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The MRX complex stabilizes the replisome independently of the S phase checkpoint during replication stress

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The Mre11–Rad50–Xrs2 (MRX) complex has an important function in the maintenance of genomic integrity by contributing to the detection and repair of chromosome breaks. Here we show that the complex is recruited to sites of paused forks where it stabilizes the association of essential replisome components. Interestingly, this function is not dependent on the S phase checkpoint or the nuclease activity of Mre11. We find that disruption of the MRX complex leads to a loss of fork recovery and a failure to properly complete DNA replication when cells are exposed to replication stress. Our data suggest that one critical function of the MRX complex during replication is to promote the cohesion of sister chromatids at paused forks, offering an explanation for why MRX deficiency leads to a loss of cell viability and high levels of chromosome rearrangements under conditions of replication stress.

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Introduction

Accurate replication of DNA is essential for the preservation of genomic integrity and the continuation of life. Sites in the genome susceptible to genomic breaks and rearrangements correspond with pauses in replication fork progression (Cha and Kleckner, 2002; Lambert *et al*, 2005). Thus, maintaining fork structures during replication stress is paramount for ensuring that DNA breaks do not occur and that synthesis can continue once the stress is overcome.

In *Saccharomyces cerevisiae*, the Mre11/Rad50/Xrs2 (MRX) complex, similar to its human counterpart Mre11/Rad50/Nbs1 (MRN), preserves genomic integrity. Mre11 interacts with itself and both Xrs2 and Rad50. Mre11 has intrinsic DNA-binding activity, associates with the ends of linear DNA molecules and duplexed DNA and has nuclease activity specified by four phosphoesterase motifs in the amino terminal end of the protein (Furuse *et al*, 1998; Usui *et al*, 1998; Paull and Gellert, 1999; de Jager *et al*, 2001; Hopfner *et al*, 2002). The overall structure of the Rad50 component is typical of a class of proteins known as the 'structural maintenance of chromosome' proteins, which are necessary for chromosome condensation and sister chromatid cohesion (SCC) (reviewed in Hirano, 2002). The ends of Rad50 contain Walker A and B nucleotide (NTP)-binding motifs (Alani *et al*, 1989), which are separated by an extended coiled-coil structure bound in the centre of the protein by a putative hinge region (Alani *et al*, 1989; Dolganov *et al*, 1996). The crystal structure of Rad50 indicates that a dimer can form between two Rad50 molecules, allowing a flexible bridge to be generated, which can tether duplexed DNA molecules over long distances of up to 1200 Å (Hopfner *et al*, 2002). Xrs2 is the most divergent member of the complex and is less well characterized.

Mutations in the genes encoding components of the MRX complex lead to well-characterized defects including telomere shortening, meiotic defects and DNA damage sensitivity (reviewed in D'Amours and Jackson, 2002). Interestingly, the nuclease activity of Mre11 has been shown to be dispensable for some of these processes including homologous recombination (HR) and telomere maintenance, indicating the complex has critical functions distinct from its DNA end-processing ability (Bressan *et al*, 1998; Moreau *et al*, 1999; Tsukamoto *et al*, 2001). Structural characterization of the MRX/MRN complex shows an elongated conformation, with tethering properties important for bridging sister chromatids during HR (Williams *et al*, 2007). Indeed, mutations in the coiled-coil domain of Rad50 that negatively influence the tethering functions of the complex render cells sensitive to genotoxic stress, underscoring the importance of complex structure preservation (Hopfner *et al*, 2002). It has been proposed that the MRX complex serves as a 'critical glue' that is necessary for cohesion establishment between sister chromatids during DNA metabolic activities (Williams *et al*, 2007).

During repair, the interconnection between the MRX complex and Tel1 kinase is well documented. The MRX complex localizes to DNA double-stranded breaks (DSBs) very rapidly (Lisby *et al*, 2004; Shroff *et al*, 2004) and recruits Tel1 (Nakada *et al*, 2003). The DSB repair function of the MRX complex is both initiated and regulated by Tel1 on a pathway parallel to Mec1, leading to Rad53 activation (Usui *et al*, 2001). Furthermore, Tel1-dependent phosphorylation of Mre11 and Xrs2 has been shown to directly influence the proper repair of DNA damage (D'Amours and Jackson, 2001; Usui *et al*, 2001). However, there are phenotypic differences

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between *mre11*Δ and *tel1*Δ cells that are most notable when cells are faced with stress during DNA replication. For example, Mre11-deficient cells show sensitivity to the replication inhibitor hydroxyurea (HU), whereas *tel1*Δ cells do not (Supplementary Figure 1; D'Amours and Jackson, 2001). Consistent with this, disruption of any component in the MRX complex leads to very high rates of gross chromosomal rearrangements (GCRs), a phenotype attributed to replication specific events (Chen and Kolodner, 1999; Myung *et al*, 2001). These data suggest a function for the MRX complex during replication that is distinct from Tel1 and its function during repair.

In contrast to the DNA damage repair pathway, the characterization of MRX during replication has remained limited even though there is precedence for the complex having a fundamental role in replication. First, in mammalian cells, the MRN complex colocalizes with proliferating cell nuclear antigen and sites of BrdU incorporation during S phase (Mirzoeva and Petrini, 2003). Second, in a cyclin-dependent kinase-mediated manner, MRN interacts with replication protein A (RPA) for recruitment to replication centres (Robison *et al*, 2004; Olson *et al*, 2007). Lastly, work in *Xenopus laevis* has shown that Mre11 promotes replication fork restart and prevents the formation of DSBs in the newly replicated DNA (Costanzo *et al*, 2001; Trenz *et al*, 2006). Currently, however, a mechanism for how Mre11 aids fork recovery, allowing replication to restart remains undefined. In this study, we have investigated the molecular function of the MRX complex during DNA replication. We show that the complex is recruited to forks during HU-induced pausing and that it stabilizes components of the replisome independently of S phase checkpoint activation and Mre11 nuclease activity. Our data suggest that the integrity of the complex is essential for replisome stability during fork pausing and for promoting cohesion between sister chromatids during replication stress. We suggest that the tethering function of the MRX complex (Hopfner *et al*, 2002; Williams *et al*, 2007) has a stabilizing influence on paused replisomes, allowing replication recovery. Our model offers one explanation for why MRX deficiency leads to replication stress sensitivity and high levels of chromosome rearrangements.

Results

In the absence of Mre11 fork progression is altered during HU exposure

Cells that lack a functional MRX complex have an unstable genome with high rates of GCRs (Myung *et al*, 2001). Because such rearrangements have been attributed to events at blocked replication forks (Lambert *et al*, 2005), we wanted to determine the function of the MRX complex functions during replication. We first visualized replication by DNA combing and compared *mre11*Δ with wild-type cells as described earlier (Tourriere *et al*, 2005). We monitored both a 'normal S phase' using asynchronous early log-phase cultures pulse labelled with BrdU for 40 min or an 'S phase during replicative stress' where asynchronous early log-phase cultures were pulse labelled with BrdU for 3 h during 0.2 M HU treatment.

DNA fibres were purified and stretched on silanized coverslips and subsequently revealed after denaturation with an anti-ssDNA antibody (red, Figure 1A). The lengths of newly replicated DNA tracks were detected with anti-BrdU (green, Figure 1A). Wild type and *mre11*Δ showed identical track lengths after 40 min of BrdU incorporation under normal conditions (Figure 1C). In contrast, we found after 3 h of BrdU incorporation that *mre11*Δ cells treated with replication stress have 25% shorter tracks (25.8 kb) than wild-type cells (32.7 kb; Figure 1A and B). These data support a role for Mre11 during replication under conditions of HU-induced pausing and suggest that in *mre11*Δ cells, forks are either more prone to collapse or continue replication but at a suboptimal rate compared with wild type.

Mre11 is necessary for replisome stability during HU-induced fork stalling

Given that the replication pattern was altered in *mre11*Δ cells during HU exposure, we wanted to assess the stability of replisome components at stalled forks by performing chromatin immunoprecipitation (ChIP). Either *mre11*Δ or wild-type cells were synchronized in G1 with α-factor and then released into S phase in the presence of HU. Under these conditions, origins fire as normal, but the rate of fork progression is significantly inhibited because of nucleotide depletion (Santocanale and Diffley, 1998; Alvino *et al*, 2007). The recovered DNA was quantified by real-time PCR with primer pairs to ARS607 and a negative control region 14 kb from the origin as described earlier (Figure 2A; Cobb *et al*, 2003; Cobb *et al*, 2005). First, we determined the association of DNA polymerase α and ε with replication forks upon HU treatment. In wild-type cells, HA-tagged DNA Polα was loaded onto forks by 20 min as cells entered into S phase and remained associated with forks for up to 60 min (Figure 2B). In *mre11*Δ cells, we were able to recover low levels of DNA Polα at both 40 and 60 min; however, there was a severe defect that was most notable at 20 min (Figure 2B). In the absence of Mre11, the defect in DNA Polε recovery was even more severe. In wild-type cells, DNA Polε associates with ARS607 from 20 to 60 min, with peak enrichment at 40 min (Figure 2C), yet in *mre11*Δ cells, there was a total loss of DNA Polε recovered at all times monitored (Figure 2C, data not shown). A similar loss of DNA Polε was observed when other MRX complex members were disrupted, with virtually no recovery observed in *rad50*Δ cells (Figure 2D). The disruption of Xrs2 also resulted in a significant decrease in DNA Polε recovery, however, the effect was slightly less pronounced (Figure 2D). These data show that disruption of any component of the MRX complex results in a considerable alteration in DNA Polε recovery at stalled forks compared with wild-type cells. Interestingly, the contribution of Mre11 to DNA Polε stabilization was dependent on fork pausing, because when cells were released from α-factor into media without added HU, the profiles were similar with only a small decrease observed in *mre11*Δ cells (Figure 2E). These data are consistent with our DNA combing data showing little discrepancy between *mre11*Δ and wild-type cells in the absence of HU.

