Rtt107 BRCT domains act as a targeting module in the DNA damage response

Grace P. Leung a, Joshua A.R. Brown a, J.N. Mark Glover b, Michael S. Kobor a, ∗

a Centre for Molecular Medicine and Therapeutics, Child and Family Research Institute, Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia V5Z 4H4, Canada
b Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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Cells are constantly exposed to assaults that cause DNA damage, which must be detected and repaired to prevent genome instability. The DNA damage response is mediated by key kinases that activate various signaling pathways. In Saccharomyces cerevisiae, one of these kinases is Mec1, which phosphorylates numerous targets, including H2A and the DNA damage protein Rtt107. In addition to being phosphorylated, Rtt107 contains six BRCA1 C-terminal (BRCT) domains, which typically recognize phospho-peptides. Thus Rtt107 represented an opportunity to study complementary aspects of the phosphorylation cascades within one protein. Here we sought to describe the functional roles of the multiple BRCT domains in Rtt107. Rtt107 BRCT5/6 facilitated recruitment to sites of DNA lesions via its interaction with phosphorylated H2A. Rtt107 BRCT3/4 also contributed to Rtt107 recruitment, but BRCT3/4 was not sufficient for recruitment when BRCT5/6 was absent. Intriguingly, both mutations that affected Rtt107 recruitment also abrogated its phosphorylation. Pointing to its modular nature, replacing Rtt107 BRCT5/6 with the BRCT domains from the checkpoint protein Rad9 was able to sustain Rtt107 function. Although Rtt107 physically interacts with both the endonuclease Slx4 and the DNA replication and repair protein Dpb11, only Slx4 was dependent on Rtt107 for its recruitment to DNA lesions. Fusing Rtt107 BRCT5/6 to Slx4, which presumably allows artificial recruitment of Slx4 to DNA lesions, alleviated some phenotypes of rtt107Δ mutants, indicating the functional importance of Slx4 recruitment. Together this data revealed a key function of the Rtt107 BRCT domains for targeting of both itself and its interaction partners to DNA lesions.

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1. Introduction

DNA is constantly damaged by internal and external agents, which must be detected and repaired to prevent genome instability. A crucial element of the cellular response to DNA damage is the activation and amplification of signaling pathways. The central components of the signaling cascades are phosphoinositol-3-kinase-related kinases (PIKK), which in Saccharomyces cerevisiae are Mec1 and Tel1 [1]. They are activated via a variety of pathways, which are initiated by the DNA damage sensors including the MRX complex and RPA protein. Mec1 and Tel1 then phosphorylate a host of targets that mediate DNA damage responses including cell cycle arrest, transcription induction, and repair pathways [1].

The cell uses post-translational modifications to respond to external stimuli and exert dynamic control over biological processes. In the DNA damage response, phosphorylation is one of the main and best-understood modifications, and has been extensively characterized. Correspondingly, many of the proteins in these pathways contain domains that recognize phosphorylated peptides [2]. One major class of these phospho-recognition domains is the BRCA1 C-terminal (BRCT) domain, which was first identified in the tumor suppressor BRCA1 [3–6]. BRCT domains preferentially recognize phosphoserine, and a pair of BRCT domains typically works together in tandem to form a binding pocket for the phosphate group [7]. There are multiple examples of BRCT domains mediating phosphorylation-dependent protein–protein interactions that are crucial for protein recruitment to DNA lesions and subsequent pathway activation [7]. Additionally, some BRCT domains can mediate phosphorylation-independent protein interactions as well as interactions with DNA [7].

One of the first targets of the checkpoint kinase Mec1 is phosphorylation of H2A S129 in yeast (termed γH2A), or the histone variant H2AX on Ser139 in humans [8–10]. In S. cerevisiae, γH2A spreads up to 50 kb away from the site of DNA damage, and is believed to form a binding platform for a number of downstream
factors [11,12]. These include the chromatin remodeling complexes SWR1-C and Ino80, and the histone acetyltransferase complex NuA4 [13]. The checkpoint adaptor Rad9 is also recruited by γH2A, and this protein–protein interaction is mediated by a pair of BRCT domains in Rad9 [14–16]. Despite the key role of γH2A in the DNA damage signaling pathways, the H2A S129A mutant is only mildly sensitive to DNA damaging agents and can still activate checkpoint arrest [8,17,18].

