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TALEN-Induced Double-Strand Break Repair of CTG Trinucleotide Repeats

Graphical Abstract

Highlights

- RAD50, SAE2, and RAD52 are involved in repairing a DSB within a CTG repeat
- POL32, DNL4, and RAD51 do not play a role in repairing a DSB within a CTG repeat
- Resection of a DSB within a CTG repeat needs the Mre11-Rad50 complex, as well as Sae2
- The double-strand break is repaired by an iterative single-strand annealing process

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In Brief

Mosbach et al. show that a TALEN could successfully contract an expanded CTG repeat tract below pathological length. The TALEN double-strand break needs Rad50, Rad52, and Sae2 to be repaired. A double-strand break end containing a long trinucleotide repeat needs both Rad50 and Sae2 to be efficiently resected.
TALEN-Induced Double-Strand Break Repair of CTG Trinucleotide Repeats

Valentine Mosbach, Lucie Poggi, David Viterbo, Marine Charpentier, and Guy-Franck Richard

INTRODUCTION

Microsatellite expansions are responsible for more than two dozen neurological or developmental disorders in humans. Among the most common sequences involved are CAG/CTG trinucleotide repeat tracts, whose expansions are the cause of Huntington’s disease, myotonic dystrophy and Huntington’s disease. Because expansions trigger the disease, contracting repeat length could be a possible approach to gene therapy for these disorders. Here, we show that a TALEN-induced double-strand break was very efficient at contracting expanded CTG repeats in yeast. We show that RAD51, POL32, and DNL4 are dispensable for double-strand break repair within CTG repeats, the only required genes being RAD50, SAE2, and RAD52. Resection was totally abolished in the absence of RAD50 on both sides of the break, whereas it was reduced in a sae2Δ mutant on the side of the break containing the longest repeat tract, suggesting that secondary structures at double-strand break ends must be removed by the Mre11-Rad50 complex and Sae2. Following the TALEN double-strand break, single-strand annealing occurred between both sides of the repeat tract, leading to repeat contraction.

SUMMARY

Trinucleotide repeat expansions involving CTG/CAG triplets are responsible for several neurodegenerative disorders, including myotonic dystrophy and Huntington’s disease. Because expansions trigger the disease, contracting repeat length could be a possible approach to gene therapy for these disorders. Here, we show that a TALEN-induced double-strand break was very efficient at contracting expanded CTG repeats in yeast. We show that RAD51, POL32, and DNL4 are dispensable for double-strand break repair within CTG repeats, the only required genes being RAD50, SAE2, and RAD52. Resection was totally abolished in the absence of RAD50 on both sides of the break, whereas it was reduced in a sae2Δ mutant on the side of the break containing the longest repeat tract, suggesting that secondary structures at double-strand break ends must be removed by the Mre11-Rad50 complex and Sae2. Following the TALEN double-strand break, single-strand annealing occurred between both sides of the repeat tract, leading to repeat contraction.

INTRODUCTION

Microsatellite expansions are responsible for more than two dozen neurological or developmental disorders in humans. Among the most common sequences involved are CAG/CTG trinucleotide repeat tracts, whose expansions are the cause of Huntington’s disease, myotonic dystrophy type 1 (DM1 or Steinert disease), and several spinocerebellar ataxias (Orr and Zoghbi, 2007). Despite having been under investigation for more than two decades, the molecular mechanism(s) leading to large expansions is not completely understood, although it is generally accepted that secondary structures formed by these microsatellites may be triggering or amplifying the expansion process (McMurray, 1999). It was shown that CAG and CTG trinucleotide repeats form imperfect hairpins in vitro (Gacy et al., 1995; Yu et al., 1995a, 1995b). In addition, there is biochemical and genetical evidence that CAG and CTG hairpins interfere with the mismatch repair machinery, an important player of the expansion process, although its precise role is not totally clear (Foiry et al., 2006; Manley et al., 1999; Owen et al., 2005; Pearson et al., 1997; Pinto et al., 2013; Savouret et al., 2004; Slean et al., 2016; Tian et al., 2009; Tomé et al., 2009, 2013; Viterbo et al., 2016; Williams and Surtees, 2015). Most trinucleotide repeat transmissions from parents to children lead to repeat tract expansion. However, it seldom happens that a large allele contracts to a shorter one. Indeed, in a family affected by DM 1, it was reported that a daughter inherited a contracted allele from her father by a mechanism likely to be gene conversion (O’Hoy et al., 1993). The daughter was followed until the age of 17 and did not develop any of the symptoms of the pathology, showing that a large repeat contraction prevented this kind of disease.

Recent attempts were made to cure trinucleotide repeat disorders by gene therapy. In Huntington’s disease pluripotent stem cells, an expanded CAG repeat in the Huntington’s disease (HD) gene was replaced by a smaller allele by homologous recombination. In corrected cells, HD disease phenotypes were reversed (An et al., 2012). Two independent groups used SpCas9 either to induce one single double-strand break upstream of the FMR1 CGG repeat (Park et al., 2015) or two double-strand breaks (DSBs) upstream and downstream of the repeat tract (Xie et al., 2016). In both cases, FMR1 reactivation was observed in edited cells. More recently, SpCas9 was used to delete the expanded CTG triplet repeat at the DM1 locus by making a DSB upstream and/or downstream of the repeat tract. Again, disease phenotypes were partially suppressed in DM1 myoblasts (van Agtmaal et al., 2017). In all of these cases, DSBs were always induced outside and never inside the trinucleotide repeat tract.

