Rtt107 Phosphorylation Promotes Localisation to DNA Double-Stranded Breaks (DSBs) and Recombinational Repair between Sister Chromatids

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Abstract

Efficient repair of DNA double-stranded breaks (DSB) requires a coordinated response at the site of lesion. Nucleolytic resection commits repair towards homologous recombination, which preferentially occurs between sister chromatids. DSB resection promotes recruitment of the Mec1 checkpoint kinase to the break. Rtt107 is a target of Mec1 and serves as a scaffold during repair. Rtt107 plays an important role during rescue of damaged replication forks, however whether Rtt107 contributes to the repair of DSBs is unknown. Here we show that Rtt107 is recruited to DSBs induced by the HO endonuclease. Rtt107 phosphorylation by Mec1 and its interaction with the Smc5–Smc6 complex are both required for efficient repair of DSBs, whereas during non-homologous end-joining (NHEJ) the broken ends of DNA are directly rejoined. Because of its nature, HR is the preferred choice in eukaryotic cells during the S and G2/M periods of the cell cycle [3]. Upon DSB induction a response that culminates in the enforcement of cohesion around the break site is triggered, which involves the recruitment of cohesin, the protein complex that maintains sister chromatids paired [4,5] to the DSB [6,7]. Increased cohesion between the broken chromatid and the intact one is thought to promote post-replicative DSB repair between them. The Smc5–Smc6 complex, related to cohesin, is also recruited to DSB sites [8,9,10] to mediate repair via SCR

Introduction

Double-stranded breaks (DSB) can arise from exposure to a variety of DNA damaging agents, but also as a consequence of cellular processes, for instance during intermediate stages in the repair of various DNA lesions or during DNA replication. DSBs trigger a cellular response that involves checkpoint, repair, chromatin and structural proteins many of which are recruited to the site of damage. This response is highly conserved, highlighting the importance of ensuring correct DSB repair to prevent genomic instability.

Cells can repair DSBs using a variety of pathways, including non-homologous end-joining (NHEJ) [1] and homologous recombination (HR) [2]. HR involves the use of similar sequences as a template to repair the break, whereas during NHEJ the broken ends of DNA are directly rejoined. Because of its nature, HR requires an intact donor DNA molecule. Sister-chromatids are the ideal templates to repair DSBs via HR because they contain an exact copy of the broken site. Therefore it is not surprising that DSB repair by sister chromatid recombination (SCR) is the preferred choice in eukaryotic cells during the S and G2/M periods of the cell cycle [3]. Upon DSB induction a response that culminates in the enforcement of cohesion around the break site is triggered, which involves the recruitment of cohesin, the protein complex that maintains sister chromatids paired [4,5] to the DSB [6,7]. Increased cohesion between the broken chromatid and the intact one is thought to promote post-replicative DSB repair between them. The Smc5–Smc6 complex, related to cohesin, is also recruited to DSB sites [8,9,10] to mediate repair via SCR [8,9]. Although the exact role of Smc5–Smc6 during SCR repair is not well understood, the fact that cohesin loading is altered in the absence of Smc5–Smc6 raises the possibility that Smc5–Smc6 mediates SCR by promoting cohesin loading to DSB sites.

In budding yeast, the checkpoint kinases Mec1p (ATM) and Tel1p (ATR) are recruited early to break sites [11]. Mec1p/Tel1p-dependent phosphorylation of a variety of proteins at the site promotes a cascade of events important to detect, signal and repair the lesion [12]. H2AX is a Mec1 target, whose phosphorylation is required for cohesin loading [7]. Rtt107, also a target of Mec1, is a phosphoprotein thought to function as a scaffold necessary for the assembly of repair proteins onto sites of damage [13,14,15,16]. Rtt107 promotes restart of DNA replication forks after DNA damage [13,14,15,17] and interacts with a number of DNA repair proteins including the Rad51 paralogs, Rad55 and Rad57 [15] as well as the Smc5–Smc6 complex [18].

Previously we demonstrated that yeast Smc5–Smc6 is recruited to DSBs to mediate SCR [8]. Here, we report that the Smc5–Smc6 interacting factor Rtt107 is also recruited to DSBs and affects SCR repair. We show that phosphorylation of Rtt107, which depends on Mec1p and Smc5–Smc6, is sufficient for its recruitment to HO-induced breaks.