We questioned whether a defect at the origin or an alteration in the initiation of replication could account for the decrease in DNA Polα and Polε recovered at forks in *mre11*Δ cells. However, the similar ChIP profiles in YPAD (Figure 2E) suggest that the loss of DNA polymerases recovered in *mre11*Δ cells during HU treatment is not likely

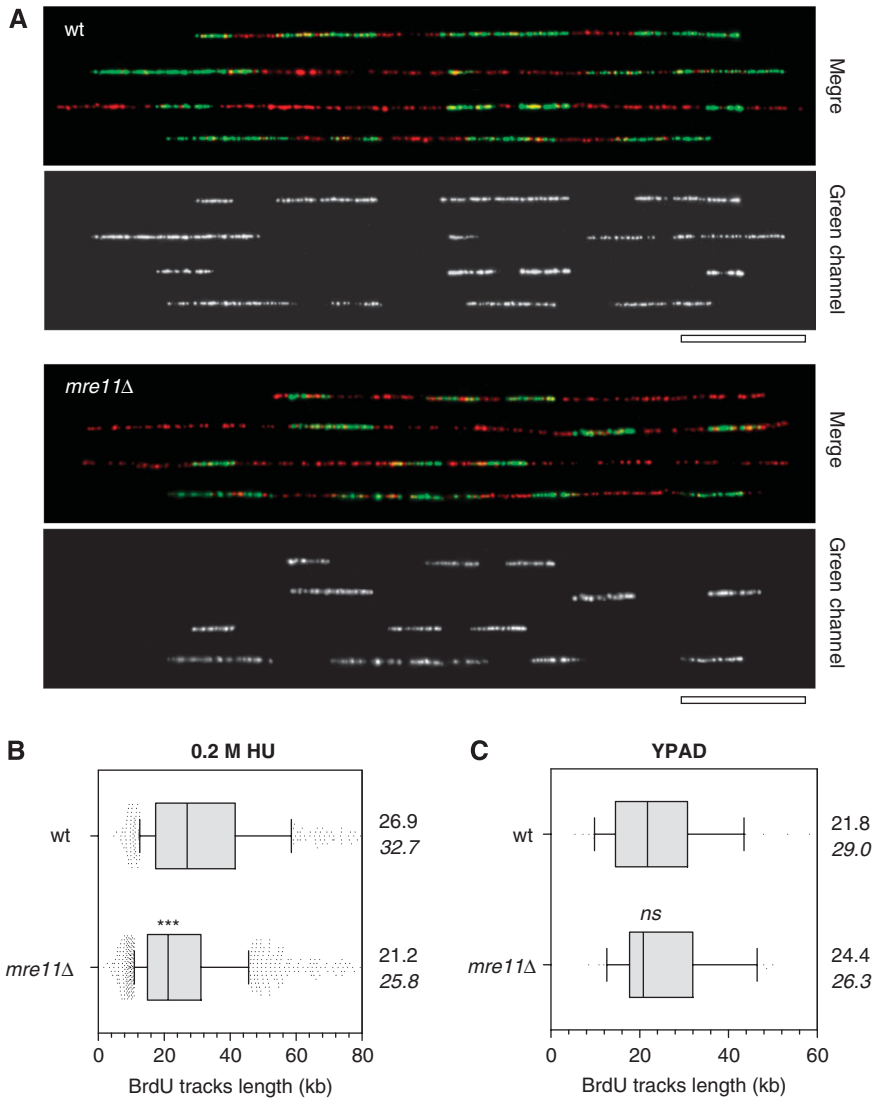

Figure 1 Mre11 is required for normal replication fork progression in the presence of HU. Exponentially growing wild-type (JC604) and *mre11Δ* (JC616) cells were labelled for 3 h with 200 μg/ml BrdU in the presence of 200 mM HU. Genomic DNA fibres were stretched on silanized coverslips by DNA combing and newly replicated DNA was detected with an anti-BrdU antibody. DNA fibres were counterstained with an anti-ssDNA antibody. (**A**) Representative fibres of wild type (top panel) and *mre11*Δ (bottom panel) are shown with DNA in red and BrdU in green; Bar: 50 kb. (**B**) Distribution of BrdU tracks length in HU-treated cells. Box: 25–75 percentile range. Whiskers: 10–90 percentile range. Median (vertical bars) and mean values (italics) are indicated in kb. ***$P<0.0001$; Mann–Whitney rank sum test. (**C**) Distribution of BrdU tracks length in untreated wt and *mre11Δ* cells. *ns*: $P=0.62$; Mann–Whitney rank sum test.

attributed to some major disruption in the initiation of replication in cells lacking Mre11 *per se*. Indeed, such an intrinsic effect on origin firing would most certainly be visible in the absence of HU as well but is not. Additionally, 2D gel analysis showed only a slight decrease in the efficiency of initiation in *mre11*Δ cells (Supplementary Figure 2A), which might help explain the small decrease in DNA Polε observed in the absence of damage (Figure 2E). Finally, the levels of Orc2 and Mcm4 recovered at replication origins in G1 are almost indistinguishable for wild-type and *mre11*Δ cells, indicating proper pre-RC assembly (Figures 2F; Supplementary Figure 2B), although we see a dramatic loss of Mcm4 recovery at the fork in *mre11*Δ cells upon entry into S phase in the presence of replication stress (Figure 2F).

During stalls in replication, the checkpoint protein complex Mec1–Ddc2 is recruited to forks (Osborn and Elledge, 2003; Cobb *et al*, 2005), and in *mec1*Δ and checkpoint defective cells, replisome components are destabilized leading to irreversible fork catastrophe (Tercero and Diffley, 2001; Lucca *et al*, 2004; Cobb *et al*, 2005). Therefore, to determine whether the loss of polymerase recovery at forks in *mre11*Δ cells could be attributed to a defect in Mec1–Ddc2 recruitment, we performed ChIP on Ddc2 during HU treatment. Surprisingly, the ChIP profile for Ddc2 looked similar for wild type and cells lacking Mre11 (Figure 2G). Ddc2 is present at stalled forks in *mre11*Δ cells at the same time points where a loss of Mcm4, DNA Polα and Polε are observed (Figures 2B, C and F). This suggests that the function of the MRX complex stabilizes replisome components but does so independently of the presence of Mec1–Ddc2 at stalled forks.

In summary, our ChIP data indicate altered recovery of replisome components when MRX-deficient cells are exposed to replication stress, but there is little or no alterations in