DSB repair is one of the molecular processes leading to trinucleotide repeat contractions and expansions. It was formerly shown that a DSB made by the I-Sce I meganuclease within a short CTG repeat tract often led to the loss of the nuclease recognition site and contraction of the repeat tract (Richard et al., 1999). In less frequent cases, it led to both expansions and contractions of the repeat tract by gene conversion during mitosis (Richard et al., 1999, 2000) or meiosis (Richard et al., 2003). Following these early experiments, zinc-finger nucleases (ZFNs) were used to direct a DSB within a CAG or CTG trinucleotide repeat tract. In two separate studies from the same lab, induction of a ZFN in Chinese hamster ovary (CHO) cells led to a 15-fold increase in repeat contractions. However, deletions in...
one or both flanking regions were observed in 20% of the cases, whereas insertions of exogenous DNA at the DSB site were found in another 24% of the cases (Mittelman et al., 2009; Santillan et al., 2014). Different authors used another ZFN expressed in HeLa cells containing CAG/CTG trinucleotide repeats integrated in the two possible orientations compared with replication fork progression. They observed contractions as well as expansions of the repeat tract when both ZFN arms were expressed, but only contractions were recovered when one single arm was expressed (Liu et al., 2010). This suggested that one arm of the ZFN was able to homodimerize and induce a DSB by itself. Using a *Saccharomyces cerevisiae* strain in which a large CTG triplet repeat from a DM1 patient was integrated in a yeast chromosome, we were recently able to show that induction of a transcription activator-like effector (TALE) nuclease (TALEN) induced contractions of a CTG triplet repeat at a high frequency. Pulse-field gel electrophoresis and genome-wide deep sequencing showed that no other mutation, duplication, or chromosomal rearrangement was induced by the TALEN outside of the repeat tract (Richard et al., 2014). These experiments demonstrated that this new family of nucleases was efficient and specific enough to envision their possible use as a future gene therapy tool in human cells (Richard, 2015). Using a different approach, Cinesi et al. (2016) recently showed that inducing single-strand breaks within a CTG repeat tract using the Cas9D10A mutant nickase also promoted contractions of the repeat tract in model human cells.

Mechanisms of DSB repair have been studied in yeast for several decades, and the main proteins involved in this process have been identified (Krogh and Symington, 2004). A large part of these advances was made possible by the use of highly specific meganucleases such as Ho or I-Sce I (Fairhead and Dujon, 1993; Haber, 1995; Plessis et al., 1992). However, the fate of a single DSB made within a long repeated and structured DNA sequence was never addressed before.

One of the goals of the present work was to study the role of several recombination genes (namely, RAD50, RAD51, RAD52, DNL4, SAE2, and POL32) in the repair of a single DSB made within a long CTG trinucleotide repeat. RAD52 encodes a mediator multimeric protein controlling homologous recombination pathways (gene conversion, single-strand annealing [SSA], and break-induced replication [BIR]) (Davis and Symington, 2004; Krogh and Symington, 2004; Sugawara and Haber, 1992). RAD51 is a RecA homolog responsible for nucleofilament formation and subsequent strand exchange and gene conversion (Shinohara et al., 1992; Sung, 1994). RAD50 belongs to the multifunctional Mre11-Rad50-Xrs2 complex involved, along with Sae2, in DSB end clamping and resection during meiosis as well as mitosis (Borde et al., 2004; Lee et al., 1998; Mimitou and Symington, 2008; Zhu et al., 2008). DNL4 encodes ligase IV, the protein responsible for the ligation step during non-homologous end joining (Wilson et al., 1997), and POL32 is part of the polymerase δ complex and was shown to be essential for BIR (Lydeard et al., 2007) as well as to be an important player in microhomology-mediated repair (Villarreal et al., 2012).

*Rad50* was found to be essential to resect both DSB ends, whereas *SAE2* was needed to resect only the DSB end that contains most of the triplet repeat tract. This observation supports the presence of secondary structures that need a functional Sae2 activity to be removed. *RAD52* was also required to repair the DSB but not *RAD51*, *POL32*, or *DNL4*, suggesting an iterative SSA process that progressively leads to repeat shortening.

**RESULTS**

A DSB induced within CTG Repeats Requires the Mre11-Rad50 Complex to be Processed

In the present work, two TALENs were used. The TALEN<sub>CTG</sub> was the same as the one used in our former publication, designed to induce a DSB within a modified *SUP4* allele containing expanded CTG triplets from the dystrophia myotonica protein kinase (DMPK) human locus (Richard et al., 2014). The TALEN<sub>noCTG</sub> was designed to induce a DSB within a modified *SUP4* allele containing an I-Sce I recognition site (Richard et al., 1999). The trinucleotide repeat tract lengths used here ranged from 20–50 triplets for short alleles to 70–90 triplets for long alleles. In a first series of experiments, the TALEN<sub>CTG</sub> was expressed in wild-type yeast cells and in isogenic strains mutated for DSB repair genes. Both TALEN<sub>CTG</sub> arms were carried on centromeric vectors, and their expression was under control of an inducible TetOFF promoter. DSB formation was followed during a time course by Southern blot analysis. When the TALEN<sub>CTG</sub> was repressed, uncut chromosomes containing CTG repeats of two different lengths were visible. When the TALEN<sub>CTG</sub> was expressed, signals corresponding to DSB formation were detected (Figure 1A). By using probes specific to each side of the repeat tract, it was possible to distinguish between signals corresponding to 5′ or 3′ ends of the DSB (Figure S1). The 5′ end of the break, containing the long CTG tract, appears like a smear. This smear corresponds to different repeat lengths because of progressive CTG repeat contraction over time. The 3′ end of the DSB appears as a sharper band because it contains only a few triplets. The DSB was not visible before 14 hr after TALEN<sub>CTG</sub> induction (time point labeled 0, Figure 1A). Quantification showed that the maximum of broken molecules was reached 4–6 hr after T0 for all strains except rad50Δ and sae2Δ (Figure 1B).