Another target of Mec1 is Rtt107, which is phosphorylated at several S/T-Q motifs [19]. In addition, Rtt107 contains six BRCT domains, suggesting that it mediates important protein–protein interactions in the DNA damage response. Rtt107 forms foci upon treatment with methyl methanesulfonate (MMS) or hydroxyurea (HU), and is also recruited locally to sites of induced DNA damage [20–22]. Using bulk chromosome spreads, a previous study reported that Rtt107 recruitment to chromatin in the presence of stalled replication forks is dependent on the acetyltransferase Rtt109 and the cullin Rtt101 [23]. Moreover, a recent study solved the crystal structure of the fifth and sixth BRCT domains of Rtt107 bound to a H2A phospho-peptide [24]. Notably, analogous findings have also been reported for the homologue of Rtt107 in Schizosaccharomyces pombe (Brc1) and humans (PTIP) [25,26]. However, the extent of the functional conservation between Rtt107 and PTIP is still unclear, despite the known role of PTIP in regulation of DNA damage response signaling pathways [27–33].

Rtt107 physically interacts with a number of proteins involved in the DNA damage response. This includes the endonuclease Slx4, which forms a complex and has a close functional relationship with Rtt107 [34]. Yeast lacking Rtt107 or Slx4 share common phenotypes such as sensitivity to DNA damaging agents and prolonged DNA damage checkpoint activation [34]. Moreover, Slx4 is required for Mec1-mediated phosphorylation of Rtt107, and vice versa [34,35]. Interestingly, the DNA replication protein Dpb11 interacts with both Rtt107 and Slx4 after exposure to DNA damage, which is proposed to counteract hyperactivation of the DNA damage checkpoint [36]. Furthermore, association of Dpb11 and Slx4 with additional endonucleases is regulated by cell-cycle dependent phosphorylation, which mediates resolution of DNA joint molecules [37,38].

In this study we sought to examine the role of the BRCT domains in Rtt107 and determine their specific contributions to Rtt107 function in the DNA damage response. We identified the fifth and sixth BRCT domains as the key region that mediated recruitment of Rtt107 to DNA lesions via binding to γH2A. Replacing Rtt107 BRCT5/6 with the BRCT domains of Rad9, which are also able to bind γH2A, restored resistance to DNA damaging agents and recruitment to a double-stranded break (DSB), albeit at lower levels. Testing the role of Rtt107 in recruiting its protein partners revealed that Slx4 recruitment to DNA lesions was dependent on Rtt107. When Slx4 was fused to Rtt107 BRCT5/6, the requirement for Rtt107 was alleviated, improving some phenotypes of rtt107Δ mutants. In contrast, Dpb11 recruitment to a DSB was only partially dependent on Rtt107, and was completely independent of Rtt107 in the case of a protein-bound nick.

2. Materials and methods

2.1. Yeast strains and plasmids

All yeast strains used in this study are listed in Table S1 and were created using standard yeast genetic techniques [39]. Complete gene deletions and integration of FLAG tags at the 3′ end of genes were achieved using one-step gene integration of PCR-amplified modules [40,41]. The Rtt107 and Rad9 BRCT swap mutants were created by one-step gene integration of DNA fragments containing the respective BRCT sequences with an in-frame 3X FLAG tag. Similarly, the Slx4-Rtt107 BRCT5/6 or K887M fusion constructs were created by one-step gene integration of DNA fragments containing the FLAG-tagged Rtt107 BRCT5/6 sequence (encoding for aa820–1070). The primers also included additional sequence encoding a GAGAGAGA linker to be placed between Slx4 and Rtt107 BRCT5/6 (primers used available upon request). Constructs were confirmed by DNA sequencing. The H2A S129* mutant strain was a generous gift from Susan Gasser (Basel).

All plasmids used in this study were created using standard molecular biology techniques. The Rtt107 pRS315 vector was created by amplification of the entire RTT107 gene, including 500 bp of the promoter sequence, with primers containing overhangs homologous to the pRS315 vector (primers used available upon request). This product was transformed into yeast containing a linearized pRS315 vector, and a 3XFLAG tag was subsequently added using one-step gene integration of PCR–amplification modules. The parental RTT107 pRS315 vector was used for all subsequent manipulations to create specified mutations using overlap extension PCR mutagenesis [42] (primers used available upon request). Overexpressed constructs were cloned into Gateway vectors containing the GPD promoter. All constructs were confirmed by DNA sequencing.