Results

Rtt107 localises to HO-induced DSBs

On the basis of the interaction between Rtt107 and the Smc5–Smc6 complex [18], we investigated whether Rtt107 localises to an HO endonuclease catalyzed DSB formed at a unique site in the
MAT locus on chromosome III [19]. Transcriptional regulation of the HO endonuclease using the galactose inducible promoter allowed us to control the timing of DSB induction [20]. DSBs at the MAT locus are repaired by homologous recombination with HML and HMR loci [21]. To prevent repair of HO-induced DSBs, both HM loci were deleted in our strains, thus maximising the persistence of the break to facilitate possible detection of Rtt107 at this site.

Cells were engineered to express an epitope tagged version of Rtt107 (\textit{RTT107-9xMYC}). Chromatin binding of this protein to sites around the DSB were assayed by chromatin immunoprecipitation (ChIP). We used different primer pairs covering at least 30 kb on either side of the DSB (Fig. 1A). DNA sequences were amplified from the input chromatin and chromatin immunoprecipitated to calculate the relative percentage of immunoprecipitated material. To control for DSB-independent effects on protein occupancy we also used a primer pair specific for sequences located in a different chromosome (chromosome VI). First we tested the efficiency of DSB induction and intact DNA damage checkpoint activation. We used the checkpoint protein Ddc2 fused to GFP as an \textit{in vivo} marker of DSB formation in our strains [22]. Two hours after galactose-mediated HO induction ~80% of the cells arrested as dumbbells with a single Ddc2 focus (data not shown) demonstrating that the HO break at the MAT locus is efficiently induced and not repaired, thus causing G2/M arrest in our experimental system.

Next we evaluated the binding of Rtt107 around the HO site. In the absence of a DSB, we found low Rtt107 binding across the region (Fig. 1A–B; uncut). After 2 hours of HO induction, we detected a general increase in binding around the regions flanking

![Figure 1. Rtt107 is enriched on regions flanking the HO-induced DSB.](http://www.plosone.org/doi/abs/10.1371/journal.pone.0020152.g001)

A DSB was induced at the MAT locus in strains expressing Rtt107-9myc. A strain containing a Myc-tagged version of Rtt107, and a galactose-inducible HO endonuclease (CCG6983) were grown at 30°C in YP raffinose. Cells were then split and one half transferred to galactose (cut), while the other half were grown in the absence of galactose (uncut). Chromatin immunoprecipitation was performed around the HO cleavage site in the MAT locus of chromosome III. The binding of Rtt107 around the locus was evaluated 2 and 4 hours post-induction (2 hrs, 4 hrs) or in the absence of the DSB (uncut). Input DNA and DNA immunoprecipitated were amplified with primers at the indicated distances from the HO site. The average of two independent experiments with the corresponding standard deviation is shown. A locus on chromosome VI was used as a control. (B) Epitope tagged Rtt107 levels were compared with an isogenic untagged strain (CCG2781) before and after HO mediated DSB.

doi:10.1371/journal.pone.0020152.g001
the break (Fig. 1A; 2 hrs). The maximum DSB-induced increase was ~7-fold and localised to regions 0.5–10 kb away from the DNA break on either side (Fig. 1A–B; 2–4 hrs). The relative binding decreased with distances greater than 20 kb from the break, however, the regions immediately adjacent to the DSB site were found to be also high in binding (Fig. 1A–B; 2–4 hrs). Thus the presence of a DSB induces a significant increase in Rtt107 binding in a domain around the DSB.