To determine how long it would take for cells to completely repair the DSB, a longer time course was run in wild-type cells over 72 hr after TALEN induction (58 hr after DSB formation; Figure 2). This experiment was set up in haploid cells to discriminate between the parental (uncontracted) allele and the contracted allele recovered after DSB repair. Cells were collected at several time points, with particular attention to the 34–46 hr time range. Total genomic DNA was extracted, and Southern blot was run as described previously (Figure 2A). Signal quantification showed that, during the first 40 hr in which ~12% of chromosomes were broken, the DSB signal stayed stable. After that time, it increased to ~20% of broken molecules and stayed at the same level until the end of the time course (Figure 2B). This result may be interpreted in two ways: (1) the nuclease was not active in all cells at the same time. Therefore, only a subfraction of repeat tracts was cleaved in the first 40 hr and another, larger, subfraction was cleaved later on. (2) A first burst of DSBs partially contracted repeat tracts in all cells. A second round of DSBs cleaved shortened repeats more efficiently because they
were more accessible to the nuclease. This experiment also showed that smear length progressively decreased over time, although the 5'0 smear intensity was too low to be reliably quantified (Figure 2A). Given the rate of decrease of the parental band, it is expected to see its complete disappearance after 6 days of induction (Figure 2C).

Time courses for dn14Δ, pol32Δ, and rad51Δ mutants were similar to the wild-type strain, with a maximum of 12.4% of broken molecules in the dn14Δ mutant (Figures 1A and 1B). We concluded that none of these mutants showed a detectable effect on DSB repair kinetics. On the contrary, in the rad50Δ mutant, an accumulation of DSBs was observed (Figure 1A). On both sides of the break, a signal increase was clearly detected (Figure 1B). This suggested that a DSB induced in CTG repeats was not correctly processed in this mutant, leading to an accumulation of unrepaired broken molecules. In the sae2Δ mutant, the 5' and 3' ends of the DSB showed an asymmetric increase compared with rad50Δ cells. The DSB end containing most of the trinucleotide repeat tract (5' end) shows the same accumulation rate as in rad50Δ, whereas the other DSB end, containing only a few triplets, accumulates more slowly (Figures 1A and 1B).

Figure 1. DSB Induction by a TALENCTG within CTG Repeats
(A) Southern blots of yeast strains during DSB induction. For each wild-type and mutant strain, cells were collected at different time points after induction (+Dox or –Dox). The time point labeled “0” represents the first time point which the DSB was detectable. For all experiments, it corresponds to 14 hr after induction (+Dox or –Dox). When the TALENCTG was repressed (OFF), no band corresponding to the DSB was visible. When the TALENCTG was induced (ON), several signals were detected: a smear corresponding to successive contractions of the large trinucleotide repeat tract located 5' of the DSB, a band corresponding to the 5' end of the DSB with only a few triplets left, and another band corresponding to the 3' end of the DSB containing 1–4 triplets. The cartoons at the left describe the different molecules detected. Blue triangles indicate the location of EcoRV sites used for restriction digestion before Southern blotting. Scissors indicate the DSB location.

(B) Quantification of 5' and 3' DSB signals. Note that the time course was run during 34 hr only in the rad50Δ and sae2Δ strains.

(C) Terminal transferase-mediated PCR. After DSB induction, dCTP was added to both 3' strands by terminal transferase. The 3' end of the break was subsequently amplified with a poly-dG oligonucleotide and another primer specific of the 3' end of the DSB. Note that additional time points and controls were present on the same gels, but only significant time points are shown here.

(D) Results of terminal transferase-mediated PCR sequences. The repeat tract is shown in the blue box, and the right TALENCTG binding site is indicated by red letters. The locations of the four TALENCTG DSBs sequenced are indicated by black arrowheads.

One time course was performed for wild-type, lig4Δ, rad51Δ, and pol32Δ strains. For the rad50Δ and sae2Δ strains, values are the average of two or three independent experiments, depending on the time points considered. Error bars correspond to one SD.
In the rad52Δ mutant, no signal corresponding to DSB formation could be detected by Southern blot (Figure S2A). TALEN expression was verified by western blot analysis (Figure S3). In the presence of doxycycline, no signal was detected in any of the strains. In the absence of doxycycline, the hemagglutinin (HA)-tagged TALEN was clearly detected in all strains. Its relative level was similar in rad50Δ, rad51Δ, and rad52Δ strains but 10-fold higher in wild-type cells. We concluded that the absence of visible DSB in the rad52Δ strain was not due to a lack of expression of the nuclease because it was present in similar amounts in the two other mutant strains, in which the DSB was clearly detected. To check whether the absence of detectable DSB was due to some mutation unrelated to the RAD52 deletion itself, we performed the same experiment in a rad52Δ/RAD52 heterozygote. In this strain, the DSB was clearly visible, showing that complementing the rad52Δ deletion with a RAD52 gene restored the wild-type phenotype (Figure S2B).

Subsequently, a terminal deoxynucleotidyl transferase-mediated PCR approach was used to amplify the DSB (Forstmann et al., 2000). PCR products were visible in the wild-type and rad50Δ strains used as positive controls at 8 hr and 10 hr, but no product was detected when the TALENCTG was repressed (Figure 1C). PCR products corresponding to DSB amplification were also visible in the rad52Δ mutant when the TALENCTG was expressed, but very faintly. We concluded that DSBs occurred as expected within the repeat tract in the rad52Δ strain but that their level was too low to be detected by Southern blot. Sanger sequencing of the terminal end of the PCR product generated by terminal deoxynucleotidyl transferase-mediated PCR products showed that the DSB occurred in the very last 1–4 CTG triplets of the repeat tract (Figure 1D).

