the size of a normal colony) was used for colony PCR using conditions described in [12]. In a subset of colonies showing partial instability, PCR amplification products >10% or >30% of the intensity of the intact band respectively, were counted as an expansion or contraction event.

Fluorescence activated cell sorting (FACS) analysis to monitor cell cycle progression

Yeast strains were grown in 1 ml of YC-Leu liquid media with 2% glucose until late-log to early stationary phase. 1 ml of the culture (~1×10⁷ cells) was pelleted, washed 3X with sterile water, resuspended in cold 70% (w/v) ethanol followed by overnight incubation at 4 °C. Cells were subsequently pelleted, resuspended in 50 mM Tris·HCl (pH 7.5) buffer containing 1 mg/ml RNaseA, followed by overnight incubation at 37 °C. FACS analysis samples were prepared by pretreatment with 55 mM HCl with 5 mg/ml pepsin, washed and resuspended in FACS buffer (200 mM Tris pH 7.5; 211 mM NaCl; 78 mM MgCl₂ adjusted to pH 7.5 with HCl) containing 1 mg/ml propidium iodide, incubated at ~20 °C for 1 hour, transferred to 1 ml of 50 mM Tris pH 7.5 and subjected to sonication. The total cellular DNA content from an average of 100,000 cells was measured using a FACS-Calibur flow cytometer and BD CellQuest software. FACS plots were generated using ModFit LT software.

Immunoblotting to detect Rad53 phosphorylation in CAG repeat-containing cells

Total cellular protein was prepared using the trichloroacetic acid (TCA) method described in [51] for immunoblotting. Briefly, ~10⁶ exponentially growing cells (as determined by OD₆₀₀ and hemocytometer counting) were pelleted, washed and resuspended in 20% TCA. Samples were vortexed with glass beads, pelleted at 3000 rpm, boiled in Laemmli buffer (BioRad) and the resulting extracts clarified by centrifugation at 3000 rpm. 15 µg of total protein (quantitated by Bradford method) was loaded per lane; proteins were resolved on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (GE Amersham). The membrane was blocked in 5% milk in TBS-T (Tween, 0.1%), incubated with polyclonal, Rad53 primary antibody (Santa Cruz Biotechnologies) followed by washes in TBS-T (0.1%) and incubation with secondary Anti-goat HRP antibody (Santa Cruz Biotechnologies). Phosphorylated isoforms
of Rad53 were visualized by chemiluminescence (Millipore). Semi-quantitative densitometry of phosphorylated Rad53 isoforms was performed using the NIH ImageJ software on film exposures where the signal fell within the linear range. Resultant values were graphed using MS Excel software.

**Chromatin immunoprecipitation (ChIP)**

A strain with TAP-tagged Mre11 [52] and a CAG-155 repeat was used for ChIP. Yeast cultures grown to an OD$_{600}$ of 0.6 were either unsynchronized (Asynch) or synchronized in G1 with α-factor, released into S phase, and samples collected at the indicated time points. Chromatin samples were cross-linked using formaldehyde and processed according to [53]. Mre11-TAP:DNA complexes were immunoprecipitated using rabbit IgG agarose beads directed against protein A on the TAP tag (Sigma) [52].

Real-time quantitative PCR was used to amplify a 150 bp fragment 186 bp proximal to the CAG repeat using CAGfor and CAGrev primers in IP and whole cell extract (WCE) fractions and an untagged control strain. Similarly, a non-repeat reference locus (ACT1) was amplified from IP, WCE and untagged control fractions (amplicon length 146 bp, amplified using Act1for2 and Act1rev2 primers; all primers available upon request). $2^{ΔΔCt}$ value, i.e. fold enrichment of Mre11-TAP at CAG repeat-containing DNA fragment was obtained by normalizing CAG locus amplification to ACT1 locus amplification in IP and WCE samples. The untagged control showed no enrichment at the CAG locus over the ACT1 locus ($2^{ΔΔCt}$ value of 1-fold). PCR was performed using SYBR-green PCR mix (BioRad) on an ABI Prism 7300 sequence detection system. Each PCR reaction was set up in triplicate; PCR cycling conditions - 95°C for 15 s, 40 cycles of 95°C for 1 s, 60°C for 30 s. Asynch, 10 min and 20 min time points represent the average of 3, 3, and 4 independent experiments respectively; 0 and 40 min values derived from one experiment.

**Statistical analyses**

Analysis of variance (ANOVA) with a three-way Fisher’s LSD post-hoc test was used to compare microcolony areas. Doublin efficiency curves were analyzed using Wilcoxon’s sum-rank test. Logistic regression analysis using Chi-square statistics was used to perform 3X2 comparisons on pedigree data sets to determine repeat-specific effects within genotypes; statistically significant (p<0.05) interactions were further subjected to a Wald’s post-hoc test (see Table S1). A 2X2 Fisher’s exact test was used to compare among genotypes to determine gene specific effects at each CAG repeat length. Analyses were performed using either SAS version 9.1 (SAS Inc. 2003) or SPSS Statistics GradPack Version 17.0, 2008 software.