2.2. Growth and DNA damage sensitivity assays

Overnight cultures grown in YPD or SC-Leu at 30 °C were diluted to 0.25 A600. The cells were tenfold serially diluted and spotted onto solid YPD plates or SC-Leu plates with MMS, CPT, or HU (Sigma) at various concentrations. The plates were then incubated at 30 °C for 2 days.

2.3. Protein extracts and immunoblot analysis

Protein levels of Rtt107, Slx4, and their respective fusion derivatives were too low to be detected clearly in whole cell extracts, therefore Western blots were carried out on immunoprecipitated material. Overnight cultures were diluted to 0.3 A600 and grown in YPD to log phase, and cells were collected for immunoprecipitation. The procedure for analytical-scale immunoprecipitation of the epitope tagged proteins was adapted from a previous report [43]. Briefly, yeast cells were harvested, and lysed in TAP-IP Buffer (50 mM Tris [pH 7.8], 150 mM NaCl, 1.5 mM MgAc, 0.15% Nonidet P-40, 1 mM DTT, 10 mM NaPpi, 5 mM EGTA, 5 mM EDTA, 0.1 mM Na3VO4, 5 mM NaF, Complete® Protease inhibitor mixture) using acid–washed glass beads and mechanically disrupting using a bead beater (BioSpec Products). FLAG tagged fusion proteins were captured using anti-FLAG M2 agarose beads (Sigma) and subsequently washed in TAP-IP buffer. Whole cell extracts were prepared from logarithmic growing cells by glass bead lysis in the presence of trichloroacetic acid. Immunoblotting was carried out with anti-FLAG M2 (Sigma), anti-H2A S129ph (Abcam), or anti-H4 (Abcam) antibodies and visualized using the Odyssey Infrared Imaging System (Licor).

2.4. Chromatin immunoprecipitation (ChIP)

For the inducible DSB experiments, cells were grown in medium containing raffinose until they reached logarithmic phase, then they were treated with galactose for 2 h to induce expression of the HO endonuclease, and subsequently collected for ChIP. Cutting efficiency was measured by quantitative real-time PCR (qPCR) using Rotor-Gene 6000 (QIAGEN) with primers spanning the HO cut site (Table S2). To introduce the protein-bound nick, cells were arrested in G1 with alpha factor in medium containing raffinose, treated with galactose to induce expression of the mutant Flp recombinase, released into S phase, and collected 2 h later for ChIP.
In brief, yeast cells (250 ml) were grown in the appropriate medium to an A₆₀₀ of 0.4–0.5 and were crosslinked with 1% formaldehyde for 20 min before chromatin was extracted. The chromatin was sonicated (Bioruptor, Diagenode; Sparta, NJ): 10 cycles, 30 s on/off, high setting) to yield an average DNA fragment of 500 bp. Anti-FLAG antibody (Sigma, 4 µl) were coupled to 60 µl of protein A magnetic beads (Invitrogen). After reversal of the crosslinking and DNA purification, the immunoprecipitated and input DNA were analyzed by qPCR using Rotor-Gene 6000 (QIAGEN). Samples were analyzed in triplicate for three independent ChIP experiments. Primer sequences are listed in Table S2. Where indicated, Student’s t-test was used to calculate the statistical significance of different enrichment levels.

3. Results

3.1. Disruption of Rtt107 BRCT domains resulted in varying phenotypes

Evidence from previous studies suggests that the four N-terminal BRCT domains are responsible for binding to the proteins that Rtt107 interacts with, including Sln4 [21,34]. Furthermore, in vitro studies demonstrated that the fifth and sixth BRCT domains of Rtt107 are able to bind γH2A [24]. Together, these data suggest that the three pairs of BRCT domains in Rtt107 potentially have distinct functions. To investigate this possibility more formally, we made three internal deletion constructs fused in-frame to a 3XFLAG epitope tag: Rtt107ΔBRCT1/2 (removed aa2-217), Rtt107ΔBRCT3/4 (removed aa261-457), and Rtt107ΔBRCT5/6 (truncated after aa820) (Fig. 1A). We first tested the DNA damage sensitivity of these mutant strains, and observed that the sensitivities of the rtt107ΔBRCT1/2 and rtt107ΔBRCT3/4 mutants were similar to that of the rtt107Δ mutant (Fig. 1B). In contrast, the rtt107ΔBRCT5/6 mutant had an intermediate DNA damage sensitivity between the wildtype and the rtt107Δ mutant. We then measured the protein levels of the mutant constructs to determine if the growth phenotypes were due to changes in protein expression. The protein levels of Rtt107ΔBRCT1/2 and Rtt107ΔBRCT3/4 were dramatically lower than that of the wildtype protein, whereas the protein level of Rtt107ΔBRCT5/6 was slightly higher than wildtype (Fig. 1C). The decreased amounts of protein were not due to reduced transcription, as the mRNA levels were not affected (data not shown). Thus deletion of the Rtt107 N-terminal pairs of BRCT domains resulted in lower protein levels, possibly due to protein instability, whereas deletion of the C-terminal pair of BRCT domains did not perturb protein levels but still resulted in DNA damage sensitivity.