**Figure 2. Expression of a TALENCTG during a 72-hr Time Course**

(A) Southern blot of a time course in a haploid strain containing only one CTG repeat allele, for 3 days. TALEN expression was verified by western blot analysis (Figure S3). In the presence of doxycycline, no signal was detected in any of the strains. In the absence of doxycycline, the hemagglutinin (HA)-tagged TALEN was clearly detected in all strains. Its relative level was similar in rad50Δ, rad51Δ, and rad52Δ strains but 10-fold higher in wild-type cells. We concluded that the absence of visible DSB in the rad52Δ strain was not due to a lack of expression of the nuclease because it was present in similar amounts in the two other mutant strains, in which the DSB was clearly detected. To check whether the absence of detectable DSB was due to some mutation unrelated to the RAD52 deletion itself, we performed the same experiment in a rad52Δ/RAD52 heterozygote. In this strain, the DSB was clearly visible, showing that complementing the rad52Δ deletion with a RAD52 gene restored the wild-type phenotype (Figure S2B).

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**DSB Accumulation in rad50Δ Depends on CTG Repeats**

To determine whether DSB accumulation was only dependent on the presence of CTG repeats at the end of the DSB, a second TALEN was designed to recognize a SUP4 allele that did not contain a repeat tract. This TALEN was called TALENnoCTG to distinguish it from the TALENCTG. In the wild-type strain, the DSB signal was weaker because only one of the two chromosomes could be cut by the TALENnoCTG (Figures 3A and 3B).
The smear detected when the TALEN<sub>CTG</sub> was induced was not visible with the TALEN<sub>noCTG</sub>, proving that it corresponds to different repeat lengths because of progressive repeat contraction over time. The number of broken molecules increased at a slower rate over time compared with the TALEN<sub>CTG</sub>. Six hours after DSB, non CTG-containing ends are four times less abundant than CTG-containing ends. Repeat-containing broken molecules are more persistent, suggesting that non CTG-containing ends are repaired faster than CTG-containing ends. In the rad50Δ mutant, the DSB also accumulates in the TALEN<sub>noCTG</sub> strain compared with the TALEN<sub>CTG</sub> strain (Figure 3C). However, the amount of non CTG-containing ends decreases slowly after 10 hr, whereas CTG-containing ends keep on accumulating. We concluded that cut fragments were greatly stabilized in the absence of RAD50 but that repair of non-repetitive ends eventually occurs at the last time point, whereas it is definitely compromised when CTG-containing ends need to be repaired. This strongly suggests that these repeats form secondary structures at DSB ends that need the Mre11-Rad50-Xrs2 (MRX) complex to be removed for repair to occur.

**Figure 3. DSB Induction by a TALEN<sub>noCTG</sub> within a Non-repeated Region**

(A) Southern blots of time courses during DSB induction of the TALEN<sub>noCTG</sub> in wild-type and rad50Δ strains. The asterisk indicates an extra band only visible in the rad50Δ strain, probably corresponding to some chromosomal rearrangement specific to this mutant.

(B) Quantifications of the TALEN<sub>noCTG</sub> 5' and 3' DSB signals and comparisons with the TALEN<sub>CTG</sub>. For each time point, the amounts of 5' or 3' signals were quantified and plotted as a ratio of the total signal in the lane. One time course was performed for the wild-type strain. For the rad50Δ strain, values are the average of two or three independent experiments, depending on the time points. Error bars correspond to one SD.

**DSB Resection within CTG Repeats Is Almost Completely Abolished in rad50Δ and sae2Δ**

DSB resection was determined by qPCR of total genomic DNA preliminarily digested by a restriction endonuclease (EcoRV in the present case) (Chen et al., 2013). Restriction sites that were resected during the course of the experiment could not be digested by EcoRV because of their single-stranded nature and could therefore be amplified by PCR primers located around the restriction site. Comparisons of cycle threshold (Ct) obtained in the fraction digested by EcoRV with Ct obtained in the undigested fraction was indicative of the amount of resection at this particular restriction site. Four EcoRV sites were studied: two of them located 800–900 base pairs (bp) upstream and downstream of the CTG repeat and two located further away, 1.8–2.9 kb upstream and downstream of the CTG repeat (Figure 4). In all experiments, raw resection and relative resection values were calculated. Raw resections were computed as a percentage of PCR product amplified in EcoRV digested compared with non-digested fractions (Experimental Procedures). Relative resection values were calculated as the ratio of raw values to DSB amounts quantified on Southern blots.
Relative values of resection with the TALEN CTG. Same as (A), except that resection values were divided by the amount of DSBs detected on Southern blots.

In the rad52Δ strain, resection was not different from the wild-type at proximal sites but increased at distal sites, suggesting that, in the absence of RAD52, resecting enzymes have a better access to DNA ends.

In the wild-type strain expressing the TALEN noCTG, raw as well as relative resection values were much higher compared with the TALEN CTG values, particularly at distal EcoRV sites. In addition, there was no detectable difference between resection values at proximal versus distal sites. In the rad50Δ mutant expressing the TALEN noCTG, resection was increased 34 hr after DSB formation compared with the TALEN CTG rad50Δ strain, but this increase was found to be significant only at distal restriction sites. In the rad50Δ strain as well as in the sae2Δ mutant to become barely detectable at early time points. Note that SAE2 was found to be more important for relative resection on the DSB end containing the longer repeat tract, whereas RAD50 was required on both DSB ends (Figure 4B; VMS20/VMAS20, p = 0.04).

Because DSB signals in the rad52Δ strain were too low to be quantified, relative resection values could not be calculated for this mutant.