Smc5–Smc6 complex contribute to Rtt107 phosphorylation

Rtt107 is extensively phosphorylated by Mec1 in response to DNA damage [13]. Consistent with this, we found that exposure to MMS, hydroxyurea, camptothecin (CPT) or an HO-induced DSB in wildtype cells expressing RTT107-9MYC led to substantial mobility changes of Rtt107 on SDS-PAGE gels. The mobility change was reversed by treatment of the extracts with λ-phosphatase (Fig. 2A and Fig. S1). Previous studies of Rtt107 phosphorylation used western blot analysis with antibodies reported to recognise phosphorylated SQ/TQ motifs, which are sites of Mec1/Tel1 phosphorylation [23]. We have been able to resolve Rtt107 phosphorylation through changes in protein mobility on SDS-PAGE gels using a 30:1 ratio of acrylamide:biacrylamide (Fig. S1). We therefore tested whether in addition to Mec1 other factors contribute to Rtt107 phosphorylation. Rtt107 was tagged in different checkpoint kinase mutant backgrounds, including the effector kinases Rad53 and Chk1 and the adaptor kinases Rad9 and Mrc1, and cells were exposed to different genotoxic agents (Fig. 2A). We found that none of these kinases contribute to Rtt107 phosphorylation (Fig. 2A). In fission yeast, the Rtt107 homologue Brcl was identified as a multi-copy suppressor of the UV hypersensitivity associated with loss of Rad18 [24], the homologue of Smc6. Recently, a physical interaction between budding yeast Smc5–Smc6 complex and Rtt107 was described [18]. We therefore investigated whether Smc5–Smc6 function contributes to Rtt107 phosphorylation in response to DNA damage. To this aim, Rtt107 was tagged in cells carrying the conditional allele for Mec1 and Smc5–Smc6 (Fig. 3A), demonstrating that Mec1 is indeed required for Rtt107 phosphorylation. Rtt107 was phosphorylated upon exposure to MMS in wildtype and smc6-9 cells at permissive temperatures (Fig. 2B). In contrast, we observed Rtt107 phosphorylation only in wildtype cells under non-permissive temperatures (Fig. 2B). These results demonstrate that Smc5–Smc6 function is required for complete phosphorylation of Rtt107 by the Mec1 kinase.

Mec1 and Smc5–Smc6 complex are necessary for Rtt107 localisation to HO-induced DSBs

Based on the observed phosphorylation of Rtt107 in response to DNA damage by Mec1 and Smc5–Smc6 [13], we investigated whether these factors are required for the localization to HO-catalyzed DSBs (Fig. 1). We examined Rtt107 levels around the MAT DSB in the temperature sensitive allele smc6-9 and the mec1D mutant. We focused our analysis in the regions immediately flanking the break (5 kb; Fig. 3). Deletion of the Mec1 kinase completely abolished the recruitment of Rtt107 to the MAT DSB (Fig. 3A), demonstrating that Mec1 is indeed required for Rtt107 recruitment. Next, we analysed the contribution of the Smc5–Smc6 complex. We observed that even at permissive temperatures for the smc6-9 allele (23°C), the recruitment of Rtt107 to the MAT DSB was partially compromised (Fig. 3B–C). The effect was more dramatic under non-permissive temperatures (37°C) where, like in mec1D cells, Rtt107 recruitment to sites flanking the MAT DSB was not observed (Fig. 3C). These results demonstrate that Mec1 and Smc5–Smc6 function are important for Rtt107 recruitment to DSBs. Furthermore, we found that deletion of the Tel1 or Rad53 kinases, which do not prevent Rtt107 phosphorylation (Fig. 2A) do not affect its recruitment to DSBs (Fig. S2), demonstrating a correlation between Rtt107 phosphorylation and its ability to be recruited to DSBs.

Rtt107 phosphorylation is sufficient for DSB recruitment

Mec1 phosphorylation occurs in Ser/Thr–Glu (SQ/TQ) motifs [23]. A number of SQ/TQ motifs are found scattered within the Rtt107 aminoacid sequence, however a cluster of SQ/TQ motifs at the C-terminal region of Rtt107 have been previously identified as critical for Mec1 phosphorylation [13]. In order to investigate whether phosphorylation in these motifs is required for Rtt107 recruitment to DSBs, we mutated the Ser743, Thr758, Thr773 and Ser806 to alanine (‘A’) residues to generate the Rtt107-AQ allele (Fig. 4A). We expressed the Rtt107-AQ allele tagged with 6 copies of the HA epitope in the C-terminal region under the control of the GAL1-10 promotor in rtt107Δ cells carrying the HO MAT DSB system. The resulting strain was sensitive to a variety of DNA damage agents (data not shown) [13]. We then evaluated the binding of Rtt107-AQ around the HO site. In the absence of a DSB at MAT, we found no Rtt107 binding across the region (Fig. 4A; uncut). After 4 hours of HO induction, we did not find evidence for Rtt107-AQ recruitment to the regions flanking the break (Fig. 4A). We therefore conclude that phosphorylation on the C-terminal SQ/TQ cluster is required for Rtt107 recruitment to the MAT DSB.