3.2. Rtt107 BRCT5/6 domains were required for recruitment to DNA lesions

BRCT domains play an important role in recruitment of proteins in the DNA damage signaling cascades, thus we tested the role of the BRCT domains in the recruitment of Rtt107 to DSBs. To this end, we used a strain containing the HO endonuclease under control of the galactose promoter, thereby allowing induction of a single DSB at the mating locus upon exposure to galactose [45]. We first focused on testing the Rtt107ΔBRCT5/6 mutant for its recruitment to DSBs since BRCT5/6 can bind γH2A in vitro [24]. Upon galactose induction, Rtt107 levels were significantly enriched at the DSB, with maximum levels at 5–10 kb from the DSB as we previously reported [21]. Consistent with the role for BRCT5/6 binding γH2A, enrichment of Rtt107ΔBRCT5/6 was dramatically reduced at DSBs (Fig. 2A). This effect was not due to impaired DSB induction, as the complete Rtt107 deletion mutant displayed the same cutting efficiency as the wildtype (Fig. S1).

Since rtt107Δ mutants are sensitive to multiple types of DNA damaging agents [19], we tested if the dependence on BRCT5/6 for Rtt107 recruitment was limited to DSBs or was generally true for other types of DNA lesions. We used an alternate system that creates protein-bound nicks at a specific locus in the genome by expressing a ligation-defective FLP recombinase that remains covalently bound to the DNA after forming a nick at its recognition target site (termed the FRT site). During S phase, DNA replication forks run into the protein-bound nick and become stalled or collapsed, thus mimicking DNA damage produced by camptothecin [46]. Rtt107 was recruited to regions near the FRT site, consistent with our previous report [21], but the Rtt107ΔBRCT5/6 mutant was no longer enriched at those same regions (Fig. 2B). Overall, our data indicated that Rtt107 was recruited to sites of DNA damage via its C-terminal BRCT domains.

3.3. Rtt107 recruitment to DSBs was mediated via the interaction of the key residue K388 with γH2A

Since internal deletions of the N-terminal BRCT domains were not well tolerated by the Rtt107 protein, we constructed point mutants that would putatively disrupt the phospho-peptide binding pocket formed by each pair of BRCT domains. K192 and K426 were chosen for BRCT1/2 and BRCT3/4, respectively, by structural modeling based on BRCT domains in other proteins with known structures, such as S. pombe Rad4 and human TopBP1. K887 was mutated for BRCT5/6 in accordance with work that described the crystal structures of this region for Rtt107 and its S. pombe homologue [24,25]. We first tested the DNA damage sensitivity of the point mutants. Consistent with previous work, the rtt107 K887M mutant was sensitive to all the drugs tested, although not to the extent of the rtt107Δ mutant (Fig. 3A) [24,25]. The rtt107 K426M
mutant was also sensitive to the DNA damaging agents, albeit to a lesser extent than the rtt107 K887M mutant, whereas the rtt107 K192M mutant grew like wildtype in all conditions (Fig. 3A). The protein levels of the point mutants were all equivalent to that of the wildtype (Fig. 3B). Surprisingly, upon exposure to MMS, phosphorylation of Rtt107 K426M and K887M were not detectable, as indicated by the lack of retarded migration through the gel (Fig. 3B). This suggested an intriguing circuitry between the Rtt107 BRCT domains and phosphorylation of the protein. We did not further analyze the rtt107 K192M mutant since it was unknown whether the lack of phenotype was due to ineffectiveness of the K192 point mutation in abrogating the function of Rtt107 BRCT1/2.