In the wild-type strain, raw resection as well as relative resection values were significantly higher at sites proximal to the DSB (VMS20/VMAS20 and VMS22/VMAS22) than at distal sites (VMS21/VMAS21 and VMS23/VMAS23) (Figures 4A and 4B; two-tailed t test p = 0.0085). Resection values dramatically dropped in the rad50Δ strain as well as in the sae2Δ mutant to repair a DSB in non-repeated DNA (Figure 5).

Survival of the DSB was determined in each strain by the ratio of colony-forming units (CFUs) when the TALEN CTG was expressed compared to when the TALEN noCTG was repressed. In wild-type diploid cells, survival was 62.5% ± 3.7%, not significantly different from pol32Δ and rad51Δ mutant strains (64.4% ± 20.4%, respectively), although a somewhat higher SD was observed for rad51Δ (Figure 5). Therefore, the absence of these genes did not significantly affect DSB repair efficacy, consistent with similarities in DSB formation and processing during time courses (Figure 1). On the contrary, the absence of RAD50 or RAD52 led to a higher mortality because only 19.9% ± 3.4% of cells survived in the rad50Δ strain and 18.2% ± 4.7% survived in rad52Δ cells, proving that the product of both genes was required to repair the break. Survival in diploid strains heterozygous for the rad50Δ or the rad52Δ deletion was not significantly different from the wild-type (Figure S2C). When a chromosome that did not contain CTG repeats was cut by the TALEN noCTG in the rad50Δ strain, survival was not significantly different from the wild-type, confirming that this gene product was not essential to repair a DSB in non-repeated DNA (Figure 5).

**Figure 5. Survival of TALEN-Induced DSBs**

Left: survival in diploid cells carrying two alleles of different trinucleotide repeat tract lengths. In these strains, both chromosomes carry trinucleotide repeats and were cut by the nuclease, as shown in the bottom cartoon. Center: survival in haploid cells carrying only one repeat tract. Right: survival in diploid cells carrying only one repeat tract. In these strains, only the chromosome that did not carry trinucleotide repeats was cut by the nuclease. Survival values are the average of two to five experiments, except for pol32Δ. Error bars are equal to one SD.

RAD50, RAD52, and SAE2 Are Needed to Repair a TALEN-Induced DSB

Survival of the DSB was determined in each strain by the ratio of colony-forming units (CFUs) when the TALEN CTG was expressed to CFUs when the TALEN noCTG was repressed. In wild-type diploid cells, survival was 62.5% ± 3.7%, not significantly different from pol32Δ and rad51Δ mutant strains (64.4% ± 20.4%, respectively), although a somewhat higher SD was observed for rad51Δ (Figure 5). Therefore, the absence of these genes did not significantly affect DSB repair efficacy, consistent with similarities in DSB formation and processing during time courses (Figure 1). On the contrary, the absence of RAD50 or RAD52 led to a higher mortality because only 19.9% ± 3.4% of cells survived in the rad50Δ strain and 18.2% ± 4.7% survived in rad52Δ cells, proving that the product of both genes was required to repair the break. Survival in diploid strains heterozygous for the rad50Δ or the rad52Δ deletion was not significantly different from the wild-type (Figure S2C). When a chromosome that did not contain CTG repeats was cut by the TALEN noCTG in the rad50Δ strain, survival was not significantly different from the wild-type, confirming that this gene product was not essential to repair a DSB in non-repeated DNA (Figure 5).
In haploid wild-type cells that are unable to repair the DSB by homologous recombination, 76.5% ± 4.3% of cells survived. This frequency slightly decreased in the dnl4Δ mutant (57.3% ± 16%) but was not significantly different from the wild-type (t test, p = 0.06). A significant decrease in survival was observed in haploid sae2Δ cells (20.5% ± 64.3%), proving that this gene was also needed to repair such a break.

**DSB Repair within CTG Repeat Tracts Is Mainly an Intramolecular Mechanism**

Repeat lengths were analyzed in several surviving colonies after TALEN<sub>CTG</sub> induction in wild-type and mutant strains by two different techniques. First, Southern blots were run on yeast colonies for each strain to determine the overall range of allele contractions (Figure 6A). Then a subset of these colonies was PCR-amplified at the repeat locus and Sanger-sequenced. When both alleles carried repeat tracts of the same exact length, the sequence was very clear before and after the repeat tract, as shown in Figure 6B. Among survivors, only 11.1% (51.6%) exhibiting very large contractions (only 4–10 CTG triplets, most of them small repeats present on both sides of the break, eventually resulting in large repeat contractions. Two arguments support iterative cycles of repeat contractions. First, progressive contractions of repeat tracts occurred between time points during TALEN induction (Figure 7A). Second, the reduction of smear length was clearly visible on Southern blots over the duration of a time course (Figures 2 and 7B).

**DISCUSSION**

**Integrity of Sae2 and of the Mre11-Rad50 Complex Is Essential for DSB Processing within CTG Repeats**

Former studies showed that Mre11 was not required to process “clean ends” such as those resulting from multiple HO DSBs in yeast (Llorente and Symington, 2004), although resection and single-strand DNA formation were delayed when the Mre11-Rad50 complex was not functional (Lee et al., 1998; Sugawara and Haber, 1992). Here we show that, when a DSB was induced in a rad50Δ strain in non-repeated DNA (TALEN<sub>noCTG</sub>), resection and repair were delayed, but survival was not significantly decreased, confirming that the Mre11-Rad50 complex was not essential to resect clean ends (Figure 4C).

On the contrary, resection and repair of a TALEN-induced DSB within a CTG trinucleotide repeat was completely abolished in a rad50Δ strain (Figures 4A and 4B). This result is consistent with a former work in which a natural chromosomal break within a long CTG repeat tract in yeast was left unrepaired and accumulated in a rad50Δ strain in such proportions that it was possible to detect the broken chromosome by pulse-field gel electrophoresis (Freudenchreich et al., 1998). It is also compatible with a recent study using Xenopus egg extracts in which Liao et al. (2016) showed that Mre11 was essential for resection of DNA with 3’ damaged nucleotides and 3’ or 5’ bulky adducts.