**Supporting Information**

**Figure S1** Repeat-containing cells show growth delays in liquid culture relative to a CAG-0 control strain. (A) and (B) represent growth curves of wild-type cells with expanded CAG-195 repeats or no-repeat control from two independent experiments. Note different X-axis intervals in graphs. Overnight cultures were diluted to a starting OD$_{600}$ of ~0.04 in YC-Leu liquid media and grown at 30°C for up to 72 hours. Growth was periodically measured using a spectrophotometer (Eppendorf) and viability is represented as optical density units (OD$_{600}$) plotted against time. Found at: doi:10.1371/journal.pgen.1001339.s001 (0.07 MB TIF)

**Figure S2** Cells with expanded CAG-70 or CAG-155 repeats experience protracted cell division cycles. (A) Wild-type, (B, C, D) DSB repair-deficient backgrounds. Graphs represent percent of divisions observed for each time window; sample size mean is 86 divisions; range of 31–183 divisions; Table S1. Data points include divisions that have completed and end divisions with indeterminate end points. Found at: doi:10.1371/journal.pgen.1001339.s002 (0.14 MB TIF)

**Figure S3** Replication stress leads to cell swelling in S and G2 phases as well as multibudded morphology. The WT CAG-0 strain was grown in YC-Leu liquid media in the presence of 0.1 M HU for 16 hours (a sub-lethal dose that causes replication stress but not full arrest); cells were classified as G1, S, G2/M or multibudded based on budding index. Numbers on graph represent data from two independent experiments; sample size scored for each treatment = 784 cells (−HU), 401 cells (+HU). * represents p<0.01 by Fisher’s exact test. Found at: doi:10.1371/journal.pgen.1001339.s003 (0.06 MB TIF)

**Figure S4** Cell cycle position of arrests in mre11Δ and dnl1Δ strains. Cell cycle position of arrests (>3.0 hrs) observed for cells dividing on YC-Leu solid media in the single cell pedigree analysis. Arrests defined as >60° for G1, >45° for S, >60° for G2/M. Not included are normal divisions <3.0 hours that show an occasional S phase or G2/M delay. Statistically significant comparisons to CAG-0 (♀) or between CAG-70 and CAG-155 strains (♀) within genotype are indicated. Statistics performed by logistic regression analysis on raw data calculated out of total number of divisions (Table S1).

Found at: doi:10.1371/journal.pgen.1001339.s004 (0.17 MB TIF)

**Table S1** Raw Numbers from Pedigree Analysis, with p-values. Found at: doi:10.1371/journal.pgen.1001339.s005 (0.17 MB DOC)

**Table S2** List of yeast strains used in the study. Found at: doi:10.1371/journal.pgen.1001339.s006 (0.13 MB DOC)

**Text S1** Differences between DNA damage checkpoint response and cellular senescences. Found at: doi:10.1371/journal.pgen.1001339.s007 (0.02 MB DOC)

**Acknowledgments**

We thank Michael Reed and Durwood Marshall for help with statistical analyses of the microcolony and pedigree data sets, Sara Lewis and Randi Rotjan for statistics discussions, Minda Berbeco for help with SPSS software, Melissa Koch and Jiahui Yang for RT-PCR analysis, Nevan Krogan for the Mre11-TAP tagged strain, and the Tufis CORE facility for FACS analysis.

**Author Contributions**

Conceived and designed the experiments: RS CHF. Performed the experiments: RS. Analyzed the data: RS CHF. Wrote the paper: RS CHF.

**References**

1. Richard GF, Kerrest A, Dujon B (2008) Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. Microbiol Mol Biol Rev 72: 686–727.
2. Orr HT, Zoghbi HY (2007) Trinucleotide repeat disorders. Annu Rev Neurosci 30: 575–621.
3. Brouwer JR, Willemsen R, Oostra BA (2009) Microsatellite repeat instability and neurological disease. Bioessays 31: 71–83.
4. Mirkin SM (2007) Expandable DNA repeats and human disease. Nature 447: 932–940.
Expanded CAG Repeats Induce DNA Damage Checkpoint

5. Samadashwily GM, Raga C, Mirkin SM (1997) Trinucleotide repeats affect DNA replication in vivo. Nat Genet 17: 298–304.

6. Pelletier R, Krasnikhova MM, Samadashwily GM, Laluse R, Mirkin SM (2003) Replication and expansion of trinucleotide repeats in yeast. Mol Cell Biol 23: 1349–1357.

7. Kerret A, Aanand RP, Sundararajan R, Bermejo R, Liberi G, et al. (2009) SRS2 and SGS1 prevent chromosomal breaks and stabilize trinucleotide repeats by restraining recombination. Nat Struct Mol Biol 16: 159–167.

8. Lu X, Kao HG, Bambara RA (2004) Flap endonuclease 1: a central component of DNA metabolism. Annu Rev Biochem 73: 589–615.

9. Panagihala GB, Lau R, Montgomery SE, Leonard MR, Pearson CE (2005) Slipped (CTG/CTG) repeats can be correctly repaired, escape repair or undergo reverse-repeat repair. Nat Struct Mol Biol 12: 654–662.