According to the crystal structure, K887 of Rtt107 forms a key bond with the phosphate group of phosphorylated H2A S129 [24], thus we tested the contribution of K887 to the recruitment of Rtt107. When K887 was mutated, Rtt107 enrichment at the DSB was completely abolished, suggesting that the bond formed by K887 was crucial to the interaction with γH2A (Fig. 4A). Conversely, to test the requirement for H2A S129 phosphorylation, we used a well-characterized non-phosphorylatable mutant of H2A, where S129 was replaced with a stop codon (H2A S129*) [47]. In the absence of H2A S129 phosphorylation, Rtt107 enrichment at the DSB was again completely lost, indicating an absolute requirement for γH2A (Fig. 4B). Deletion or mutation of RTT107 did not have a notable effect on γH2A levels (Fig. S2), suggesting that Rtt107 was dependent on γH2A and not vice versa. Taken together, these data provide a solid support for the model that Rtt107 is recruited to sites of DNA lesions via its fifth and sixth BRCT domains binding to γH2A.

Since the rtt107 K426M mutant was also sensitive to DNA damaging agents, we tested whether the third and fourth pair of BRCT domains also contributed to Rtt107 recruitment to DSBs, or if the fifth and sixth BRCT domains were solely responsible. Rtt107 K426M was still recruited to the DSB, but at a lower level than the wildtype protein (Fig. 4C). Interestingly, Rtt107ΔBRCT3/4 under the control of the GPD promoter, overexpressed to circumvent the problem of low protein levels, was enriched at the DSB to the same level as that of the overexpressed full-length Rtt107 (Fig. 4D and E). This suggests that although BRCT5/6 was completely required for Rtt107 recruitment, BRCT3/4 also made a secondary contribution to this process, which could be bypassed by overexpression of Rtt107.

3.4. The function of Rtt107 BRCT5/6 could be recapitulated with the Rad9 BRCT domains

We then asked whether the structure of BRCT5/6 was uniquely required for its functions, or whether it could be replaced by other pairs of BRCT domains. In S. cerevisiae, the pair of BRCT domains in Rad9 have been shown to bind γH2A in vitro, although the structure has not been solved [14]. We tested whether substituting Rtt107 BRCT5/6 with the Rad9 BRCT domains could complement its function. Fusion proteins were constructed so that the FLAG-tagged Rad9 BRCT domains (aa881-1309) were fused in-frame.
to Rtt107ΔBRCT5/6, or in the reverse construct the FLAG-tagged Rtt107 BRCT1/2, BRCT3/4, or BRCT5/6 domains were fused in-frame to Rad9ΔBRCT (Fig. 5A). All of the constructs were expressed, with the exception of Rad9ΔBRCT, although all three of the Rad9-Rtt107BRCT fusions had lower protein levels than the wildtype Rad9-FLAG protein (Fig. 5B and C). Strikingly, the fusion of Rad9 BRCT to Rtt107ΔBRCT5/6 greatly reduced the sensitivity to DNA damaging agents of the mutant strain compared to the rtt107ΔBRCT5/6 mutant alone (Fig. 5D). Fusion of Rtt107 ΔBRCT5/6 to Rad9ΔBRCT also reduced the mutant strain’s sensitivity to DNA damaging agents, whereas this effect was not observed for the rad9-rtt107 BRCT1/2 or rad9-rtt107 BRCT3/4 mutants, although this may have been due to a slightly lower protein level of these two constructs (Fig. 5E). Next, we tested whether the complementation achieved by fusing Rtt107 BRCT5/6 to Rad9ΔBRCT was linked to the ability of Rtt107 BRCT5/6 to bind to γH2A. We constructed the rtt107ΔBRCT5/6-rad9 BRCT mutant in a strain containing the HO endonuclease, and evaluated enrichment of the fusion protein at a DSB. Importantly, the Rtt107ΔBRCT5/6-Rad9BRCT mutant was indeed recruited to a DSB, although we note that at 5 kb and further from the DSB, the enrichment level of the mutant was lower than the full-length protein (Fig. 5F). These results suggest that although the levels of Rtt107 recruitment were lower than wildtype, this was sufficient to confer substantial resistance to DNA damaging agents.
Fig. 5. Rtt107 BRCT5/6 and Rad9 BRCT domains were able to replace each other's function. (A) Diagram of Rtt107 and Rad9 constructs. Boxes indicate BRCT domains, numbered in order along the protein for Rtt107. Analytical-scale immunoprecipitations of (B) Rtt107-FLAG or (C) Rad9-FLAG were performed on whole cell extracts of the indicated strains and analyzed by immunoblotting with anti-FLAG antibodies. Cross-reaction bands were used as a loading control. (D) Fusion of Rad9 BRCT domains to the Rtt107ΔBRCT mutant improved resistance to DNA damaging agents. (E) Fusion of Rtt107 BRCT5/6 but not BRCT1/2 or BRCT3/4 to the Rad9ΔBRCT mutant greatly improved resistance to DNA damaging agents. 10-fold serial dilutions of the indicated strains were plated onto media containing various DNA damaging agents and incubated at 30 °C for 2 days. (F) The Rtt107ΔBRCT5/6-Rad9BRCT fusion protein was recruited to sites near the DSB, albeit at levels lower than that of the wildtype protein. ChIP-qPCR experiments were analyzed as described in Fig. 2. *p<0.1 for comparisons to the corresponding non-induced control.
3.5. Rtt107 was completely required for recruitment of Slx4, but not Dpb11, to DNA lesions