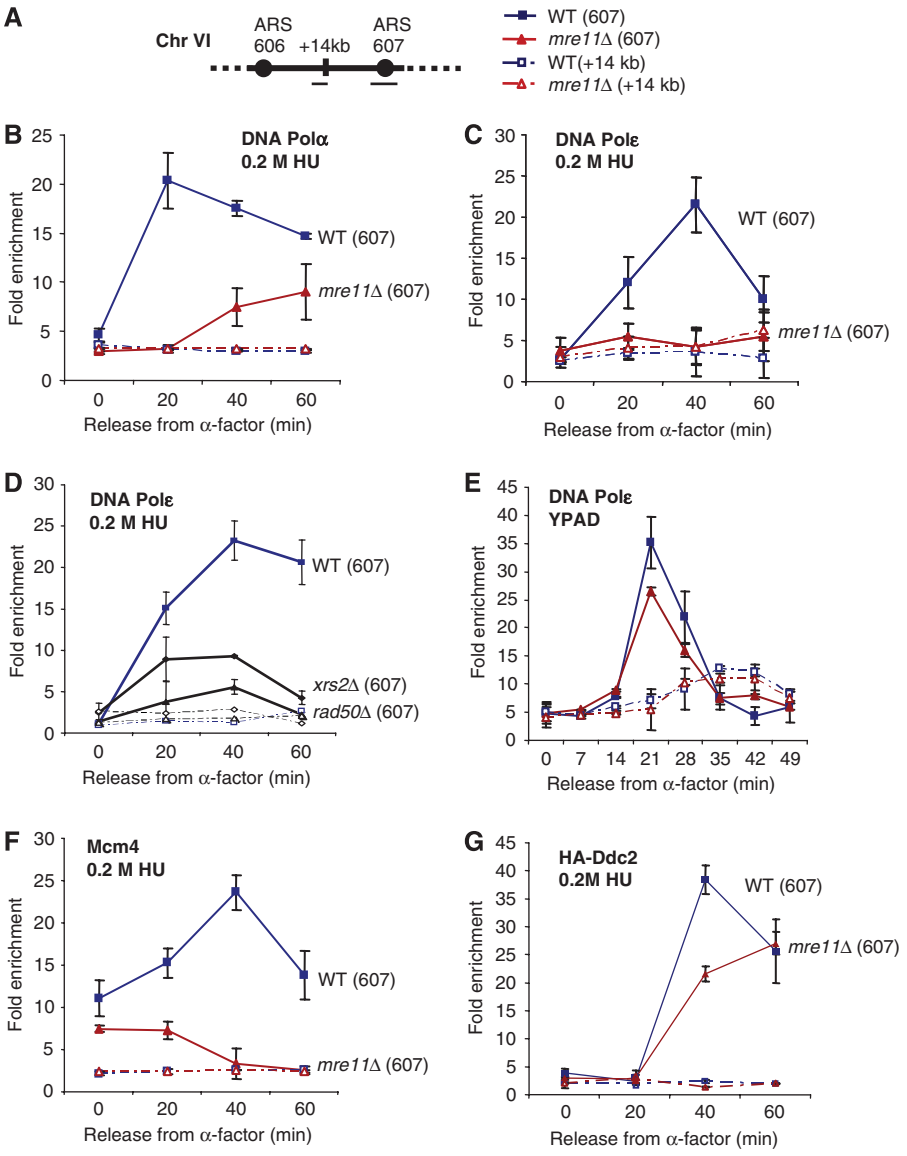

Figure 2 Stability of replisome components in *mre11*Δ cells. (**A**) Genomic regions amplified for ChIP analysis on Chr VI are as described earlier (Cobb *et al*, 2003, 2005) and correspond to early firing origin ARS607 (filled symbol) and a nonorigin site, +14 kb (open symbols). ChIP was performed using the following strains with either αMyc (9E10) or αHA (F-7, Santa-Cruz) antibodies on cells released from α-factor into YPAD + 0.2 M HU (**B**) for HA-Polα in wild-type (JC539) and *mre11*Δ (JC538) cells; (**C**) Myc-Polε was monitored after released into 0.2 M HU comparing wild-type (JC285) with *mre11*Δ (JC388) and (**D**) *rad50*Δ (JC796) and *xrs2*Δ (JC757) cells. (**E**) Myc-Polε in wild-type (JC285) and *mre11*Δ (JC388) cells was monitored after released into YPAD without HU at 19°C. (**F**) Myc-Mcm4 was monitored after released into 0.2 M HU comparing wild type (JC171) with *mre11*Δ (JC559). (**G**) HA-Ddc2 in wild-type (JC257) and *mre11*Δ (JC549) cells was determined after release into 0.2 M HU. Error bars represent the standard deviation in fold enrichment of multiple runs of at least three different ChIP experiments.

either the recruitment of Mec1–Ddc2 under these conditions or in the timing of origin activation in *mre11*Δ cells.

The MRX complex is recruited to stalled forks and promotes replication recovery after fork pausing

We then determined whether the function of the MRX complex during HU-induced replication stress is a result of direct interaction of the complex with paused forks. We performed ChIP as described in Figure 2 (Cobb *et al*, 2003, 2005) using a strain containing both an epitope tagged HA-DNA Polα and Myc-Rad50. Cellular extracts were divided and immunoprecipitations were performed with either α-HA to detect DNA Polα as a marker for the location of the replication fork or αMyc to detect Rad50 as a marker for the complex (ChIP on Rad50 gave more reliable results than Mre11). Upon release into S phase, we consistently observed a 5–6-fold enrichment for Rad50 that correlated with the presence of DNA Polα at ARS607 (Figure 3A), indicating that the MRX complex is present at paused forks. The recovery of Rad50 at forks was dependent on replication stress because no preferential recovery was observed in cells released into media without HU (data not shown).

We then wanted to determine whether the loss of viability in *mre11*Δ cells could be attributed specifically to defects in replication upon HU treatment. We monitored replication progression in *mre11*Δ cells after replication stress by FACS analysis. Cells lacking Mre11 traverse S phase by 75 min after 3 h HU exposure and looked similar to wild-type cells

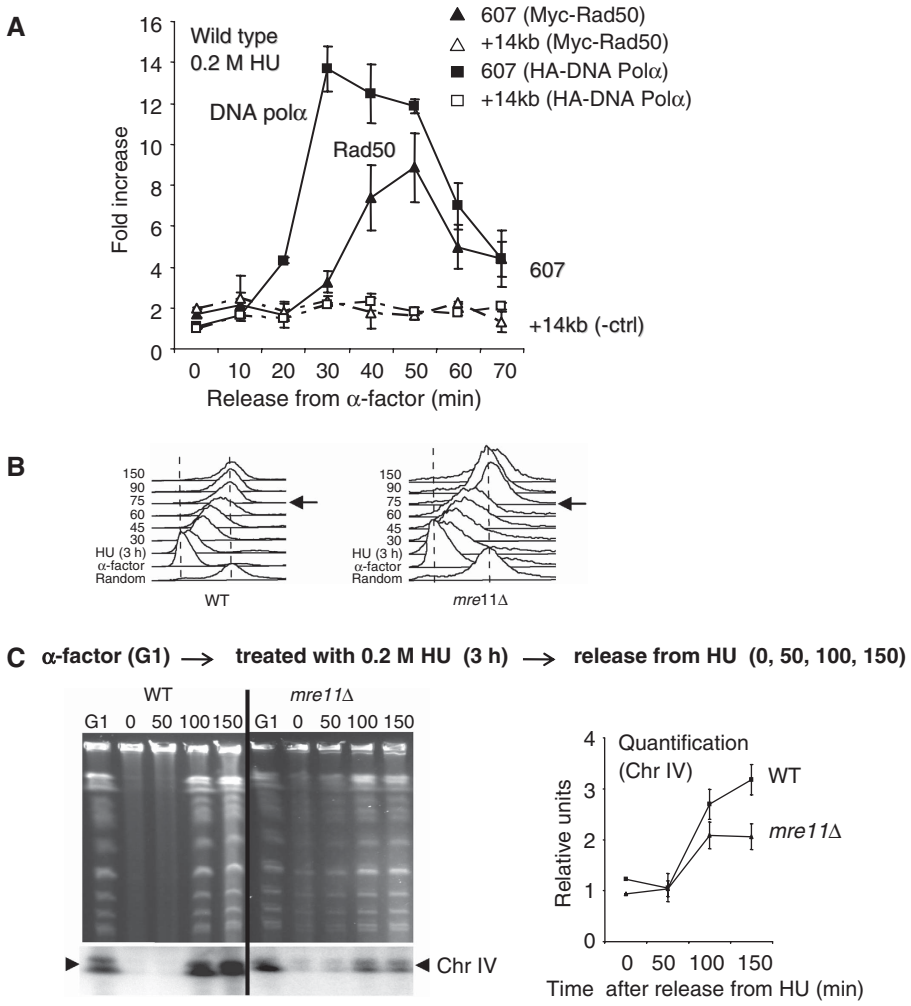

Figure 3 The MRX complex is recruited to forks and promotes recovery during pauses in replication. ChIP using αMyc (9E10) and αHA (F-7, Santa-Cruz) antibodies as described in Figure 2 was performed on (**A**) Myc-Rad50 and HA-Polα in a wild-type background (JC528), where enrichment at ARS607 (solid bar) and + 14 kb from ARS607 (speckled bar) is compared at the indicated time points after release into 0.2 M HU. (**B**) FACS analysis was performed as described in Cobb *et al* (2003). Wild-type (JC467) and *mre11*Δ (JC371) cells were blocked with α-factor for 90 min (G1) and released into S phase with 0.2 M HU for 3 h before cells were washed and resuspended in YPAD to monitor the progression through S phase. Samples are taken at indicated time points (30, 45, 60, 75, 90, 150) after HU removal. (**C**) Ethidium bromide stained PFGE for samples arrested in G1 with α-factor for 90 min (G1) before treatment with 0.2 M HU for 180 min (0 min time point). HU was removed and cells were washed and released into YPAD media with samples taken at 50, 100 and 150 min. Southern blot analysis was performed to monitor ChrIV replication (lower panel) with quantification of triplicate experiments for Chr IV replication shown in relative units compared with the signal in G1 using Quantity One (Biorad) comparing wild type (JC467) and *mre11*Δ (JC371).

(Figure 3B). However, FACS analysis only measures bulk DNA content, and it cannot determine whether a small percentage of the DNA remains unreplicated or whether the apparently replicated chromosomes are intact. Therefore, to examine the integrity of the chromosomes during replication, we performed pulse-field gel electrophoretic (PFGE) analysis to monitor the recovery of DNA synthesis upon HU exposure as described earlier (Desany *et al*, 1998).

DNA was prepared in agarose plugs from an equal number of cells that were blocked in α-factor (G1 sample) then released into S phase + 0.2 M HU for 3 h. The 0 time point indicates samples after 3 h HU treatment with subsequent samples taken 50–150 min after HU removal (Figure 3C). The replicative state of the genome was visualized by the appearance of fully replicated chromosomes in the pulse-field gel and by Southern blot analysis with a radio-labelled probe to detect fully replicated chromosome IV (bottom arrows, Figure 3C). The quantification indicates the signal relative to G1 levels (Quantity One (BioRad); Figure 3C). In G1 the chromosomes have not started replication and are visualized in the gel and on the blot (Figure 3C). Upon release into S phase + 3 h HU treatment (0 min), the chromosomes are in the process of replication and do not migrate into the gel. In wild-type cells, replication is complete and chromosome re-entry is observed by 100–150 min post-HU removal (Figure 3C). In contrast, the cells lacking Mre11 always exhibited a reduced pattern of chromosome re-entry after HU treatment. Quantification of three independent experiments show that approximately half the level of replicated Chr IV can be detected by 150 min compared with G1 levels in *mre11*Δ cells (Figure 3C), suggesting a defect in replication recovery upon HU-induced stress. We note that in *mre11*Δ mutants, we consistently observed a small subpopulation that never blocked in G1 with α-factor and migrated into the gel at time point 0 (Figure 3C), possibly indicating a defect in the G2-M transition. Taken together, the MRX

complex is recruited to forks during replication stress (Figure 3A) and in *mrx*-deficient cells, the replisome components show altered association with sites of replication (Figure 2). This ultimately leads to defects exhibited by a decrease in replication recovery after fork pausing (Figure 3C).