10. Freudenreich CH (2007) Chromosome fragility: molecular mechanisms and cellular consequences. Front Biosci 12: 4911–4924.

11. Kim HM, Narayanan V, Mieraczkowski PA, Peters TD, Krasnikhova MM, et al. (2008) Chromosome fragility at GAA tracts in yeast depends on repeat orientation and requires mismatch repair. EMBO J 27: 2896–2906.

12. Sundararajan R, Gellon L, Zander RM, Freudenreich CH (2010) Double-strand break repair pathways protect against CAG/CTG repeat expansions, contractions, and repeat-mediated chromosomal fragility in Saccharomyces cerevisiae. Genetics 184: 65–77.

13. Krogsh BO, Nyminngton LS (2004) Replication proteins in yeast. Ann Rev Genet 38: 233–271.

14. Libby M, Rosenstein R (2008) Chevronography of replication complexes during the DNA damage response. DNA Repair (Amst) 7: 1068–1076.

15. Soja GM, Lopes M, Foiani M (2002) Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science 297: 599–602.

16. Harrison JC, Haher JE (2006) Surviving the breakup: the DNA damage response. Annu Rev Genet 40: 209–235.

17. Putnam CD, Jahnig EJ, Kolodner RD (2009) Perspectives on the DNA damage and replication checkpoint responses in Saccharomyces cerevisiae. DNA Repair (Amst) 8: 974–982.

18. Paulus and G, Hartwell LH (1995) A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell 82: 841–847.

19. Lopes M, Cotta-Ramusino G, Pellicoli A, Liberi G, Plevani P, et al. (2001) The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412: 557–561.

20. Redon C, Pilch DR, Roagkou EP, Orr AH, Lowndes NF, et al. (2003) Yeast replication proteins that increase CAG/CTG expansions also increase instability by at least two mechanisms. DNA Repair (Amst) 7: 633–640.

21. Toczyski DP, Galgoczy DJ, Hartwell LH (1997) CDC5 and CKII control checkpoints and replication checkpoint responses in Saccharomyces cerevisiae. Hum Mol Genet 6: 2341–2348.

22. Kuzminov A (2001) DNA replication meets genetic exchange: chromosomal damage and its repair by homologous recombination. Proc Natl Acad Sci U S A 98: 8461–8468.

23. Balakumaran BS, Freudenreich CH, Mirkin SM (2009) Repair-induced fragility, replication checkpoint and replication-activated DNA damage checkpoint. DNA Repair (Amst) 8: 1068–1076.

24. Freudenreich CH (2007) Chromosome fragility: molecular mechanisms and cellular consequences. Front Biosci 12: 4911–4924.

25. Weinert TA, Hartwell LH (1986) The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science 234: 317–322.

26. Lee SE, Moore JK, Colombo R, Kolodner RD, et al. (1998) Saccharomyces Ky70, mrr1/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell 94: 399–409.

27. Perini JH, Stracker TH (2003) The cellular response to DNA double-strand breaks: defining the sensors and mediators. Trends Cell Biol 13: 458–462.

28. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

29. Hartwell LH, Culotti J, Pingle JK, Reid BJ (1974) Genetic control of the cell division cycle in yeast. Science 183: 46–51.

30. Entezam A, Usdin K (2008) ATR protects the genome against CGG.CCG triplet repeats in mice. Mol Biol Cell 19: 1116–1124.

31. Freudenreich CH, Kastrow SM, Zakian VA (1998) Expansion and length-dependent fragility of CTG repeats in yeast. Science 279: 853–856.

32. Lin Y, Long M, Wan M, Wilson JH (2010) Convergence of transcription through a Long CAG Tract Destabilizes Repeats and Induces Apoptosis. Mol Cell Biol. 31: 674–684.

33. Balakumaran BS, Freudenreich CH, Mirkin SM (2009) Repair-induced fragility, replication checkpoint and replication-activated DNA damage checkpoint. DNA Repair (Amst) 8: 1068–1076.

34. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

35. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

36. Enserink JM, Smulski MB, Zhou H, Kolodner RD (2006) Checkpoint proteins control morphogenetic events during DNA replication stress in Saccharomyces cerevisiae. J Cell Biol 175: 729–741.

37. Jiang YW, Kang CM (2003) Induction of S. cerevisiae filamentous differentiation by slowed DNA synthesis involves Mec1, Rad53 and Swi1 checkpoint proteins. Mol Biol Cell 14: 5116–5124.

38. Freudenreich CH, Kastrow SM, Zakian VA (1998) Expansion and length-dependent fragility of CTG repeats in yeast. Science 279: 853–856.

39. Lin Y, Long M, Wan M, Wilson JH (2010) Convergence of transcription through a Long CAG Tract Destabilizes Repeats and Induces Apoptosis. Mol Cell Biol. 31: 674–684.

40. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

41. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

42. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

43. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

44. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

45. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.

46. Vaze MB, Pellicoli A, Lee SE, Iza G, Liberi G, et al. (2002) Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Mol Cell 10: 373–385.