In addition to investigating the requirements of Rtt107 recruitment, we were also interested in the proteins that depend on Rtt107 for their own recruitment to DNA lesions. We analyzed the recruitment of Slx4 and Dpb11 because the physical interactions between Rtt107 and Slx4 or Dpb11 have both been characterized [34,36]. We first tested the recruitment of Slx4 to a DSB in the presence or absence of Rtt107. Slx4 was recruited to the DSB, and the highest enrichment levels occurred around 5 kb from the DSB (Fig. 6A). In the absence of Rtt107, Slx4 enrichment was completely abolished. Similarly, in the Rtt107ΔBRCT5/6 mutant, Slx4 enrichment at the DSB was also lost (Fig. 6B), suggesting that Rtt107 recruitment to the DSB was specifically required, rather than a general function of Rtt107. Dpb11 was also recruited to the DSB, with peak levels around 5 kb from the DSB, consistent with a previous study [48]. In contrast to Slx4, Dpb11 enrichment was decreased but not completely lost in the rtt107Δ mutant or the Rtt107ΔBRCT5/6 mutant (Fig. 6C and D). Notably, the levels of Dpb11 at 1 kb or less from the DSB were unaffected. Therefore Dpb11 was dependent on Rtt107 only at distances further from the DSB, and likely relied on additional mechanisms for recruitment to regions near the DSB.

Aside from the DSB, we again utilized the protein-bound nick system to determine if the requirements extended to a different type of DNA lesion. Slx4 was also recruited to the protein-bound nick, and was enriched up to 12 kb away from the FRT site (Fig. 7A). Mirroring the situation at a DSB, loss of Rtt107 completely eliminated Slx4 recruitment to the protein-bound nick. Dpb11 was also enriched at regions up to 12 kb from the FRT site, but in contrast, the protein levels were not affected by loss of Rtt107 (Fig. 7B).

Given that Slx4 was completely dependent on Rtt107 for its recruitment, we hypothesized that some of Rtt107’s functions could be bypassed by artificial recruitment of Slx4. To test this, we fused Rtt107 BRCT5/6 in-frame to the C-terminus of Slx4, which would presumably allow artificial recruitment of Slx4 to sites of DNA lesions via the Rtt107 BRCT5/6 domains. This construct was compared to a parallel construct wherein the Rtt107 BRCT5/6 K887M mutant was fused to Slx4. The protein levels of both the fusion constructs were lower than wild-type Slx4 (Fig. 8A). Despite the lower protein levels, the presence of the Slx4-Rtt107BRCT5/6 fusion improved the growth of the rtt107Δ mutant in MMS conditions, whereas this was not observed for the Slx4-Rtt107BRCT5/6 K887M mutant fusion (Fig. 8B). Interestingly, this effect was particular to MMS conditions, and did not extend to HU or CPT conditions. Exposure to MMS induces phosphorylation of Slx4, which is dependent on Rtt107 [35]; however, fusion of Rtt107BRCT5/6 to Slx4, but not Rtt107BRCT5/6 K887M, was able to partially bypass this requirement (Fig. S3). Taken together, this data suggests that an important component of Rtt107 function was the recruitment of Slx4, as artificial recruitment was able to alleviate some phenotypes of rtt107Δ mutants.
at sites of DNA lesions. Although it is possible that there are intermediary factors involved, we favor a model of direct interaction because it is consistent with the in vitro crystal structure data showing Rtt107 BRCT5/6 bound to the phosphorylated H2A peptide [24]. The primary function of Rtt107 BRCT5/6 is likely for binding γH2A, since the Rad9 BRCT domains, which are also able to bind γH2A [14], were able to replace the function. However, the Rad9 BRCT domains did not fully restore Rtt107 enrichment levels, which could be due to a lower affinity of the Rad9 BRCT domains for γH2A or suboptimal recruitment of the artificial fusion protein.