The integrity of the MRX complex but not Mre11 nuclease activity is required for the maintenance of DNA Polε with paused forks

To show that Rad50 recruitment can be used as an indicator for the localization of the entire complex, we performed ChIP on Rad50 in *mre11*Δ cells (Figures 4A and B). We used Orc2 as a positive control because we had determined that in *mre11*Δ cells, the levels of Orc2 were indistinguishable from wild type in both G1 and through S phase (Supplementary Figure 2). In the absence of Mre11, there is a complete loss of Rad50 recovery compared with wild-type cells (Figures 4B and C). This strongly suggests that it is the MRX complex that is recruited and not something unique to Rad50.

The nuclease activity of Mre11 degrades DNA ends and hairpins by processing misfolded DNA at broken ends (Furuse *et al*, 1998; Paull and Gellert, 1998; Connelly *et al*, 1999; Trujillo and Sung, 2001; Lobachev *et al*, 2002). Because such DNA intermediates likely exist at least transiently at paused or stalled forks, we determined if the nuclease activity of Mre11 and the structural integrity of the MRX complex contributed to polymerases recovery. We introduced into *mre11*Δ cells mutants of Mre11 were either deficient for complex formation *mre11*-2 ($56–58^{DLF\rightarrow FLS}$) or only nuclease deficient *mre11*-3 ($125–126^{HD\rightarrow LV}$) but still MRX complex assembly proficient (Bressan *et al*, 1998). We compared isogenic *mre11Δ* and wild-type strains carrying empty vectors, and *mre11Δ* cells expressing either plasmid-borne wild-type pMRE11 or one of the mutants p*mre11*-2 (structurally deficient and nuclease dead) or p*mre11*-3 (only nuclease dead). Transformation with either pMRE11 or p*mre11*-3 in *mre11*Δ cells rescued the phenotype and DNA Polε association with stalled forks, with levels returning to that observed in wild-type cells (Figure 4D). As a control, we showed that p*mre11*-3 cells were sensitive to growth on media containing 0.01% methyl methanesulfonate, which forms DNA lesions

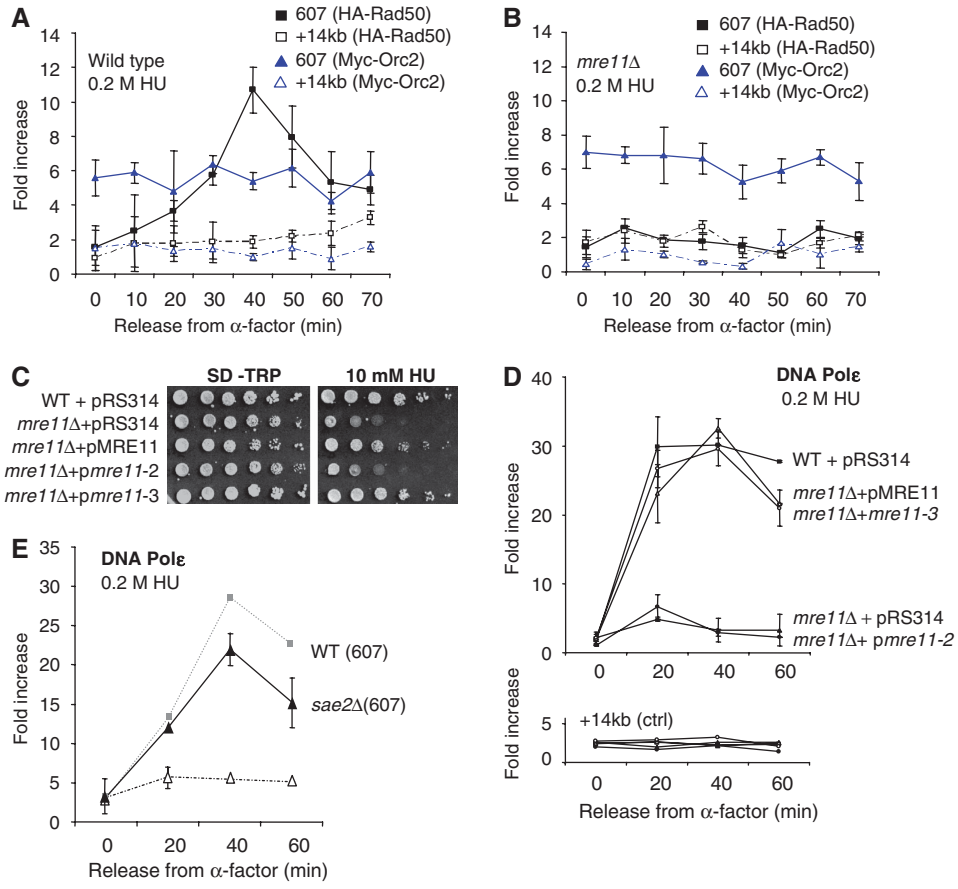

Figure 4 The MRX complex functions at the fork independently of Mre11 nuclease activity. ChIP using αMyc (9E10) and αHA (F-7, Santa-Cruz) antibodies as described in Figure 2 was performed on (**A**) on HA-Rad50 and Myc-Orc2 in wild-type (J764) and (**B**) *mre11*Δ (JC743) cells, where enrichment at ARS607 (solid line) and +14 kb from ARS607 (speckled line) is monitored at the indicated time points after release into 0.2 M HU. (**C**) Drop assays on exponentially growing cells of 1:10 serial dilutions on SC-TRP plates +10 mM HU at 30°C or (**D**) ChIP on Myc-Polε using αMyc (9E10) as described in Figure 2 were performed with wild-type (JC285) cells after transformation with pRS314 vector alone (WT +pRS314, ■), and in *mre11Δ* (JC388) cells after transformation with pRS314 vector alone (*mre11Δ* + pRS314, ▲), or pRS314 carrying either full length *MRE11* (*mre11Δ* + pMRE11, △) or *mre11-3* (*mre11Δ* + p*mre11-3*, ○) and *mre11-2* (*mre11Δ* + p*mre11-2*, ●) constructs from cultures grown in SD-TRP media. (**E**) ChIP using αMyc (9E10) was performed on Myc-Polε in wild-type (JC285) and *sae2*Δ (JC686) cells as described in Figure 2.

and is consistent with nuclease deficiency (Supplementary Figure 3). In stark contrast to p*mre11*-3, transformation with p*mre11*-2 did not complement DNA Polε recovery and levels remained indistinguishable from those in *mre11*Δ cells (Figure 4D). This indicates that the integrity of the MRX complex is essential for replisome stability at the fork, but that the nuclease activity of Mre11 is not required either for DNA Polε maintenance or cell viability during exposure to HU (Figure 4C and D).

Sae2 is also involved in processing meiotic and mitotic double-strand breaks, and it has been shown to cooperatively function with the MRX complex to process hairpin DNA structures (Lengsfeld *et al*, 2007). Therefore, we performed ChIP on Myc-Polε in *sae2Δ* cells and observed a similar profile to wild-type cells upon release into HU (Figure 4E). Taken together, these data suggest that while the MRX complex is recruited to stalled forks, replisome maintenance is independent of DNA processing by the complex or its partners.

Genetic interactions between Mre11 and components of the replisome

We have determined that the MRX complex has a role during replication, but it was unclear how it was functioning. To investigate a potential mechanism for the MRX complex, we took a genetic approach and combined the loss of Mre11 with temperature sensitive (ts) replisome alleles. These mutants show defects in replication and cell viability at a non-permissive temperature (37°C) and include *pol2*-12 and *pol2*-16, which compromise the catalytic subunit of DNA Polε, as well as *mcm10*-43 and *mcm5*-3 (Budd and Campbell, 1993; Kesti *et al*, 1999; Kawasaki *et al*, 2000; Dziak *et al*, 2003). Mcm5 is a component of the MCM2-7 helicase and is important for the loading of Mcm10, which in turn is important for the loading of additional replication factors including RPA and DNA Polα. After the initiation of replication, the Mcm2-7 helicase, Mcm10 and the replicative polymerases including DNA Polε, migrate with the fork during elongation.

Strains were monitored for cell growth in the presence of the replication inhibitor HU. A slight defect was observed in *mre11*Δ cells as well as in the *mcm5*-3 and *pol2*-12 ts alleles at the semipermissive temperature; 30°C (Figure 5A). Strikingly, a synergistic loss of viability was observed when *mre11*Δ was coupled with either the *mcm10*-43 or *pol2*-12 allele that was most evident in the *mre11*Δ/*pol2*-12 double mutant (Figure 5A). Consistent with the drop assays, *mre11*Δ/*mcm10*-43 and *mre11*Δ/*pol2*-12 cells showed extremely poor recovery after transient exposure to 0.2 M HU, with viability dropping to 10.7 and 0.3%, respectively, after 8 h (Figure 5B).

