

# Regulation of Rtt107 Recruitment to Stalled DNA Replication Forks by the Cullin Rtt101 and the Rtt109 Acetyltransferase

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***RTT107 (ESC4, YHR154W)* encodes a BRCA1 C-terminal domain protein that is important for recovery from DNA damage during S phase. *Rtt107* is a substrate of the checkpoint kinase *Mec1*, and it forms complexes with DNA repair enzymes, including the nuclease subunit *Slx4*, but the role of *Rtt107* in the DNA damage response remains unclear. We find that *Rtt107* interacts with chromatin when cells are treated with compounds that cause replication forks to arrest. This damage-dependent chromatin binding requires the acetyltransferase *Rtt109*, but it does not require acetylation of the known *Rtt109* target, histone H3-K56. Chromatin binding of *Rtt107* also requires the cullin *Rtt101*, which seems to play a direct role in *Rtt107* recruitment, because the two proteins are found in complex with each other. Finally, we provide evidence that *Rtt107* is bound at or near stalled replication forks in vivo. Together, these results indicate that *Rtt109*, *Rtt101*, and *Rtt107*, which genetic evidence suggests are functionally related, form a DNA damage response pathway that recruits *Rtt107* complexes to damaged or stalled replication forks.**

## INTRODUCTION

Evolutionarily conserved DNA repair and checkpoint processes ensure accurate DNA replication in eukaryotic cells. The DNA damage checkpoint pathway is a signal transduction pathway that responds to DNA damage to facilitate cell cycle delay, promote DNA repair, and induce transcription when DNA lesions are present (Melo and Toczyński, 2002; Carr, 2002; McGowan and Russell, 2004). Mutation of DNA damage response pathways causes a failure to accurately transmit the genome from one generation to the next, resulting in genomic instability, which is a hallmark of cancerous cells (Kastan and Bartek, 2004).

In *Saccharomyces cerevisiae*, the DNA damage checkpoint is made up of a signaling cascade that includes the essential protein kinase *Mec1* (Weinert *et al.*, 1994; Sanchez *et al.*, 1996). When DNA damage is encountered in S phase, stalling, uncoupling, and collapse of replication forks can result (Tercero and Diffley, 2001; Katou *et al.*, 2003; Lopes *et al.*, 2006). *Mec1* together with its binding partner *Ddc2* is thought to localize to stalled forks by binding to single-stranded (ss) DNA coated with the ssDNA-binding protein replication protein A (Zou and Elledge, 2003). Recruitment of *Mec1* and *Ddc2* to stalled replication forks leads to activation of the S phase checkpoint, and it is thought to delay the onset of mitosis until replication is complete (Osborn *et*

*al.*, 2002). Several other DNA replication and repair proteins are also thought to act at stalled replication forks to prevent fork collapse, remove or bypass DNA lesions, and ultimately restart DNA replication (for review, see Tourrière and Pasero, 2007).

*Rtt107*, a target of *Mec1* phosphorylation, is one protein that has been proposed to promote the restart of DNA replication after DNA damage in S phase (Rouse, 2004; Chin *et al.*, 2006; Roberts *et al.*, 2006). *RTT107 (ESC4; YHR154W)* was originally identified in a genetic screen for increased Ty transposon mobility and contains several BRCA1 C-terminal (BRCT) homology domains (Scholes *et al.*, 2001). The BRCT domain was first noted in the protein encoded by the human breast cancer susceptibility gene *BRCA1* (Koonin *et al.*, 1996; Bork *et al.*, 1997), and it is a protein–protein interaction domain frequently found in proteins that have roles in the response to DNA damage, in cell-cycle control, and in checkpoint-mediated DNA repair (Callebaut and Mornon, 1997; Bork *et al.*, 1997). BRCT domains are thought to facilitate assembly of signaling complexes in response to DNA damage, and they may direct repair proteins to the site of damage, resulting in removal of DNA lesions (El-Khamisy *et al.*, 2003). Mutations in BRCT proteins frequently confer sensitivity to a variety of DNA-damaging agents (Callebaut and Mornon, 1997).

