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# Mrc1, Tof1 and Csm3 inhibit CAG·CTG repeat instability by at least two mechanisms

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ABSTRACT

Trinucleotide repeats frequently expand and contract in humans and model organisms. Protein factors that modulate this process have been found by candidate gene approaches or mutant screens for increased expansion rates. To extend this effort, *Saccharomyces cerevisiae* mutants with higher CAG·CTG repeat contraction rates were sought using a disruption library. This screen identified Mrc1, the homolog of human Claspin, which mediates the replication and DNA damage checkpoints, and also couples the replicative helicase and polymerase. Genetic analysis showed that Mrc1, along with Tof1 and Csm3, inhibits instability in two distinct ways. Contraction rates of $(CAG)_{20}$ tracts are elevated by loss of Mrc1, Tof1 or Csm3, but not by defects in most replication checkpoint or DNA damage checkpoint proteins. The three proteins likely inhibit contractions primarily through their coupling activity, which would prevent accumulation of single-strand template DNA prior to the formation of aberrant secondary structure. In contrast, expansion rates of $(CTG)_{13}$ are elevated in strains defective for Mrc1, Tof1, Csm3, Mec1, Ddc2, Rad24, Ddc1, Mec3, Rad17, Rad9, Rad53 or Chk1, suggesting that the DNA damage checkpoint inhibits expansions after formation of repeat-dependent structures. Together, these results indicate that at least two Mrc1-dependent mechanisms function to reduce CAG·CTG repeat instability.

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## 1. Introduction

Trinucleotide repeats (TNRs) are unstable repetitive DNA elements found in both coding and non-coding regions of numerous human genes. Expansions in specific TNRs cause at least 15 heritable neurodegenerative human diseases, including Huntington's disease and fragile X syndrome [1,2]. Expansion patterns follow non-Mendelian inheritance patterns in afflicted families [3], indicating that complex and unique molecular mechanisms underlie the propensity of triplet repeats to expand and contract [1,2]. Disease-causing TNRs almost exclusively have the sequence $(CNG)_n$, and single-stranded DNA containing these repeats readily forms secondary structures *in vitro* that correlate strongly with the genetic instability of these sequences *in vivo* [4,5]. Furthermore, DNA polymerases *in vitro* [6] and replication forks in *E. coli* [7] and yeast [8] have difficulty synthesizing G–C-rich TNRs. These and other observations led to well supported replication-based models for TNR instability in proliferating cells that are all founded on the premise that aberrant replication of the lagging strand is linked to secondary structure formation in single-stranded DNA (ssDNA) [1,2,9]. Generation

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of ssDNA on the nascent strand of the Okazaki fragment may trigger hairpin formation, allowing formation of this crucial structured intermediate that ultimately yields an expansion. Similarly, generation of excess ssDNA on the template strand is thought to permit collapse into a hairpin, and aberrant synthesis past this hairpin would result in contraction on one strand. Thus, for both expansions and contractions, the availability of ssDNA at TNRs is a critical factor determining the likelihood of hairpin formation and subsequent genetic instability.

Expansions and contractions in somatic cells can exhibit differing levels of instability in various tissues [10–13], suggesting that tissue-specific *trans*-acting factors modulate TNR instability. In accordance with this idea, several pathways in yeast modulate TNR mutagenesis, including Okazaki fragment maturation [14–16] and post-replication repair [17,18]. To identify novel *trans* factors, we performed a blind screen for *S. cerevisiae* mutants that increase rates of TNR contractions. This screen revealed an *mrc1* mutation, suggesting that Mrc1 protein normally prevents contractions in wild type cells. We focused on Mrc1 because of recent findings implicating it and associated proteins in limiting accumulation of ssDNA, discussed below, and also in prevention of chromosome fragility and instability in yeast with a long, disease-length $(CAG)_{85}$ tract [19,20].