These data might indicate that the p*ol2*-12 and *mcm10*-43 mutant alleles generate damage during replication that is then recognized and processed through the canonical DNA damage repair cascade by the MRX complex together with Tel1, a pathway characterized earlier and termed as the TM checkpoint pathway (Usui *et al*, 2001). However, drop assays showed that in contrast to *mre11*Δ, no mutants containing the deletion of TEL1 were sensitive to HU; and the double-mutant phenotypes observed with *mre11*Δ were not recapitulated in *tel1*Δ/*mcm10*-43 and *tel1*Δ/*pol2*-12 cells (Figure 5A). As well, to determine whether the loss of viability in the *mre11*Δ/*mcm10*-43 and *mre11*Δ/*pol2*-12 double mutants was due to a failure in the replication checkpoint, we also monitored Rad53 phosphorylation when cells were synchronously released into S phase in the presence of 0.2 M HU. We observed similar kinetics in wild-type and *mcm10*-43 cells for Rad53 activation peaking at 40 min (Figure 5C). When Mre11 was deleted, a slight defect in full Rad53 activation was observed, which is consistent with earlier reports (D'Amours and Jackson, 2001; Nakada *et al*, 2004) and the phenotype of the double mutant *mre11*Δ/*mcm10-43* resembled that of *mre11*Δ. Also in agreement with earlier reports (Navas *et al*, 1995), *pol2-12* cells were deficient in the S phase checkpoint after release into 0.2 M HU (Figure 5C), therefore, we do not interpret the additive loss of viability observed in *mre11Δ/mcm10-43 and mre11*Δ/*pol2-12* cells as solely a deficiency in the Rad53 checkpoint activation. This is particularly clear for *mre11*Δ/*pol2-12* cells where the dramatic loss of viability cannot be explained simply by a defect in checkpoint activation because *pol2*-12 alone shows complete abrogation of Rad53 phosphorylation (Figure 5C). Taken together, these data suggest a function for Mre11 during HU-induced fork arrest that is distinct from and not correlating with Tel1 or Rad53 checkpoint signalling.

Consistent with an independence of the intra-S checkpoint after HU treatment, no additive loss of recovery was observed when *pol2*-12 and *mcm10*-43 were combined with a *rad9*Δ mutation (Figures 6A and C). The viability after 8 h in the double-mutant cells *mcm10*-43/*rad9*Δ (89%) and *pol2*-12/*rad9*Δ (70%) was similar to the single mutants *mcm10*-43 (84%), *pol2*-12 (62%) and *rad9*Δ (87%; Figure 5B). As well, the percentage of viable cells after 8 h of HU treatment for the triple-mutant cells *mre11*Δ/*mcm10*-43/*rad9*Δ (7.5%) and *mre11*Δ/*pol2*-12/*rad9*Δ (7.1%) looked similar to the double mutants *mre11*Δ/*mcm10*-43 and *mre11*Δ/*pol2*-12, with a small alleviation in cellular death observed when Rad9 was disrupted in the *mre11*Δ/*pol2*-12 (0.3%) mutant background (compare Figures 5B with 6A and C).

In contrast to Rad9-mutant cells, a severe additive loss of viability was observed upon the disruption of Rad51. The double mutants *rad51*Δ/*mcm10*-43 (11.1%) and *rad51*Δ/*pol2*-12 (0.3%) showed a loss of viability after 8 h of HU exposure similar to double mutants with *mre11*Δ (compare Figures 5B with 6B and D), and the triple mutant *mre11*Δ/*mcm10*-43/rad51Δ (7.5%) showed a similar loss of viability as both double mutants *mre11*Δ/*mcm10*-43 (10.7%) and *mre11*Δ/*rad51*Δ (6.12%), suggestive of an epistatic relationship. These genetic interactions indicate the importance of Mre11 when DNA replication is compromised and suggest that the function of Mre11 might principally be within the HR pathway for recovery after HU exposure. The triple mutant *mre11*Δ/*pol2*-12/*rad51*Δ did show the most severe phenotype with no survivors after 8 h of HU (Figure 6B), suggesting in the *pol2*-12 background that Mre11 is not functioning solely within the Rad51-dependent HR pathway. However, we note the identical and severe loss of viability in both double mutants *mre11*Δ/*pol2*-12 (0.3%) and *rad51*Δ/ *pol2*-12 (0.3%; Figures 5B and 6B).

The MRX complex and SCC factors provide support to forks during stalls in replication

The genetic studies showed that disrupting Mre11 in combination with the alleles *pol2*-12 and *mcm10*-43 leads to synergistic HU sensitivity (Figure 5A). Interestingly, common to

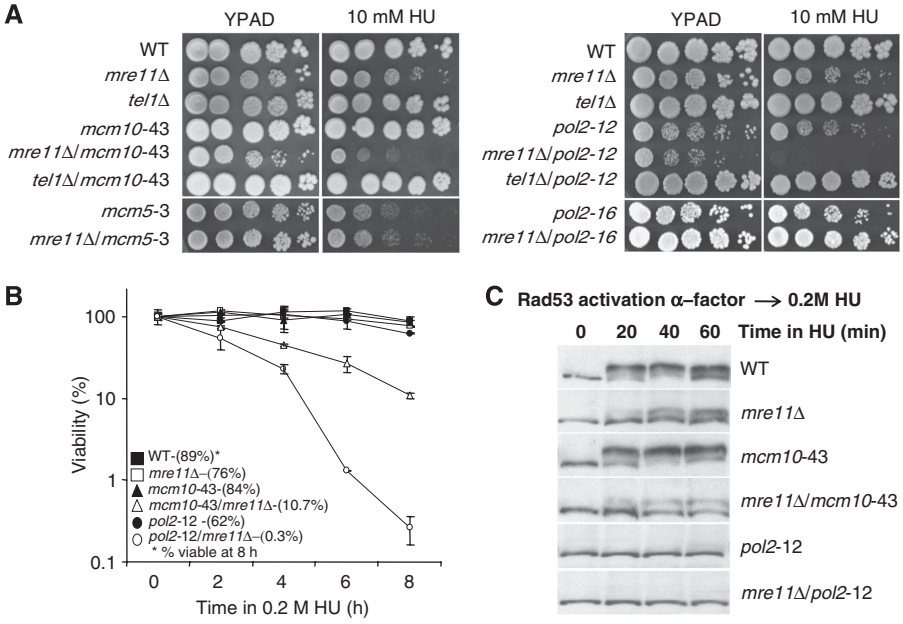

Figure 5 Mre11 shows synergistic interactions with replisome components. (**A**) Drop assays on YPAD ± 10 mM HU were performed with exponentially growing cultures at permissive temperature (25°C) with 1:5 serial dilutions before 2 days incubation at 30°C (**B**) Cell viability was monitored as colony outgrowth from asynchronous cultures grown at permissive temperature (25°C) before transient exposure to 0.2 M HU at 30°C for the indicated times with values normalized to survival at time point 0 and (**C**) the phosphorylation status of Rad53 was monitored by western blot analysis after cells were blocked in G1 with α-factor and released in 0.2 M HU for the indicated time at 30°C using anti-Rad53 antibodies (yC-19, Santa Cruz) for the following strains: wild type (JC467), *mre11*Δ (JC371) *mcm10*-43 (JC216), *mcm10*-43/*mre11*Δ (JC554), *pol2*-12 (JC617), *pol2*-12/*mre11*Δ (JC553), *mcm5*-3 (JC227), *mcm5*-3/*mre11*Δ (JC564), *pol2*-16 (JC583) *pol2*-16/*mre11*Δ (JC619), *tel1*Δ (JC65), *mcm10*-43/*tel1*Δ (JC591), *pol2*-12/*mre11*Δ (JC612).

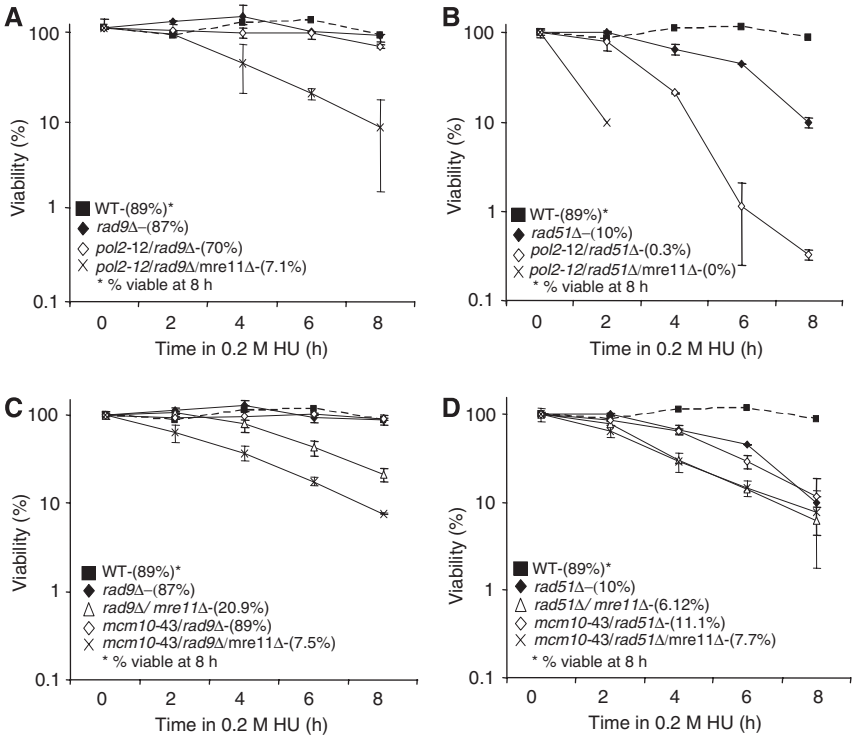

Figure 6 Epistatic analysis between replisome alleles with *mre11*Δ and *rad9*Δ or *rad51*Δ. (**A**–**D**) Cell viability was monitored as colony outgrowth from asynchronous cultures grown at permissive temperature (25°C) before transient exposure to 0.2 M HU at 30°C for the indicated times with values normalized to survival at time point 0. All experiments were performed at the same time as Figure 5B for the following strains: *rad9*Δ (JC176), *rad9*Δ/*mre11*Δ (JC568) *mcm10*-43/*rad9*Δ (JC569), *mcm10*-43/*rad9*Δ/*mre11*Δ (JC570), *pol2*-12/*rad9*Δ (JC618), *pol2*-12/*rad9*Δ/*mre11*Δ (JC572), *rad51*Δ (JC445), *rad51*Δ/*mre11*Δ (JC633) *mcm10*-43/*rad51*Δ (JC634), *mcm10*-43/*rad51*Δ/*mre11*Δ (JC629), *pol2*-12/*rad51*Δ (JC630), *pol2*-12/*rad51*Δ/*mre11*Δ (JC683).