A secondary role for Rtt107 K426 (BRCT3/4) in recruitment to DSBs was also observed. K426 likely plays a supporting role because enrichment of the Rtt107 K887M single mutant at the DSB was essentially abolished, indicating that K426 alone without the presence of K887 was insufficient for recruitment of Rtt107. It is tempting to speculate that Rtt107 K426 contributes to recruitment by stabilizing Rtt107 at DSBs, although it is unclear whether Rtt107 BRCT3/4 binds directly to γH2A or to an intermediary protein. Consistent with the idea that Rtt107 K426M recruitment at the DSB was less efficient, overexpression of the Rtt107 ΔBRCT3/4 mutant resulted in wildtype enrichment levels, suggesting that providing large amounts of Rtt107 molecules to the DSB can overcome the poorer recruitment of the mutant. In contrast, overexpression of the Rtt107 ΔBRCT5/6 mutant did not improve the enrichment level at the DSB (data not shown), consistent with the model that K887 is absolutely required for recruitment. Both the Rtt107 ΔBRCT5/6 and Rtt107 K887M mutants showed an intermediate sensitivity to DNA damaging agents, indicating that the Rtt107 mutants that completely lacked recruitment to DSBs still contributed functionally to cellular resistance to DNA damage. The Rtt107 K426M mutant was also sensitive to DNA damaging agents, but less so than Rtt107 K887M, suggesting that the difference in sensitivities could be due to the partial enrichment of Rtt107 K426M. Ultimately, solving the structure of the entire Rtt107 protein would provide insight into the importance of the residues mutated in this study, as well as potentially reveal additional residues in the N-terminal BRCT domains that mediate key interactions.

Previous studies have described other modes of recruitment of Rtt107 in addition to the model presented here. One such study reported that Rtt107 recruitment to chromatin spreads after exposure to DNA damaging agents is dependent on the acetyltransferase Rtt109 and the cullin Rtt101 [23]. However, the relationship between Rtt109, Rtt101, and γH2A in the regulation of Rtt107 recruitment to sites of DNA damage is unclear. Surprisingly, a separate study found that Rtt101 is dispensable for Rtt107 recruitment to an HO endonuclease-induced DSB [22]. One possible explanation is that regulation of Rtt107 recruitment to specific sites of DNA damage is distinct from global recruitment to chromatin. Thus while γH2A is solely responsible for localizing Rtt107 to the DNA lesion, where the histone modification itself is localized, Rtt109 and Rtt101 could be affecting general chromatin enrichment of Rtt107. That being said, genome-wide studies have shown additional enrichment sites of γH2A [49, 50], thus it would be interesting to investigate how those sites regulate Rtt107 recruitment.

Whereas the C-terminal BRCT domains of Rtt107 mediated recruitment of Rtt107 to DNA lesions, the N-terminal portion of Rtt107 is sufficient for interacting with Slx4, as well as the chromosomal maintenance complex SMCS5/6 [21, 34]. This suggests that Rtt107 could function in a modular manner whereby the C-terminal portion of the protein mediates its recruitment to DNA lesions while the N-terminal region binds to the interaction partners to facilitate their recruitment. The region of Rtt107 responsible for interacting with Dpb11 is still unknown, but our model would predict that Dpb11 also binds to the N-terminal portion of Rtt107. The N-terminal BRCT domains also appeared to have a great influence on the stability of the protein, since the protein levels were dra-

![Fig. 7](image-url)
matically decreased upon deletion of these domains, as well as if the C-terminal BRCT domains were replaced with either Rtt107 BRCT1/2 or BRCT3/4 tandem domains (data not shown). Further investigation into regulation of Rtt107 turnover, as well as characterization of the Rtt107 tertiary structure, would help provide an explanation for this surprising phenomenon.