Mrc1 was initially identified as a mediator of the replication checkpoint [21], which responds to stalled replication forks arising from treatment with hydroxyurea (HU). In the presence of a stalled fork, ssDNA coated with RPA stimulates the recruitment of Mec1/Ddc2 (in yeast) or ATR/ATRIP (in humans) to the replication fork [22,23]. Mec1 phosphorylates and activates Mrc1, which recruits and facilitates the activation of the effector kinase Rad53 (Chk2 in humans). Activated Rad53 then phosphorylates a variety of downstream targets, resulting in the inhibition of late origin firing and the upregulation of genes involved in DNA repair [24]. The loss of Rad53, combined with HU treatment, leads to excess ssDNA formation at the replication fork that is detectable by electron microscopy [25]. Mrc1 is also involved in a second checkpoint, the intra-S phase DNA damage response. A number of proteins in the DNA damage response overlap with those in the checkpoint response, including Mrc1, Mec1/Ddc2, Rad53 and others [26]. This overlap may be due to damage sensing through promotion of single-strand gaps. However the DNA damage response also requires additional factors, such as the alternative clamp loader Rad24 and the alternative clamp Rad17/Mec3/Ddc1 (9-1-1 in humans) [26]. Thus phenotypes associated with defects in Rad24, Rad17, Mec3 or Ddc1 distinguish the DNA damage response from the replication checkpoint. In addition to signaling, Mrc1 also has a structural role at the replication fork that is central to normal replisome function [27]. Mrc1 functions with Tof1 and Csm3 to form the replication pausing complex, which maintains fork stability and prevents the uncoupling of helicase and polymerase activities under conditions of replication stress [28,29]. In cells lacking Mrc1, Tof1 or Csm3, helicase activity occurs without polymerization, and leads to accumulation of excess ssDNA [30]. Thus Mrc1 is involved both in preventing accumulation of ssDNA through its structural role, and response to ssDNA via the replication checkpoint and the DNA damage response [29].

The evidence summarized above shows that genetic instability at TNRs is potentially suppressed by Mrc1 either through replicational coupling to avoid ssDNA and secondary structure formation, or to checkpoint response(s) after structure formation to reduce the likelihood of completing the mutagenic process. Previous work showed that long CAG·CTG tracts, which are disease-causing in humans, can be further destabilized by defects in the DNA damage response [19] or Mrc1 [20]. Our independent discovery of an *mrc1* mutant that also destabilized shorter CAG·CTG runs, more similar to those seen in normal humans, suggested that checkpoint activities help prevent triplet repeat mutations between genetically stable, subthreshold alleles and the longer, unstable tracts that can give rise to further mutation and disease in humans. Furthermore we found that Mrc1, Tof1 and Csm3 are highly selective in protecting TNRs from instability, and that they use two distinct mechanisms to help avoid CAG·CTG repeat expansions and contractions. Together these results significantly extend what is known about Mrc1, Tof1 and Csm3 and their action at TNRs.

## 2. Materials and methods

### 2.1. *Saccharomyces cerevisiae* strains

Most strains used in this study were derived from BY4741 (*MAT-a his3*$\Delta$*1 leu2*$\Delta$*0 met15*$\Delta$*0 ura3*$\Delta$*0*), a derivative of *S. cerevisiae* strain S288C (Open Biosystems). Mutants used in this study were created by targeted deletion and confirmed by PCR, and when possible, by phenotypic traits such as UV or hydroxyurea sensitivity. TNR-containing plasmids were digested and integrated into the yeast genome; single integrants were confirmed as described previously [31].

### 2.2. Plasmids

The pBL94 vector was used to construct all TNR-containing plasmids as described previously [32]. The dinucleotide repeat-containing plasmid, pSH44 [33], was a gift from Tom Petes, Duke University. The *CEN*/*ARS*-based recovery plasmid p*MRC1* and the p*mrc1*$^{AQ}$ mutant plasmid [34] were gifts from Stephen Elledge, Harvard University.

### 2.3. Genetic assays and molecular analysis of mutated TNR alleles

Expansion and contraction rates were measured by fluctuation analysis as described previously [31,32] and as shown in Fig. 1. Mutation rates were calculated by the method of the median [35]. Single-colony PCR analysis of expansions and contractions was performed as previously described [32,36], and rates were corrected by multiplying the percent bona fide expansions/contractions by the apparent mutation rates obtained by fluctuation analysis [31]. Dinucleotide mutation rates were measured as described previously [33]. Forward mutation rates for the *CAN1* gene were determined by fluctuation analysis using selection for canavanine resistance. At least two independent clones were tested for all the above assays to ensure reproducibility. Statistical analyses for data

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