both Pol2 and Mcm10 are their interactions with factors necessary for SCC. The *pol2*-12 allele has a 22 amino acid truncation in the C-terminal domain and results in the disruption of SCC (Edwards *et al*, 2003). Also, Mcm10 has been shown to bind Ctf4, a factor that associates with replication forks and is critical for the establishment of SCC (Wittmeyer and Formosa, 1997; Hanna *et al*, 2001; Mayer *et al*, 2001; Bermudez *et al*, 2003; Gambus *et al*, 2006; Lengronne *et al*, 2006; Skibbens *et al*, 2007; Zhu *et al*, 2007).

Interestingly, Pan *et al* (2006) reported that *ctf4*Δ/*mre11*Δ double-mutant cells exhibit growth defects. This in combination with the loss of viability we observed in *mre11*Δ/*mcm10*-43 cells (Figures 5 and 6) led us to investigate Mre11 and Ctf4 during S phase in the presence of replication stress. We report here an additive decrease in cell viability in *mre11*Δ/*ctf4*Δ double mutants after transient exposure to HU, suggesting at least a partial overlap in function for these proteins in response to replication stress (Figure 7A). To determine what effect the deletion of Ctf4 would have on the replisome, we performed ChIP on Myc-Polε in *ctf4Δ* cells and observed a similar profile to the recovery levels observed in *mrx*-mutant cells (Figure 7B). Because the loss of DNA Polε was so pronounced in the single mutants, determining an additive affect for replisome stability was precluded in *mre11*Δ/*ctf4*Δ mutant cells.

However, to determine whether *mre11*Δ/*ctf4*Δ mutants had an additive defect during replication, we monitored recovery from HU treatment by performing PFGE analysis as in Figure 3C. In contrast to *mre11*Δ cells, which did not recover by 150 min post-HU treatment, cells lacking Ctf4 resumed replication with kinetics similar to wild-type cells and full reentry was observed at 100–150 min (Figures 7C). This suggests that the altered association of DNA Polε during HU treatment is reversible in *ctf4*Δ cells and that the fork remains in a competent state to resume replication once the HU is removed. Strikingly, at 150 min post-HU treatment, the *mre11*Δ/*ctf4*Δ cells showed a severe recovery defect with the level of fully replicated chromosomes remaining unchanged from 0 to 150 min (Figure 7C). In summary, these data suggest that Mre11 and the SCC establishment factor Ctf4 share a partially redundant function for recovery from HU-induced fork pausing.

Drop assays show growth defects in *mre11*Δ/*ctf4*Δ double mutants on YPAD and sensitivity to growth on HU (Figures 7D; Pan *et al*, 2006). As our data suggest a structural role for the MRX complex to promote cohesion between sister chromatids, we reasoned that a nuclease dead Mre11 would not exhibit the same additive phenotype as *mre11*Δ when combined with a disruption in Ctf4. Indeed this was the case, and when using the nuclease dead *mre11*-D56N separation of function allele (Krogh *et al*, 2005) there was no additive sensitivity in *ctf4*Δ/*mre11*-D56N double-mutant cells on 5 mM HU (Figure 7D).

We have interpreted the additive loss of viability after exposure to HU in *mre11*Δ/*ctf4*Δ and *mre11*Δ/*mcm10*-43 double mutants to be independent of checkpoint activation and a result of disruptions in cohesion between sister chromatids at paused forks. Indeed, it has been proposed that one essential function of the MRX complex is to provide bridging cohesion between two strands of DNA (Hopfner *et al*, 2002). To address this more directly, we monitored cellular viability in these double mutants during transient exposure to HU while overexpressing the cohesin factor Scc1; which is a cohesin complex member regulated in a cell cycle dependent manner, peaking in S phase (Michaelis *et al*, 1997). We reasoned that an increase in cohesin complex assembly from Scc1 overexpression would promote SCC and restore cell viability if *mre11*Δ/*ctf4*Δ and *mre11*Δ/*mcm10*-43 double mutants exhibit cohesion-related defects.

Scc1 was placed under control of the galactose inducible GAL1 promoter and integrated into the genome at the LEU2 locus in wild-type and mutant cells (Sullivan *et al*, 2004). Cultures were grown overnight in YPLG glycerol before transfer to media containing either 2% galactose or glucose followed by 0.2 M HU treatment. Cultures containing 2% galactose showed robust Scc1 expression (Supplementary Figure 4). As a positive control, we show that overexpression of Scc1 during HU treatment restored viability in cells harbouring the ts allele *scc1*-73 (Ciosk *et al*, 2000). Similarly, the overexpression of Scc1 restored viability in both *mre11*Δ/*mcm10*-43 (51.3%) and *mre11*Δ/*ctf4*Δ (55.8%) double-mutant cells to the level of *mre11*Δ (55.4%) cells, providing direct evidence that SCC defects are a major contributor to cell death in these double mutants during replication stress (Figure 7E).

Discussion

We describe here a role for the MRX complex during DNA synthesis and show in three different ways by: DNA combing, ChIP and PFGE analysis that in the absence of Mre11, replication is altered upon exposure to replication stress. During pauses in replication, the MRX complex is recruited to forks (Figures 3A) but its role there appears distinct from DNA end processing and checkpoint signalling events. Our work presents *in vivo* data for the MRX complex that is specific to replication forks and which supports the earlier reported structural function of the complex (Chen *et al*, 2001; Hopfner *et al*, 2002; Williams *et al*, 2008). We suggest that upon its recruitment to paused forks, the MRX complex provides cohesive support to sister chromatids, and in SCC compromised mutant cells, the MRX complex becomes important for efficient fork recovery in the face of replication stress. Through MRX scaffold abilities (Chen *et al*, 2001; Hopfner *et al*, 2002; Williams *et al*, 2008), the complex promotes replication recovery possibly through a Rad51- and HR-dependent mechanism.

The MRX complex is recruited to stalled forks stabilizing the replisome

When cells are exposed to HU-induced replication stress, forks do not collapse but remain in a competent conformation to resume high fidelity and rapid replication once the stress is overcome. The MRX complex has a role in this process (compare WT and *mre11*Δ, Figure 3C). The complex is recruited to stalled forks in wild-type cells (Figure 3A) and functions either during a stall or in aiding fork recovery or both; however, distinguishing between these two events is difficult to demonstrate. Nonetheless, its presence at HU stalled forks in wild-type cells suggests that the complex has a function before and distinct from a function in the repair of DNA DSBs arising from forks that have collapsed. Indeed, support of this is demonstrated