Deletion of *rtt107* confers sensitivity to the DNA alkylating agent methyl methane sulfonate (MMS), the replication inhibitor hydroxyurea (HU), and camptothecin (CPT), an agent that induces replication dependent double-strand breaks (Chang *et al.*, 2002; Hanway *et al.*, 2002; Rouse, 2004). *Rtt107* is required for normal DNA synthesis in the presence of DNA damage and for the resumption of DNA replication during recovery from DNA damage (Chang *et al.*, 2002;

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Rouse, 2004; Roberts *et al.*, 2006). Rtt107 binds the Slx4/Slx1 nuclease complex through the N-terminal, BRCT-containing half of Rtt107, and Slx4 is required for the Mec1 phosphorylation of Rtt107 (Roberts *et al.*, 2006; Zappulla *et al.*, 2006). Slx4, like Rtt107, is important for the resumption of DNA synthesis after DNA damage (Rouse, 2004; Roberts *et al.*, 2006). Two-hybrid and high-throughput studies have found Rtt107 in complex with a number of DNA repair proteins, including Mms22, the DNA helicase Sgs1, and the homologous recombination proteins Rad55 and Rad57 (Ho *et al.*, 2002; Chin *et al.*, 2006; Zappulla *et al.*, 2006). The fission yeast homologue of Rtt107, Brc1, is thought to act in the postreplication repair pathway through the Slx1 and Mus81 nucleases (Sheedy *et al.*, 2005) and in a pathway with Mms22 to manage DNA damage in S phase (Dovey and Russell, 2007). These data indicate that Rtt107 functions with distinct DNA repair proteins to tolerate DNA damage that stalls replication forks. One possibility is that Rtt107 acts as a scaffold for the assembly of DNA damage response and repair proteins at stalled DNA replication forks, thereby promoting replication fork restart.

Here, we report that Rtt107 is recruited to chromatin in the presence of agents that cause replication fork stalling. The binding of Rtt107 to chromatin after DNA damage is regulated by a pathway comprising the acetyltransferase Rtt109 and the cullin Rtt101. Results from chromatin immunoprecipitation on tiling microarray analysis were consistent with Rtt107 functioning at stalled replication forks *in vivo*. We propose that Rtt109 acetylation of an unidentified target results in the Rtt101-dependent recruitment of Rtt107 to stalled replication forks. The interaction between Rtt107 and Slx4, and perhaps other DNA repair proteins, promotes their assembly at sites of replication fork stalling, leading to resumption of DNA synthesis.

## MATERIALS AND METHODS

### Yeast Strains and Media

Yeast strains used in this study were derivatives of W303 or BY4741 (Thomas and Rothstein, 1989; Brachmann *et al.*, 1998), and they are listed in Supplemental Table 1. Nonessential haploid deletion strains marked with the kanamycin (G418) resistance gene were made by the *Saccharomyces* Gene Deletion Project (Winzler *et al.*, 1999). Deletion strains marked with nourseothricin (nat) resistance gene were constructed by switching the kanamycin resistance gene with the nat resistance gene as described previously (Tong *et al.*, 2001). Standard yeast media and growth conditions were used (Sherman, 1991).

### Chromosome Spreads and Microscopy

Cells were grown in YPD at 30°C to early-log phase, and they were treated with no drug, 0.03% MMS (Sigma-Aldrich, St. Louis, MO), 150 mM HU (Bioshop, Toronto, ON, Canada), or 15  $\mu$ g/ml CPT (Sigma-Aldrich) for 1 h. For  $\alpha$  factor-treated cells, cultures were treated with 8  $\mu$ g/ml  $\alpha$  factor for 2 h, and then 0.03% MMS and 8  $\mu$ g/ml  $\alpha$  factor were added for an additional hour. Cells were harvested and prepared as described previously (Michaelis *et al.*, 1997). To detect proteins immunofluorescently, microscope slides were treated with mouse anti-vesicular stomatitis virus (VSV) antibodies (Roche Diagnostics, Indianapolis, IN) and goat anti-mouse Alexa Fluor 546 (Invitrogen, Carlsbad, CA). Before examination, VECTASHIELD mounting media with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) was added. Fluorescence intensity was measured relative to background intensity for at least 100 nucleoids in two independent experiments by using Openlab 5.0.2 (Improvision, Coventry, United Kingdom).