To investigate whether phosphorylation on the Rtt107 C-terminal SQ/TQ cluster is sufficient for DSB recruitment, we mutated Ser743, Thr758, Thr773 and Ser806 to phosphomimicking aspartic acid (‘D’) residues to generate the Rtt107-DQ allele (Fig. 4B). We expressed the tagged Rtt107-DQ in mec1Δ cells, where wildtype Rtt107 is neither phosphorylated in response to DNA damage [13] (Fig. 2A) nor recruited to HO-induced DSBs (Figs. 3A and 4B). Unlike wildtype Rtt107, Rtt107-DQ was recruited to the regions flanking the break following DSB induction at MAT in the absence of Mec1 (Fig. 4B). These results show that phosphorylation on the C-terminal SQ/TQ cluster of Rtt107 is sufficient for its recruitment to DSBs.

Rtt107 regulators are dispensable for DSB recruitment

The N-terminal region of Rtt107 contains four tandem BRCT domains, which are characteristic of proteins involved in cell cycle checkpoint functions related to DNA damage [26]. These domains have been shown to interact with a number of proteins, including the Slx4/Slx1 nuclease complex [14]. In addition, Rtt107 is recruited to stalled replication forks via interaction with the cullin Rtt101 protein [17]. We therefore wished to address whether these interactions are important for the observed recruitment of Rtt107 to DSBs. First, we investigated whether Rtt107 is recruited to DSBs in the absence of Slx4. Slx4 largely prevents Rtt107 SQ/TQ phosphorylation [14], a requirement for Rtt107 DSB recruitment (Fig. 4). Surprisingly, we found no significant defects in Rtt107 recruitment to MAT DSBs in the absence of Slx4 (Fig. 4A). Next we tested whether the cullin Rtt101 is required for the localization of Rtt107 to breaks. We found Rtt107 recruitment to HO-induced DSBs to be normal in rtt101Δ cells (Fig. 5B). Therefore, we conclude that while the Slx4/Slx1 and Rtt101 interactions might be important for Rtt107 functions at damaged forks [14,17] they are not essential for its recruitment to DSBs induced by HO.

Rtt107 contributes to sister chromatid recombination

The role of Smc5–Smc6 complex at DSBs is to promote repair of the lesion by sister chromatid recombination [8,9]. Deletion of
Rtt107 has been shown to have a small effect on sister chromatid exchange (SCE) rates in S-phase cells exposed to DNA damage [13]. To investigate whether phosphorylation is important for Rtt107 function in SCR, we used an assay that can measure spontaneous recombination between sister chromatids in the absence of DNA damage [27]. The assay is based on two ade2 marker genes separated by a TRP1 gene. Recombination between the ade2 alleles that generates a wildtype ADE2 gene and retains the TRP1 gene is indicative of gene conversion events between sister chromatids [27]. Deletion of the Rad51 paralogs, Rad57 and Rad55, has been shown to significantly decrease spontaneous recombination events between sister chromatids in this assay [27].

First, we investigated whether the smc6–9 conditional mutant, known to be important for SCR, shows defects in the formation of DSBs.
Figure 3. Mec1 kinase and the Smc5–Smc6 complex are required for Rtt107 recruitment to induced MAT DSB. (A) Phosphorylation of Rtt107 upon DNA damage is dependent on the ATR kinase, Mec1. A DSB was induced at the MAT locus in a wildtype strain expressing Ddc2-GFP (CCG6983) and Ddc1-GFP in a mec1Δ mutant (CCG7143) both also expressing Rtt107-9myc. Cells were collected at indicated times after HO induction and scored for formation of Ddc2 and Ddc1 foci. The binding of Rtt107 around the MAT DSB locus on chromosome III was assayed by chromatin immunoprecipitation.
immunoprecipitation at the indicated time points, after (cut-2 hours) and before (uncut) DSB induction. The averages of two independent experiments with the corresponding standard deviation are shown. A locus on chromosome VI was used as a control. (B) Rtt107 enrichment to regions flanking the HO-induced DSB is dependent on the function of the Smc5–Smc6 complex. A DSB was induced at the MAT locus in wildtype (CCG6983) and smc6–9 (CCG6985) strains expressing Ddc2-GFP and Rtt107-9myc tagged at permissive (25°C) temperature. The binding of Rtt107 around the MAT DSB locus on chromosome III was assayed by chromatin immunoprecipitation at the indicated time points, after (cut-2 hours) and before (uncut) DSB induction in wildtype and smc6–9 cells at 25°C. The averages of two independent experiments with corresponding standard deviations are shown. A locus on chromosome VI was used as a control.

doi:10.1371/journal.pone.0020152.g003

Ade2+Trp1+ recombinants. We measured the rate of Ade+ Trp1+ recombinants in wildtype and smc6–9 cells growing at 30°C, which is semi-permissive for the smc6–9 allele [25], and found a 6-fold reduction of recombinant formation in the smc6–9 mutants (Fig. 6A). Consistent with previous reports, rad55Δ caused a 1000-fold reduction in gene conversion events between sister chromatids [27] (Fig. 6A). The reduction in Ade2+Trp1+ recombinants in rtt107Δ was found to be similar to that observed for smc6–9 cells (Fig. 6A).