Sae2 as well as the Mre11-Rad50 complex were previously shown to be required to resolve hairpin-capped natural DSBs in yeast cells (Lobachev et al., 2002). Consistent with this study, the purified Sae2 protein was shown to exhibit endonuclease activity on DNA gaps close to a hairpin structure, and this activity was stimulated by the Mre11-Rad50 complex (Lengsfeld et al., 2007). Later on, Sae2 was shown to be involved in resection at the MAT locus following HO DSB only in the absence of the single-strand binding protein Rfa1. In that particular case, Sae2 was required to remove hairpin-like folded back structures at DSB ends (Chen et al., 2013). In our present experiments, a clear absence of resection was observed in the sae2Δ mutant on the 5’ DSB end that contained most of the repeat tract
Figure 6. Molecular Analysis of CTG Repeat Length after DSB Repair

(A) Representative Southern blot showing 14 haploid colonies in which the TALEN_CTG was induced. In each lane, total genomic DNA was extracted from one single yeast colony and analyzed. Two molecular weight markers were used: the GeneRuler 1-kb ladder (Sigma-Aldrich) on the left and a homemade CTG repeat tract length on the right (Viterbo et al., 2016). The red dotted line shows the parental CTG repeat tract length. In colonies after TALEN_CTG induction, one or more bands containing contracted CTG repeat tracts were detected.

(B) Molecular analysis of cells after TALEN_CTG induction. DNA was extracted from colonies after DSB induction, and the repeat containing-locus was sequenced. After Sanger sequencing of the PCR product, two outcomes could be obtained. When the two alleles contained the same exact number of triplets, one unique sequence was clearly read (homozygous, in orange); when the two alleles contained different numbers of triplets, the sequence was blurry and unreadable after the shortest of the two repeat tracts (heterozygous, in blue). Repeat lengths are given in number of triplets. The number of colonies sequenced in each strain was as follows: WT, 36; pol32Δ, 16; rad50Δ, 31; rad51Δ, 29; rad52Δ, 53. Chi-square test values (degrees of freedom [ddl] = 3) of comparisons between wild-type and mutant distributions are indicated above each graph. Only two distributions (in red) are significantly different from the wild-type: rad50Δ and rad52Δ.

(C) The same as (B), except that repeat tract lengths were compared between the wild-type strain and dnl4Δ or sae2Δ haploid strains, so only one trinucleotide repeat allele was present. The number of colonies sequenced in each strain was as follows: WT, 32; dnl4Δ, 40; sae2Δ, 18. Chi-square test values of comparisons between wild-type and mutant distributions are indicated above each graph (ddl = 2).
On the 3' end, though, resection was reduced at the proximal site compared with the wild-type but was higher than in the rad50Δ mutant. It is possible that the few triplet repeats left at the 3' end after DSB induction are sufficient to form a small hairpin that decreases or delays resection. This delay might also explain why resection at the distal EcoRV site is decreased on the 3' end of the DSB. We concluded that DSB ends of CTG trinucleotide repeats most probably harbor some kind of secondary structures, are therefore not clean, and absolutely require a functional Mre11-Rad50 complex as well as Sae2 to be processed for repair to start.

On the opposite, resection was increased at longer distances in the rad52Δ mutant (Figure 4A). This suggests that binding of the Rad52 protein on early recombination intermediates interferes with DNA end resection, as already shown in former publications (Van Dyck et al., 1999; Frank-Vaillant and Marcand, 2002; Parsons et al., 2000; Ristic et al., 2003; Sugawara and Haber, 1992; White and Haber, 1990). It is at the present time unclear how competition occurs and is resolved between Rad52p and resection proteins.

Differences between Yeast and Mammalian Systems
Former works showed the role of the Mre11-Rad50 complex on natural CTG trinucleotide repeat expansions in yeast (Sundararajan et al., 2010; Ye et al., 2016). When an I-Sce I DSB was repaired using a long CTG trinucleotide repeat as a template, expansions occurring during DSB repair were larger in strains overexpressing MRE11 or RAD50 (Richard et al., 2000). In the present experiments, DSB induction in long CTG repeats only led to contractions of the repeat tract, in wild-type as well as in rad50Δ strains (Figure 6B), and no expansion was ever observed. Cinesi et al. (2016) reported some expansions when inducing a DSB within a CTG repeat tract in human cells using either wild-type Cas9 or the mutant Cas9D10A nickase. This suggests that DSB repair mechanisms within CTG repeats exhibit subtle differences between yeast and human cells. The chromatin environment in human cells is different and may affect the way a DSB within a CTG repeat tract will be repaired (reviewed in House et al., 2014). In addition, although human cells contain RAD51 and RAD52 homologs, two additional genes, BRCA1 and BRCA2, involved in breast cancer, play a central role in DSB repair.
role in homologous recombination, whereas yeast cells lack these genes (Moynahan et al., 1999, 2001). Comparing results obtained in yeast and in human cells will also hopefully help our understanding of the respective roles of these factors during DSB repair of CTG repeats.