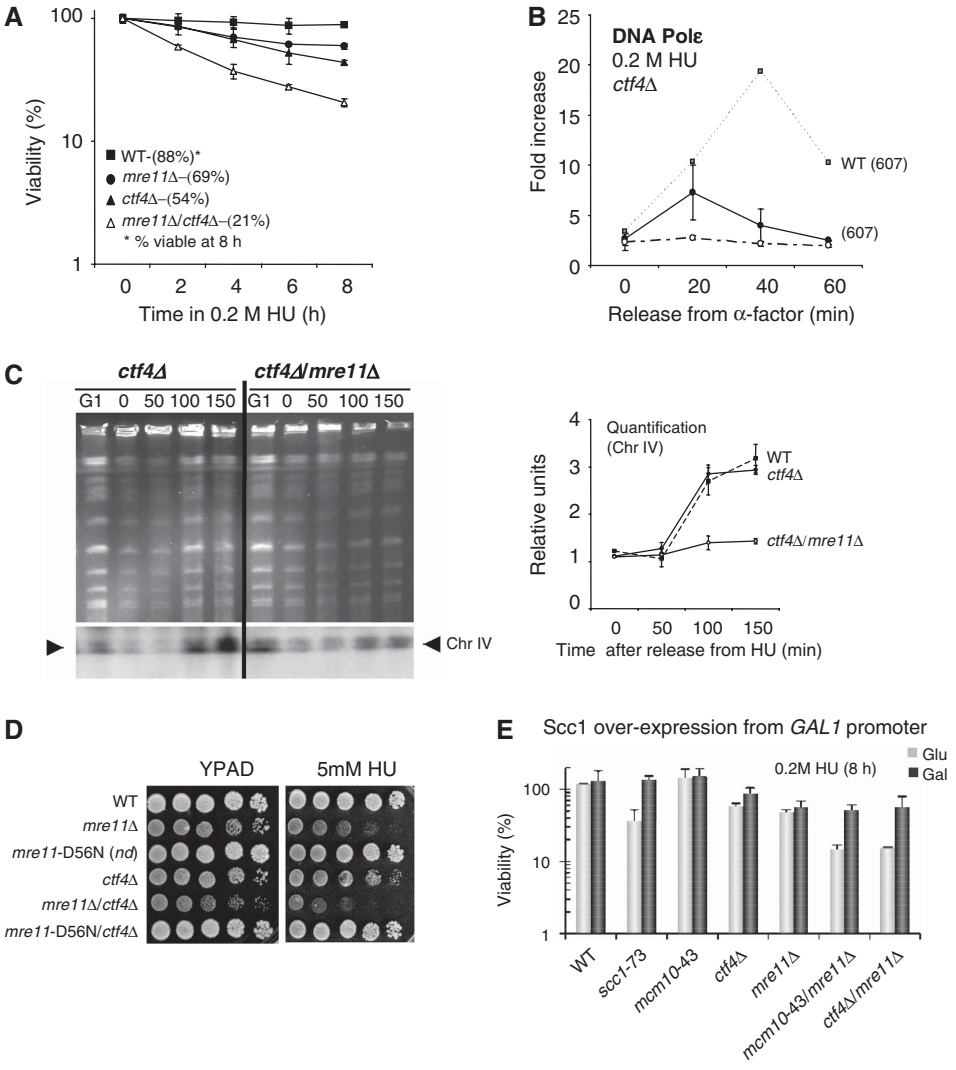

Figure 7 The MRX complex functions on a pathway parallel to SCC factors to maintain forks during replication stress. (**A**) Cell viability was monitored as described earlier in Figure 5B for the following strains: wild type (JC467), *mre11*Δ (JC371), *ctf4*Δ (JC648), *mre11*Δ/*ctf4*Δ(JC662). (**B**) ChIP using αMyc (9E10) was performed on Myc-Polε in wild-type (JC285) and *ctf4*Δ (JC670) cells as described in Figure 2. (**C**) Ethidium bromide stained PFGE for samples arrested in G1 with α-factor for 90 min (G1) before treatment with 0.2 M HU for 180 min (0). HU was removed and cells were washed and released into YPAD media for 50, 100 and 150 min. Southern blot analysis was performed to monitor ChrIV replication (lower panel) with quantification of triplicate experiments for Chr IV replication shown in relative units compared with the signal in G1 using Quantity One (Biorad) strains used are *ctf4*Δ (JC648) and *ctf4*Δ/*mre11*Δ (JC662) with all experiments perform at the same time as Figure 3C. (**D**) Drop assays on YPAD ± 5 mM HU were performed from exponentially growing cultures with 1:10 serial dilutions at 30°C comparing wild type (JC467), *mre11*Δ (JC371), *mre11*D56N(JC606), *ctf4*Δ (JC648), *mre11*Δ/*ctf4*Δ (JC662) and mre11D56N/*ctf4*Δ (JC647). (**E**) Cell viability was monitored as colony outgrowth on SCC1overexpression induced (gal) or repressed (glu) from asynchronous cultures grown at permissive temperature (25°C) before transient exposure to 0.2 M HU at 30°C for 8 h. Galactose-induced SCC1 overexpression and glucose repressed SCC1 started 90 min before HU exposure and was maintained throughout all the experiments. The percentage survival is normalized to the cellular viability at time 0 for the following strains: wild type (JC784), *scc1-73* (JC786), *mcm10*-43 (JC787), *ctf4*Δ (JC795), *mre11*Δ (JC801), *mre11*Δ/*mcm10*-43 (JC803), *mre11*Δ/*ctf4*Δ (JC797).

by alterations in replisome association and the loss of replication recovery in *mre11*Δ cells upon HU treatment (Figures 2 and 3C).

We have proposed a function for the MRX complex during replication based on EM structural data, which showed the complex can tether sister chromatids (Hopfner *et al*, 2002). In contrast to mammals and Xenopus, live cell imaging detected no Mre11 focus formation in wild-type cells upon HU treatment, but only after forks collapse in checkpoint defective cells (Lisby *et al*, 2004). We do not think our ChIP results showing Rad50 recruitment to forks (Figure 3A) directly contradicts these reports and suggest that its function at HU-paused forks might involve fewer or more dispersed MRX complex molecules when 'tethering' sister chromatids, which would be difficult to detect by microscopy. One can also speculate that because Mre11 can interact with itself (Nairz and Klein, 1997; Paull and Gellert, 1998), having the complex present at stalled forks in anticipation of collapse might also aid in the rapid recruitment of additional MRX complex molecules to repair/prevent the formation of DNA DSBs upon breakdown.

One question still remaining is what signals MRX recruitment and what does it interact with at stalled forks. We see an interaction between RPA and Mre11 by co-immunoprecipita-

tion that might have functional significance, yet this interaction did not increase upon HU treatment (data not shown). This is different from the scenario in human cells where a physical interaction between components of the MRN complex and RPA has been reported to increase during HU treatment and signal MRN localization to replication centres (Olson *et al*, 2007). Also, we did not detect a physical interaction between the MRX complex and either DNA Polε or Polα (Supplementary Figure 5). This suggests that the influence of MRX on replisome stability is likely indirect in nature, and also argues that the MRX complex is not recruited to forks through direct interaction with these polymerases.

During HU exposure replication is impaired in the absence of Mre11

Fork progression was altered in m*re11*Δ cells upon exposure to HU replication stress but not in the absence of damage. This alteration in progression did not, however, indicate a complete cessation of replication (Figure 1A and B), and the ChIP data should be interpreted within this context. One explanation for the lack of DNA Polε (Figure 2C and D) detected at stalled forks in the absence of MRX complex members is that aberrant DNA conformations are generated that preclude efficient antibody access to replisome components in *mrx*-deficient cells, resulting is the loss of detectable enrichment. Second, DNA Polε is present but with reduced stoichiometry and below the levels of detection, however this is difficult to demonstrate. Finally, DNA Polε might very well be lost from forks. This would be consistent with experiments performed in *X. laevis* egg extracts showing DNA Polε association with chromatin during replicational stress dependent on Mre11 (Trenz *et al*, 2006). Currently, we are trying to determine whether replication continues with a compensating polymerase such as DNA Polδ; however, these experiments have proven to be quite difficult because the inclusion of an HA epitope tag on DNA Polδ in *mre11*Δ cells (but not wild type) renders cells extremely sick (Supplementary Figure 6). This prevents ChIP but underscores the importance of DNA Polδ in *mre11*Δ cells.

The nuclease activity of Mre11 is not required for replisome maintenance during pauses in replication

GCRs are attributed to polymerase destabilization and mishaps at replication forks (Kolodner *et al*, 2002; Cobb *et al*, 2005; Lambert *et al*, 2005). The results presented here are consistent with the relatively low rates of chromosomal rearrangement reported for the nuclease dead *mre11*-3 allele compared with the high rates seen in *mre11*Δ cells and *mrx* alleles that disrupt complex formation (Smith *et al*, 2005), such as *mre11*-2. Our data support a structural function for the MRX complex that is dependent upon the integrity of the interactions among the Mre11, Rad50 and Xrs2 components. We show that the nuclease activity of Mre11 is dispensable for replisome maintenance at paused forks, but that the structural integrity of the complex is essential. This is evident from DNA Polε complementation assays where the ectopic expression of *mre11*-3 in *mre11*Δ restored DNA Polε recovery to wild-type levels, but with *mre11*-2 the recovery of DNA Polε remained as in *mre11*Δ cells (Figure 4D). Similar to cells transformed with *mre11*-3, the endogenous nuclease dead *mre11*-D56N allele is not sensitive to low levels of HU (Krogh *et al*, 2005) and showed no additive sensitivity when coupled with a disruption of the SCC establishment factor Ctf4 during replication stress (Figure 7D). Taken together, these data indicate that Mre11 nuclease activity does not contribute in any detectable way to MRX function at stalled forks for recovery from replication stress.

Together SCC factors and the MRX complex promote the resumption of DNA replication

Our data support a model where under conditions of replication stress maintaining newly synthesized daughter strands promotes fork stability (Figure 8). We propose that preser-

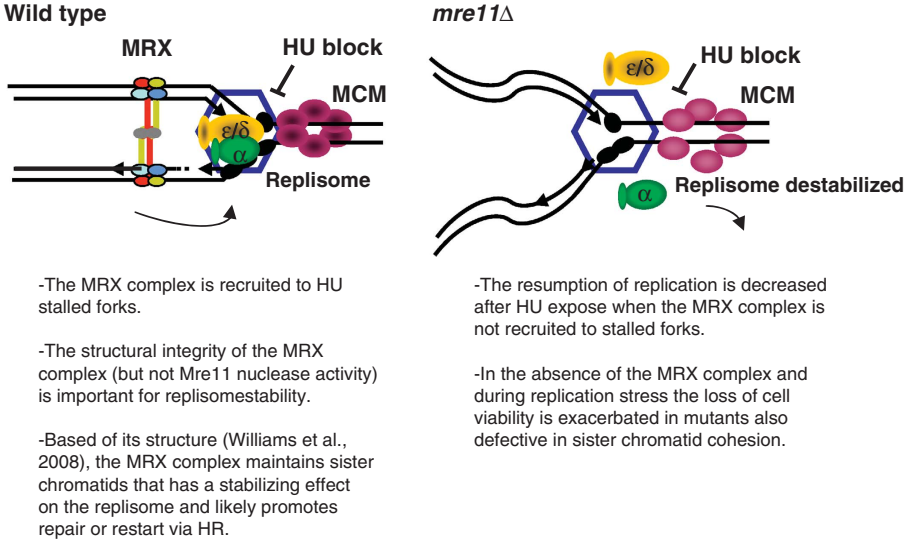

Figure 8 Model for role of the MRX complex at stalled forks. The MRX complex is present at stalled replication forks and is important for fork recovery. The MRX complex is important for the association of DNA polymerase α and ε and possibly other replisome factors (represented by the hexagon) with stalled forks. In the absence of the MRX complex and of properly established SCC, sister chromatids are not held together, and this has a destabilizing influence on the fork resulting in a loss of fork recovery and cell viability.