To evaluate whether Rtt107 phosphorylation promotes gene conversion events between sister chromatids, we used the Rtt107–AQ and Rtt107–DQ alleles in the genetic assay. Expression of wildtype Rtt107 from the GAL1–10 promoter in rtt107Δ cells yielded similar levels of Ade2+Trp1+ recombinants to wildtype cells (Fig. 6B). Recombinant levels in cells expressing the phospho-defective mutant Rtt107–AQ were comparable to those found for rtt107Δ cells (Fig. 6B). In contrast, we observed partial rescue of Ade2+Trp1+ recombinant formation in rtt107Δ cells expressing the phospho-mimicking Rtt107 allele, Rtt107–DQ (Fig. 6B). These results demonstrate that phosphorylation of Rtt107 is involved in the function of this protein promoting sister chromatid recombination events.

Discussion

Genomes are constantly being challenged by lesions on their DNA that are either induced as a consequence of the action of exogenous agents, such as different drugs causing DNA damage, or as a consequence of the cell’s own metabolism, for instance during DNA replication. DSBs are one of the most serious lesions in DNA and can be lethal if not repaired or can generate deleterious effects to the genome if repaired improperly. Therefore, accurate mechanisms for DNA double-strand break repair (DSBR) are important for all living organisms. Repair generally occurs in a stepwise manner and begins with the recruitment of different factors to the break site to orchestrate a coordinated response that involves signalling and repair activities. It is therefore important to understand the order of events at DSBs as well as the dependencies between the factors that are recruited.

The checkpoint kinase Mec1 is recruited to break sites early during the repair response [11]. Mec1 phosphorylation then acts on a variety of proteins at the site [12]. One of Mec1 targets is the Rtt107 scaffold protein [13], however, prior to this study Mec1-dependent phosphorylation of Rtt107 had only been studied in the context of damaged replication forks [13]. Previous investigation of Rtt107 homologues in fission yeast [24] and mammalian cells, raised a possible function for Rtt107 at DSBs; PTIP (mammalian Rtt107 homologue) is indeed recruited to DNA damage sites formed by ionizing radiation [28]. Here, we have investigated the role of Rtt107, and its phosphorylation, at DNA double-stranded breaks. We observed that following the induction of an irreparable break by the HO endonuclease, Rtt107 is recruited to regions surrounding the break. We demonstrated that the Mec1 kinase is required for both Rtt107 phosphorylation in response to a single DSB as well as its recruitment, as both events are absent in meclA mutants. This result indicates that the recruitment of Rtt107 to DSBs is controlled by Mec1 phosphorylation. We confirmed this hypothesis showing that the phospho-mutant allele of Rtt107 (Rtt107–AQ) is unable to be recruited to breaks in the presence of Mec1 while a phospho-mimicking allele (Rtt107–DQ) is indeed recruited even when Mec1 is non-functional. The dependency of Rtt107 recruitment to DSB on phosphorylation by the Mec1 kinase is in contrast to Rtt107 recruitment to stalled forks, which is independent of Mec1 [17], and thus phosphorylation.

A number of protein interactions have been described for Rtt107 [14,15,17,18]. We investigated whether some of these interactions are important for Rtt107 recruitment to DSBs. We found that recruitment was drastically reduced in the smc6–9 mutant allele, suggesting that intact Smc5–Smc6 function is a requirement for Rtt107 DSB-loading. Furthermore, we observed that Rtt107 phosphorylation is impaired in smc6–9 mutants, confirming that Rtt107 phosphorylation is important for recruitment. Surprisingly, deletion of Slx4 did not prevent Rtt107 recruitment to DSBs, despite the fact that Slx4 is required for Mec1-dependent phosphorylation [14]. Interestingly, Slx4 is also not essential for Rtt107 binding to stalled replication forks [17]. It is possible that low-level Rtt107 phosphorylation is retained in slx4Δ cells and that this might be sufficient to promote detectable Rtt107 recruitment to damaged forks and/or DSBs.