Between the N-terminal and C-terminal BRCT domains is a 300 amino acid region, which contains the S/T-Q motifs that are phosphorylated by Mec1. Importantly, Rtt107 phosphorylation is crucial for some of its functions under consideration here, namely Rtt107 recruitment to a DSB and interaction with Dpb11 [22,36]. Paradoxically, both the Rtt107 K426M and K887M mutants were no longer phosphorylated in response to DNA damaging agents, suggesting that loss of recruitment to DSBs or binding of the BRCT domains to its target could impact the phosphorylation status of Rtt107. Perhaps phosphorylation of Rtt107 stabilizes its interactions at DSBs, thus the non-phosphorylatable mutant of Rtt107 is not retained at DSBs, although it may be initially recruited. In this situation, a snapshot of Rtt107 enrichment at DSBs at a relatively late time point (2 h post-induction), as is true of the experiments both in this study and a published report [22], would record low enrichment levels of Rtt107 whether it has mutations in the BRCT domains or the phosphorylation sites. How phosphorylation of the 300 amino acid linker region (and possibly at other sites) affects the function of the BRCT domains is still unknown and further structure-function analyses and careful examination of the kinetics of recruitment to DSBs are needed to shed light on this question.

Rtt107 forms a complex with Slx4 and Dpb11 after exposure to DNA damage, wherein Slx4 is thought to directly interact with Dpb11, and Rtt107 stabilizes this interaction [36]. However in an rtt107Δ mutant, Slx4 recruitment to a DSB was completely abolished, whereas Dpb11 remained enriched close to the break, although its enrichment dropped to lower levels further away from the DSB. The differential dependencies of Slx4 and Dpb11 on Rtt107 for recruitment to DNA lesions indicated the existence of a separate pool of Dpb11 molecules that did not interact with Slx4 and Rtt107 and that depended on a separate pathway for recruitment to sites of DNA damage. Indeed, Dpb11 is recruited to DNA lesions via its interaction with Rad9 and Mec1 [51–53], which may constitute an Rtt107-independent pathway. The skewed distribution of Dpb11 enrichment at the DSB in the absence of Rtt107 suggests the possibility that distinct pathways are responsible for recruiting Dpb11 to regions close to the break versus spreading away from the DSB. Intriguingly, in the case of a protein-bound nick, Dpb11 recruitment was unaffected by loss of Rtt107, suggesting that only the alternative recruitment pathways for Dpb11 are active in that context, or that they are able to fully compensate for loss of the Rtt107-dependent pathway.

Rtt107 recruitment of Slx4 to sites of DNA damage was functionally important, as shown by the artificial recruitment of Slx4. Fusion of Rtt107 BRCT5/6 to Slx4 was able to rescue the phosphorylation of Slx4 in the absence of Rtt107, suggesting that Rtt107 mediates Slx4 phosphorylation by recruiting it to DNA lesions. This artificial fusion also improved the MMS resistance of rtt107Δ mutants, suggesting that the MMS sensitivity of rtt107Δ mutants was partially due to loss of Slx4 recruitment. We observed only a mild effect of the fusion protein on the DNA damage sensitivity phenotype, which could be in part explained by the lower protein levels of the fusion protein compared to the wildtype. Interestingly, the effect of the fusion protein varied depending on the type of DNA damage. The suppression was observed only for MMS conditions, but not CPT or HU conditions, suggesting that Slx4 recruitment was more important in certain contexts than others. This is consistent with the idea that the cell uses distinct responses for different types of DNA damage, as suggested by large-scale genetic interactions measured under multiple DNA damaging conditions [54,55].

Evidence from S. pombe and human cell lines suggest that the regulation of Rtt107 recruitment to sites of DNA damage is likely conserved. The S. pombe homologue of Rtt107 is BrC1 (BRCT containing protein 1) and the putative human homologue of Rtt107 is PTIP (Pax2 transactivation domain interaction protein). Both BrC1 and PTIP contain six BRCT domains and are involved in the DNA damage response [56–58]. Crystal structures of BrC1 or PTIP BRCT5/6 show that they bind to γH2A in vitro, and formation of either protein depends on γH2A [25,26,29]. Although Slx4 and Dpb11 both have conserved human homologues, it is still unclear whether the interaction with Rtt107 is also conserved. Further work on the regulation of Rtt107 in the DNA damage response is needed.
to fully understand its roles, which can then be compared to the homologous pathways in humans.

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Appendix A. Supplementary data

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References