ving the architecture behind the fork is important for replication recovery, possibly through an HR-dependent restart mechanism. The model we present here suggests that cohesion between sister chromatids during times of replication stress involves the MRX complex. This model is supported by our observations that the MRX complex is recruited to paused forks (Figures 3A and 4A and B) as well as our genetic data that indicate the MRX complex has overlapping functions with factors important for SCC. Indeed, the loss of Mre11 in combination with SCC-deficient mutants cells results in a loss of viability when cells were confronted with replication stress. This model is also supported by structural data showing that the MRX complex can bridge two strands of duplexed DNA, the distance of newly synthesized sister chromatids (Hopfner *et al*, 2002). In summary, we present here a novel function for the MRX complex during replication that involves conformational preservation of newly synthesized DNA behind stalled forks. During pauses in replication if sister chromatids are not maintained, the consequence is disruption of the replisome at the fork, and ultimately the loss of chromosome integrity, a hallmark of *mrx*-deficient cells.

Materials and methods

Strains and plasmids

All strains are listed in Table I. The *mre11Δ*::HIS3 and *ctf4Δ*::TRP1 disruptions were constructed using pFA6a PCR-based cassettes (Longtine *et al*, 1998) and verified by PCR and phenotypic analyses. pRS314-MRE11-FL-TRP-HA, -*mre11*-2-TRP-HA, and -*mre11*-3-TRP-HA were kind gifts from the Dr J Petrini at the Sloan-Kettering Institute (Bressan *et al*, 1998). The plasmid YIplac128 GAL-Scc1-3HA was kindly provided by F Ulhmann at the Cancer Research, UK, and used for integration of SCC1 gene under control of the galactose inducible GAL1 promoter at the LEU2 locus in JC-770 (Sullivan *et al*, 2004).

Survival and drop assays

Survival assays were performed on cultures grown overnight in YPAD at 25°C before exposure to 0.2 M HU at 30°C for the indicated times. In total, 500 cells were plated in triplicate and the percentage of viability was determined from the 0 time point after release from α-factor into HU. Drop assays were performed with 1:5 serial dilutions of cultures grown at 25°C and plated on YPAD ± HU and incubated at 30°C. For complementation experiments, cultures were grown on -TRP selective media and drop assays were performed with 1:5 dilutions on SD-TRP +/− HU and incubated at 30°C. Survival assays with SCC1 overexpression were performed on exponentially grown cultures in YPLG glycerol at 25°C before shifting to YPAD or YPA galactose (2%) for 90 min before 8 h HU treatment at 30°C in the same media. In all, 500 cells were plated in duplicate on YPAD or YPA galactose media with the percentage viability determined from 0 time point after release.

Chromatin immunoprecipitation

ChIP was performed as described earlier in Cobb *et al* (2003, 2005) except that washes buffer contained 0.01% SDS. DNA was quantified by real-time PCR using the ABI 7900 Sequence Detector System in the Southern Alberta Cancer Research Institute (provided by the Alberta Cancer Foundation). Primer sequences are available upon request. α-mouse Dynabeads were coupled with monoclonal antibodies against Myc (9E10) or HA (F-7; SC-7392 Santa-Cruz). Uncoupled Dynabeads were used for background controls.

Table I *S. cerevisiae* strains used in this study

Strain	Genotype	Source
JC-65	JC-141 with *tel1*::*URA*3	This study
JC-141	MATα, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100	R Rothstein (W303-1A)
JC-171	JC-467 with CDC54- 13Myc::*KanMX6*	This study
JC-176	MATa *can1*-100, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *rad9*::*LEU2*	
JC-216	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100, *mcm10-43*	Kawasaki *et al*, 2000
JC-227	MATα, *leu2*-3,-112ura3-52, his4-34,*mcm5-3*	Dziak *et al*, 2003
JC-257	JC-467 *with RPA1-13Myc*::*TRP, Ddc2-3HA*::*URA3*	This study
JC-285	JC-467 with *POL2*-13Myc::*KanMX6*	Bjergbaek *et al*, 2005
JC-371	JC-467 with *mre11*::*HIS3*	This study
JC-388	JC-285 with *mre11*::*HIS3*	This study
JC-445	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *rad51*::*URA3*	This study
JC-467	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100, *pep4*::*LEU2*	R Rothstein (W303-1A)
JC-528	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100, *RAD50-13Myc*::*KanMX6, CDC17-3HA*::*TRP1*	This study
JC-538	JC 539 with *mre11*::*HIS3*	This study
JC-539	JC 467 with *CDC17-3HA*::*TRP1*	This study
JC-549	JC-257 with *mre11*::*HIS3*	This study
JC-553	JC-617 with *mre11*::*HIS3*	This study
JC-554	JC-216 with *mre11*::*HIS3*	This study
JC-559	JC 171 with *mre11*::*HIS3*	This study
JC-564	JC-227 with *mre11*:: *HIS3*	This study
JC-568	JC-371 with *rad9*::*TRP1*	This study
JC-569	JC-216 with *rad9*::*LEU2*	This study
JC-570	JC-569 with *mre11*::*HIS3*	This study
JC-572	JC-618 with *mre11*::*HIS3*	This study
JC-583	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *pol2*-16	Kesti *et al*, 1999
JC-591	JC-216 with *tel1*::*URA*3	This study
JC-604	MATa *ade2-1,trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3-1, GAL, psi+,RAD5,URA3*::*GPD-TK*	Tourriere *et al*, 2005
JC-606	MATa *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 with *mre11-D56N*	Krogh *et al*, 2005
JC-612	JC-617 with *tel1*::*URA*3	This study
JC-616	JC-604 with *mre11*::*HIS3*	This study
JC-617	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100, *pol2*-12	This study derived from Budd and Campbell, 1993

Table I continued

Strain	Genotype	Source
JC-618	JC-617 with *rad9*::*LEU2*	This study
JC-619	JC-583 with *mre11*::*HIS3*	This study
JC-629	JC-633 with *mcm10*-43	This study
JC-630	JC-617 with *rad51*::*URA3*	This study
JC-633	JC-371 with *rad51*::*URA3*	This study
JC-634	JC-216 with *rad51*::*URA3*	This study
JC-647	JC-606 with *ctf4*::*TRP1*	This study
JC-648	JC-467 with *ctf4*::*TRP1*	This study
JC-662	JC-648 with *mre11*::*HIS3*	This study
JC-670	JC-285 with *ctf4*::*TRP1*	This study
JC-683	JC-633 with *pol2*-12	This study
JC-686	JC-285 with *sae2*::*URA3*	This study
JC-743	JC764 with *mre11*::*HIS3*	This study
JC-757	JC-285 with *xrs2*::*HIS3*	This study
JC-764	MATa, *RAD50-3HA*::*TRP1*, *ORC2-9Myc*::*LEU2*, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112	This study
JC-770	MATa, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100	This study
JC-784	MATa, *GAL-SCC1-3HA*::*LEU2*, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100,	This study
JC-786	JC-784 with *scc1-73*	This study
JC-787	JC-784 with *mcm10*-43	This study
JC-795	JC-784 with *ctf4*::*URA3*	This study
JC-796	JC-285 with *rad50*::*KanMX6*	This study
JC-797	JC-795 with *mre11*::*HIS3*	This study
JC-801	JC-784 with *mre11*::*HIS3*	This study
JC-803	JC-787 with *mre11*::*HIS3*	This study

All strains are W303 background unless otherwise indicated.

For complementation experiments, cultures were grown on -TRP selective media before synchronization with α-factor in YPAD pH 5.0 and released in YPAD + 0.2 M HU. The subsequent steps of ChIP were performed as described earlier.

PFGE and 2D gel electrophoresis

Yeast cells were embedded in agarose plugs (3×10^7 cells/plug), and genomic DNA was extracted as described (Lengronne *et al*, 2001). Agarose plugs were used for PFGE. Yeast chromosomes were separated by PFGE (Chef DRII, BioRad) for 18 h at 6V/cm 60″/60″, stained with ethidium bromide and transferred to Hybond XL (GE Healthcare). Southern blot analysis for chromosome IV was detected using a probe to the Rad9 gene. All experiments were performed in a least triplicate, and quantification was carried out using Quantity One (BioRad).

DNA combing

DNA combing was performed as described (Michalet *et al*, 1997). HU was added 30 min before the addition of BrdU. BrdU was detected with a rat monoclonal antibody (BU1/75, AbCys) followed by treatment with a secondary antibody coupled to Alexa 488 (Molecular Probes). DNA molecules were counterstained with an anti-ssDNA antibody (MAB3034, Chemicon) and an anti-mouse IgG coupled to Alexa 546 (Molecular Probes). DNA fibres were analysed on a Leica DM6000B microscope equipped with a CoolSNAP HQ CCD camera (Roper Scientifics) and a 40× objective. Image acquisition was performed with MetaMorph (Roper Scientifics). Representative images of DNA fibres were assembled from different fields of view with Adobe Photoshop as described (Pasero *et al*, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal. org).

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