Unlike bacterial models, the role of recombination at stalled forks is poorly understood in eukaryotes. It is presently unclear why recombination at collapsed forks can, under some circumstances, rescue replication while in other cases it might generate genomic rearrangements. Prompted by the positive role of the Smc5–Smc6 complex in sister chromatid recombination [8,9] and its interaction with Rtt107 [18], we explored a potential role of Rtt107 in promoting repair by the SCR pathway. Importantly, we found that rtt107Δ cells exhibit a defect in the formation of recombinant products between sister chromatids (an assay measuring unequal exchange between sister chromatids). The defect was similar to that observed for smc6–9 mutants in the same assay. Furthermore, we showed that Rtt107 phosphorylation contributes to its role in SCR since the Rtt107–DQ but not the Rtt107–AQ allele could partially restore the formation of recombinant products between sister chromatids in rtt107Δ cells.

A future question is the role of the Rtt107 scaffold protein at DSBs in the recruitment of downstream repair factors. The Rad55-Rad57 complex is known to play a role in the stabilization of the Rad51 nucleoprotein filament, and rad55−/rad57Δ have strong defects in SCR [27]. In fission yeast, Rhp55/Rhp57 (homologues of Rad55/Rad57) are required for Brc1 (Rtt107 homologue) suppression of smc6–74 mutants [29]. In budding yeast, the Rad55/Rad57 heterodimer interacts with Rtt107 [15] and Rad55 is a
known target of the Mec1 kinase [30]. Interestingly, Rad55 phospho-mutants show similar phenotypes to both Rtt107 and smc6–9 mutants, i.e. inability to complete replication and failure to re-enter pulsed-field gels after treatment with the DNA damage agent MMS [13,30,31,32]. It is tempting to speculate that the phosphorylation of the BRCT-domains of Rtt107 could attract phosphorylated Rad55 and other repair factors to mediate repair of DNA lesions by the error-free sister recombination pathway.

Figure 4. Rtt107 phosphorylation is required for its recruitment to the MAT DSB. (A) The phospho-mutant allele of Rtt107, Rtt107-AQ is not recruited to HO-induced DSBs. A DSB was induced at the MAT locus in a strain expressing wildtype Rtt107-6HA from the GAL1-10 promoter (CGG7290) or the phospho-mutant version Rtt107-AQ-6HA (CCG7291) is shown. The binding of Rtt107 and Rtt107-AQ around the MAT DSB locus on chromosome III was assayed by chromatin immunoprecipitation at the indicated time points, after (cut- 4 hours) and before (uncut) DSB induction. The averages of two independent experiments with corresponding standard deviation are shown. A locus on chromosome VI was used as a control. Western blot analysis of Rtt107-6HA and Rtt107-AQ-6HA upon DSB induction is also shown. No band-shift was observed for Rtt107-AQ, suggesting that this protein is not phosphorylated upon DSB induction. (B) The phospho-mimicking allele of Rtt107, Rtt107-DQ is recruited to HO-induced DSB in the absence of the Mec1 kinase. A DSB was induced at the MAT locus in a mec1Δ strain expressing wildtype Rtt107-6HA from the GAL1-10 promoter (CGG7558) or the phospho-mimicking version Rtt107-DQ-6HA (CCG7556). The binding of Rtt107 and Rtt107-DQ around the MAT DSB locus on chromosome III was assayed by chromatin immunoprecipitation at the indicated time points, after (cut- 4 hours) and before (uncut) DSB induction. The averages of two independent experiments with corresponding standard deviations are shown. A locus on chromosome VI was used as a control.

doi:10.1371/journal.pone.0020152.g004
Methods

Yeast, Media and Cell growth conditions

Yeast strains used are listed in supplementary materials (Table S1). Media for yeast growth, both complete YP (1% yeast extract, 2% peptone) and synthetic drop out media lacking various amino acids were prepared according to standard protocols. Media were mixed with different carbon sources, depending on the experiment: glucose (D), galactose or raffinose all at 2% final concentration. Carbon sources were sterilised by filtration. To detect Rtt107 phosphorylation for the various experiments, liquid cultures were grown overnight to a final concentration of 0.7<OD<0.4 and were then incubated in the presence of different DNA damage agents; methyl methanesulfonate (MMS), camptothecin (CPT) or hydroxyurea (HU) and grown for 2 hours more before processing. Final concentration of MMS was 0.03%, final concentration of CPT was 5 μg/ml and the final concentration of HU was 50 μM. The drugs were added to the cultures as in [13]. Cultures growing in liquid media were incubated at 25°C, 30°C or 37°C in flasks (the volume of the flask was 5 times the volume of the culture), shaken at high speed [250 rotations per minute (rpm) in a New Brunswick G25 shaking incubator (GMI