**Single-Strand Annealing Is the Main Mechanism of DSB Repair within a CTG Repeat Tract**

Former studies of SSA requirements on direct repeats showed that its efficacy relied on three factors: homology length between the two repeated sequences, resection rate, and proximity on the DNA molecule, with closer sequences recombining more easily than distant ones (Lazzaro et al., 2008; Sugawara and Haber, 1992). In addition, RAD52 was shown to be important for SSA reaction between 15- to 18-bp microhomologous sequences but strongly inhibited SSA between 6- to 13-bp microhomologies (Villarreal et al., 2012). In the present case, the DSB was made close to the 3′ end of the repeat tract, leaving only 1–4 repeat units (3–12 bp) on the 3′ end of the break but a much longer stretch of repeats on the 5′ end (around 70 triplets). SSA between triplet repeats was partially RAD52-dependent (survival was 3-fold decreased), suggesting that 15-bp microhomologies were sometimes present and used for SSA between triplet repeats. These results are in good accordance with our former work in which repair occurred in 67% of the cases by annealing two short repeats flanking an I-Sce I restriction site (Richard et al., 1999).

Although no effect of POL32 and RAD51 on DSB repair of a CTG repeat tract was detected in the present experiments, it is interesting to mention that both genes were involved in spontaneous expansions of CAG repeats, probably by a BIR-related mechanism. However, expansions were sometimes present and used for SSA between triplet repeats. These results are in good accordance with our former work in which repair occurred in 67% of the cases by annealing two short repeats flanking an I-Sce I restriction site (Richard et al., 1999).

**A Model Supporting Progressive Repeat Contractions Associated with TALEN-Induced DSB Repair**

We propose a model involving iterative SSA between short repeat-containing DNA ends after DSB induction (Figure 7C). In this model, the Mre11-Rad50 complex and Sae2 are essential to process DSB ends, after which Rad52 annealing activity catalyzes the SSA reaction. Given that the DSB occurs only a few base pairs from the end of the repeat tract, leaving only 1–4 repeat units (3–12 bp) on the 3′ end of the break but a much longer stretch of repeats on the 5′ end (around 70 triplets), SSA between triplet repeats was partially RAD52-dependent (survival was 3-fold decreased), suggesting that 15-bp microhomologies were sometimes present and used for SSA between triplet repeats. These results are in good accordance with our former work in which repair occurred in 67% of the cases by annealing two short repeats flanking an I-Sce I restriction site (Richard et al., 1999).

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**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**

All mutant strains were built from strains GYM6162-14A and GYM6162-3D by classical gene replacement method (Orr-Weaver et al., 1981) using KANMX4 as a marker (Table S1), amplified from the European Saccharomyces cerevisiae archive for functional analysis (EUROSCARF) deletion library, using primers located 1 kb upstream and downstream of the cassette (Table S2). The VMY350 strain was used to construct the VMY650 strain by mating-type switching, using the pHJ132 vector carrying an inducible HO endonuclease (Homes and Haber, 1999). Plasmid pCLS9996 carrying the TALEN<sub>CTG</sub> right arm was digested by Ncol (New England Biolabs) and EagI (Takara). The fragment containing the right arm was cloned in the centromeric pCMha182, digested by BamHI (NEB) and PstI (NEB) using two oligomeric adaptors of 16 bp (BamH1-Ncol) and 19 bp (EagI-Pst). The resulting vector, pCMHa182KN9996, was transformed in the haploid strain GYM6162-14A and its mutant derivatives. Plasmid pCLS16715 carrying the TALEN<sub>CTG</sub> left arm was digested by Ncol (NEB) and EagI (Takara). The fragment containing the left arm was cloned in the centromeric pCMHa188, digested by BamHI (NEB) and PstI (NEB) using two oligomeric adaptors of 16 bp (BamH1-Ncol) and 19 bp (EagI-Pst). The resulting vector, pCMHa188KN16715, was transformed in the GYM6161-3D haploid strain and its mutant derivatives. Haploid transformants were crossed on rich medium (yeast extract peptone dextrose medium [YPD]) supplemented with doxycycline (10 μg/mL) and diploids containing both TALEN<sub>CTG</sub> arms were selected on synthetic complete medium lacking uracil and tryptophan (SC-Ura-Trp) with doxycycline (10 μg/mL). For the dna4A mutant, both TALEN<sub>CTG</sub> arms were transformed in haploid strains GYM6162-3D and VMY104 because NHEJ is downregulated in diploid cells (Frank-Vaillant and Marcard, 2001; Valencia et al., 2001).

The TALEN<sub>noCTG</sub> was designed by the Museum National d’Histoire Naturelle platform. The target sequence was chosen to be an I-Sce I recognition site integrated in the SUP4 gene (Richard et al., 1999). Plasmid pR1 was used as a template to PCR-amplify the TALEN<sub>noCTG</sub> right arm using primer pairs VM525/VMS525, containing a 50-bp tail homologous to sequences flanking a KpnI site on pCMHa182KN. The PCR product and pCMHa182KN linearized by KpnI (NEB) were directly cloned in yeast cells. Plasmid pL1 was used as a template to PCR-amplify the TALEN<sub>noCTG</sub> left arm using primer pairs VM525/VMS525, containing a 50-bp tail homologous to sequences flanking a KpnI site on pCMHa188KN. The PCR product and pCMHa188KN linearized by KpnI (NEB) were also directly cloned in yeast. Centromeric vectors pCMHa182KN1 and pCMHa188KN1, carrying respectively, the TALEN<sub>noCTG</sub> right arm and the TALEN<sub>noCTG</sub> left arm, were transformed in diploid strains VMY001 and VMY002.