![diagram](http://example.com/diagram.png)

**Figure 5. Slx4 and Rtt101 are not required for Rtt107 recruitment to the MAT DSB.** (A) Slx4 deletion does not prevent Rtt107 recruitment to HO-induced DSB. A DSB was induced at the MAT locus in wildtype (CCG6983) and slx4Δ (CGG7970) cells expressing Rtt107-9myc. The binding of Rtt107 around the MAT DSB locus on chromosome III was assayed by chromatin immunoprecipitation at the indicated time points, after (cut- 4 hours) and before (uncut) DSB induction. The averages of two independent experiments with corresponding standard deviations are shown. A locus on chromosome VI was used as a control. (B) Rtt101 deletion does not prevent Rtt107 recruitment to HO-induced DSBs. A DSB was induced at the MAT locus in wildtype (CCG6983) and rtt101Δ (CGG7970) cells expressing Rtt107-9myc. The binding of Rtt107 around the MAT DSB locus on chromosome III was assayed by chromatin immunoprecipitation at the indicated time points, after (cut- 4 hours) and before (uncut) DSB induction. The averages of two independent experiments with corresponding standard deviations are shown. A locus on chromosome VI was used as a control. doi:10.1371/journal.pone.0020152.g005
Inc.), with 4 cm rotation diameter, or at 150 rpm in a SM1003 shaking incubator [Kulner], with 44 cm rotation diameter. For growth of Rtt107 alleles under the \textit{GAL1–10} promoter, cells were grown in 20 ml YP media with raffinose (supplemented with 0.01% glucose) overnight to 0.3 OD\textsubscript{595} < 0.5 at 25°C. Expression of the constructs was subsequently induced by addition of galactose at a final concentration of 2% w/v. The cultures were then grown for 2–4 hours, before processing for western blotting or immunoprecipitation. To detect Rtt107 in \textit{smc6–9} temperature sensitive strains, cells were grown overnight at 25°C in YP media with raffinose overnight to 690.3 OD\textsubscript{595} < 0.5 and then transferred to 37°C for 1 hour to inactivate the \textit{smc6–9} allele, before the addition of MMS.

Culture conditions for HO endonuclease induction

The HO endonuclease was induced in strains carrying a stably integrated \textit{GAL1–10:HO} construct [33]. Cells were grown in YP media with raffinose (supplemented with 0.01% glucose) overnight to 0.3 OD\textsubscript{595} < 0.5 at 25°C. HO expression was subsequently induced by addition of galactose at a final concentration of 2% w/v. The cultures were then grown for 2–4 hours and samples were taken to check Ddc2-GFP foci formation to detect the efficiency of the DSB induction in the population. To induce the HO endonuclease in \textit{smc6–9} temperature sensitive alleles, strains were grown overnight at 25°C in YP media with raffinose to 0.3 OD\textsubscript{595} < 0.5 and then transferred to 37°C for 1 hour to inactivate the \textit{smc6–9} allele, before HO induction at 35°C.

Construction of Rtt107 phosphorylation mutants

The Rtt107 constructs cloned for this study were made using gene synthesis (GeneCust). Three constructs were synthesized; Rtt107, Rtt107-AQ and Rtt107-DQ. All constructs contained 6HA epitopes in the C-terminal region and were expressed using the \textit{GAL1–10} promoter in Rtt107-AQ, Ser743, Thr758, Thr773 and Ser806 were changed to alanine, while in Rtt107-DQ these sites were changed to aspartic acid. All constructs were cloned in the integrative yeast plasmid pRS416 containing the URA3 selectable marker. For transformation, constructs were digested by \textit{Sau} I and integrated into the genome at the endogenous \textit{ura3} locus.

SDS-PAGE and Western blot

To detect phosphorylation shifts of Rtt107, gels with different ratios of acrylamide: bisacrylamide were prepared. Gels were run at 100 V in Tris-glycine-SDS running buffer (National Diagnostics) and were transferred to polyvinylidene fluoride transfer membrane (Hybond-P, Amersham Biosciences) in a Biorad blotting system in Tris-glycine transfer buffer (National Diagnostics) containing 20% methanol. Transfer was for 1.5 hours at 250 mA or overnight at 30 V. Membranes were blocked in 5% skimmed milk powder in PBS with 0.1% Tween 20 (PBST) for at least 30 minutes, then incubated with mouse monoclonal anti-Myc IgG1 antibody 9E10 (Roche), at a 1/5000 dilution (from stock 5 mg/ml) in blocking solution for 1 hour at room temperature or overnight at 4°C in 2.5% milk powder in PBST. Following several washes in PBST, membranes were incubated with sheep anti-mouse IgG horseradish-peroxidase-linked antibody (GE Healthcare) at 1/10000 in PBST, followed by washing in PBST, the ECL Plus Western Blotting Detection System (GE Healthcare) was used to detect the secondary antibody.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation experiments were conducted as in [34]. Primers sites used were as in [8]. Real-time PCR was
used to quantify enrichment. PCR reactions were performed using the SensiMix NoRef Kit (Quatance). Reactions were carried out according to the manufacturer’s instructions in a total volume of 20 µl, containing 1 µl of immunoprecipitated or input DNA and 1.5 µl of 10 µM oligonucleotide primer pairs. Reactions were amplified using a DNA Engine Opticon2 thermal cycle and were analysed using Opticon software (MJ Research). Each PCR reaction was reproduced in duplicate: reactions in which the difference between the two duplicates was bigger than 0.5 cycles were not considered. PCR amplifications were analysed for the melting curve profile to confirm the absence of contaminant PCR products. Mean “threshold cycle number” (or Ct value) was calculated for each PCR, selected in the window of exponential amplification phase. Enrichment was calculated using the following formula: enrichment = 2(Ct IP DNA – Ct input DNA), where Ct IP is the Ct value for the immunoprecipitated sample, and Ct input is the Ct value for the input DNA. The specificity of the enrichment was tested by comparing the values from tagged and untagged strains.

Recombination assays for sister chromatid exchanges

The sister chromatid exchange used has been described before [27]. In brief, yeast strains were grown on YPD plates for 2–3 days at 30°C. 5 independent colonies were inoculated into 5 ml of YPD, and cultures were grown overnight at 30°C. For the Rtt107 phosphorylation mutants, strains were grown on YPD plates for 2–3 days at 30°C. Colonies were inoculated into 5 ml of YPD, and cultures were grown overnight at 30°C. Cells were then washed and transferred to medium containing galactose, and the constructs were expressed for 2 hours, before washing and aliquoting into dilutions. Cells were then plated on SC medium, and SC medium minus adenine and tryptophan supplemented with galactose. Cells were pelleted and resuspended in 1 ml of sterile water. Aliquots of appropriate dilutions were plated onto SC medium to determine the number of viable cells in each culture and onto SC medium minus adenine and tryptophan for the recombination assay, to determine the total number of recombinants in each culture. Plates were incubated for 3–5 days, after which the colonies were counted. For each strain, recombination rates were measured three times on independent isolates and the mean values are shown.

Supporting Information

Figure S1 Western blot analysis showing different conditions to detect Rtt107 phosphophorylation upon DNA damage. Logarithmically growing cultures were treated with 0.3% MMS, 50 µM HU and 50 µg CPT for 90 minutes. Samples were then fixed with RIPA buffer. Extracts were run on SDS-PAGE gels with different concentrations of acrylamide:bis-acrylamide as shown. (A) SDS-PAGE gels with a 37:1 ratio of acrylamide:bisacrylamide. Immunoblots were probed with anti-Myc antibodies. (B) SDS-PAGE gels with a 30:1 ratio of acrylamide:bisacrylamide. Immunoblots were probed with anti-Myc antibodies.

Table S1 Yeast strains used in this study.

Acknowledgments

We thank members of the Aragon laboratory for discussions and critical reading of the manuscript. We also thank Jim Haber and Patrick Sung for providing materials.

Author Contributions

Conceived and designed the experiments: LA. Performed the experiments: PU. Analyzed the data: PU. Contributed reagents/materials/analysis tools: FV-M AJ. Wrote the paper: LA.

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