**TALEN Inductions**

Before nuclease induction, Southern blot analyses were conducted on several independent subclones to select cells with different CTG repeat tract lengths on both chromosomes. Yeast cells were grown at 30°C in liquid SC-Ura-Trp medium complemented with 10 μg/mL of doxycycline. Cells were washed with sterile water to eliminate doxycycline and then split in two cultures at 9 x 10<sup>6</sup> cells/mL, one in SC-Ura-Trp medium complemented with 10 μg/mL of doxycycline (TALEN-repressed) and the other in SC-Ura-Trp (TALEN-induced). For each time point, 2 x 10<sup>6</sup> cells were collected at time point (T) = 0, 14, 16, 18, 20, 22, 24, or 48 hr afterward, rapidly centrifuged, washed with water, and frozen in dry ice before DNA extraction. To determine viability after DSB induction, cells were plated at 24 hr on SC-Ura-Trp plates supplemented with doxycycline (10 μg/mL) for the TALEN-repressed culture and on SC-Ura-Trp plates for the TALEN-induced culture. CFUs were counted after 3-5 days of growth at 30°C.
DSB Analysis and Quantification

Total genomic DNA (4 μg) of cells collected at each time point was digested for 6 hr by EcoRV (20 U) (NEB) and analyzed by Southern blot as described previously (Viterbo et al., 2018). Alternatively, a terminal transferase-mediated PCR assay (Förstemann et al., 2003) was used to amplify the TALEN-induced DSB. Genomic DNA (100 ng) of cells collected at different time points was heat-denatured and treated with 7 U of terminal deoxynucleotidyl transferase (Takara) in a volume of 10 μL (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.2; 40 mM MgCl₂; 0.5 mM DTT; 0.1% BSA; and 1 mM deoxycytidine triphosphate [dCTP]) for 30 min at 37°C to add polyC tails to 3’OH free ends. The enzyme was inactivated for 10 min at 65°C and 5 min at 94°C. Then 30 μL of PCR mix was added to each reaction to obtain a final volume of 40 μL containing 67 mM Tris HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 5% glycerol, 0.01% Tween, 200 μM each deoxynucleotidyl triphosphate (dNTP), and 40 nM of each primer (G18 and VMS14). The following PCR program was used: 94°C for 2 min (94°C for 20 s, 62°C for 12 s, and 72°C for 20 s) for 45 cycles and then 72°C for 5 min. For each reaction, 20 μL was loaded on a 1% analytical agarose gel, and 20 μL was sent for Sanger sequencing.

Trinucleotide Repeat Length Analysis

Several colonies from each induced or repressed plates were picked, total genomic DNA was extracted, and 4 μg was digested for 6 hr by Sspl (20 U) (NEB) and analyzed by Southern blot as described previously (Viterbo et al., 2018). Repeat tracts in some of the survivors were subsequently amplified using primers su3 and su7 and sequenced using a third internal primer, su2 (Table S2). Sanger sequencing was performed by GATC Biotech.

Analysis of DSB End Resection

A real-time PCR assay using primer pairs flanking EcoRV sites 0.81 kb and 2.94 kb away from the 5’ end of the CTG repeat tract (VMS29/VMAS20 and VMS21/VMAS21, respectively) and 0.88 kb and 1.88 kb away from the 5’ end of the CTG repeat tract (VMS22/VMAS22 and VMS23/VMAS23, respectively) was used to quantify end resection. Another pair of primers was used to amplify a region of chromosome X near the ARG2 gene to serve as an internal control of the DNA amount (JEM11–JEM1r). Genomic DNA of cells collected at T = 0 hr, T = 18 hr, T = 24 hr, and T = 48 hr was split in two fractions; one was used for EcoRV digestion and the other one for a mock digestion in a final volume of 15 μL. Samples were incubated for 5 hr at 37°C and then the enzyme was inactivated for 20 min at 80°C. DNA was subsequently diluted by adding 55 μL of ice-cold water, and 4 μL was used for each real-time PCR reaction in a final volume of 25 μL. PCRs were performed with Absolute SYBR Green Fluorescent Mix (Thermo Scientific) in a Mastercycler S Realplex (Eppendorf) using the following program: 95°C for 15 min, 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s repeated 40 times, followed by a 20-min melting curve. Reactions were performed in triplicate, and the mean value was used to determine the amount of resected DNA using the following formula: raw resection = 2/(1+2^ΔCt) with ΔCt = Ct,EcoRV − Ct,EcoRVmock. Relative resection values were calculated by dividing raw resection values by the percentage of DSB quantified at the corresponding time point. All t tests were performed using the R package (Milott, 2011).

Western Blots

Liquid cultures were grown to exponential phase in the presence or absence of 10 μg/mL doxycycline. Proteins were extracted on 2 × 10⁶ cells in 200 μL Laemmli solution with 100 μL glass beads. Proteins were separated on a 10% acrylamide gel under standard conditions and blotted to a nitrocellulose membrane (Optitran BA-55 83 reinforced NC, Schleicher & Schuell). For TALEN detection, a polyclonal anti-HA antibody was used (ab9110, Abcam, 0.25 μg/mL final concentration). For Msh2 detection, the primary antibody was a polyclonal rabbit antibody directed against an internal part of the yeast Msh2 protein (N3C2, GeneTex, 1 μg/mL final concentration) (Viterbo et al., 2018). A secondary goat anti-rabbit antibody conjugated to horseradish peroxidase was used for detection in both cases (Thermo Scientific, 0.16 μg/mL final concentration). Quantification was performed using a ChemiDoc MP Imager (Bio-Rad) with the dedicated Image Lab software. The molecular weight marker used was the Precision Plus Protein Standards All Blue (Bio-Rad).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.083.

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AUTHOR CONTRIBUTIONS

V.M. designed and performed most of the TALEN experiments and all resection experiments, analyzed the data, and wrote the manuscript. L.P. performed some TALEN experiments, analyzed the data, and contributed to the manuscript. D.V. contributed to building mutant strains. M.C. designed and built the TALENconting. G.-F.R. designed the project, analyzed the data, and wrote the manuscript.

DECLARATION OF INTERESTS

There is a patent related to this work, held by the Institut Pasteur (WO 2015/078935 A1).

REFERENCES