### Immunoblotting and Immunoprecipitation

Cultures were grown in the absence or presence of 100 mM HU or 0.03% MMS. Cells ( $1 \times 10^8$ ) were collected and treated with 10% trichloroacetic acid, and extracts were prepared essentially as described previously (Pellicoli *et al.*, 1999). Proteins were resolved on 7.5% or 15% SDS-polyacrylamide gels, and they were subjected to immunoblot analysis with rabbit anti-VSV-G (Bethyl Laboratories, Montgomery, TX), anti-H3-K56-Ac (a kind gift from Alain Verreault), or rabbit anti-histone H3 antibody 1791 (Abcam, Cambridge, MA). Immunoblots were developed using SuperSignal ECL (Pierce Chemical,

Rockford, IL). Immunoprecipitation was performed essentially as described previously (Roberts *et al.*, 2006). Anti-hemagglutinin (HA) antibody (Covance Research Products, Princeton, NJ) and protein G-agarose (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were used to immunoprecipitate HA-tagged proteins. After extensive washing, proteins were subjected to immunoblot analysis with anti-HA or anti-myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA).

### In Situ Kinase Assay and Ddc2-Yellow Fluorescent Protein (YFP) Foci

Rad53 *in situ* kinase assays were carried out as described previously (Pellicoli *et al.*, 1999), by using extracts from cells growing in mid-logarithmic phase in the absence of DNA-damaging agents. Imaging of Ddc2-YFP foci was carried out as described previously (Roberts *et al.*, 2006). At least 200 cells were counted in two independent experiments.

### Growth Measurements

For temperature sensitivity assays, cells were grown in YPD or SD-leucine, diluted serially, spotted onto YPD or SD-leucine plates, and incubated at 30°C or 37°C for 2–3 d. For DNA damage sensitivity assays, plates contained 0.03% (vol/vol) MMS, 150 mM HU, or 40  $\mu$ M CPT in YPD, and they were used within 24 h of preparation.

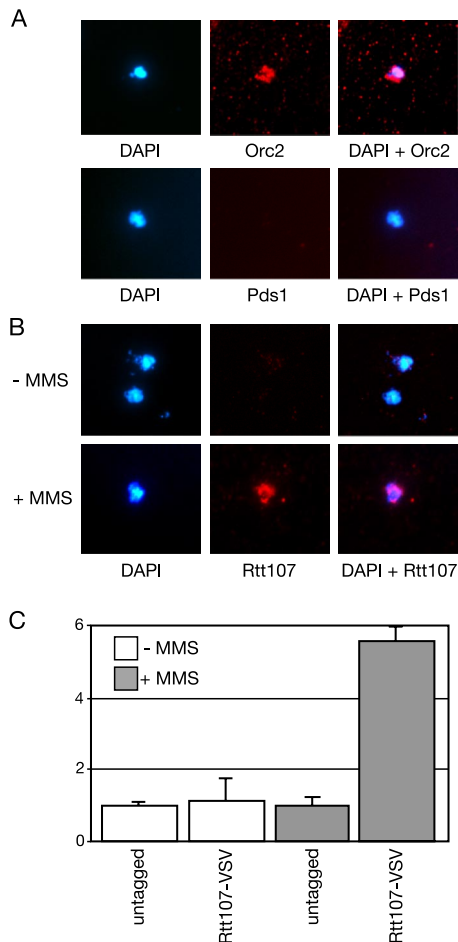
### Chromatin Immunoprecipitation (ChIP)-Chip

Chromatin immunoprecipitation on microarray (ChIP-chip) experiments were performed essentially as described previously (Katou *et al.*, 2003; Katou *et al.*, 2006). Cells were arrested for 2 h in 200 mM HU before cross-linking. After labeling, bound and unbound DNA was hybridized to whole-genome tiling arrays (P/N 520055; Affymetrix, Santa Clara, CA), and arrays were washed and scanned as described previously (Lee *et al.*, 2007). For the analysis in Figure 7A, raw data from the Affymetrix GCOS software (.CEL format) was analyzed to judge the statistical significance of regions enriched in the bound fraction based on the continuity of significant change of -fold change value (Katou *et al.*, 2006; Bermejo *et al.*, 2007). For the analysis in Figure 7, B and C, raw data from Affymetrix GCOS software (.CEL format) were analyzed with Affymetrix Tiling Analysis software, version 1.1, and they were visualized with Affymetrix Integrated Genome Browser. The data were normalized with the built-in quantile normalization and probe-level analysis with perfect match probes, and they were run with a bandwidth of 60. Each probe was given a value calculated from the average of all probe intensities within the specified window (defined by the bandwidth parameter). Then, a ratio was calculated for the two-sample analysis. The resulting  $\log_2$  ratio was then output for each probe position, where the probe position is defined as the center (13th) base coordinate for each 25-mer probe.

## RESULTS

### Rtt107 Is Recruited to Chromatin in Response to Replication Fork Stalling

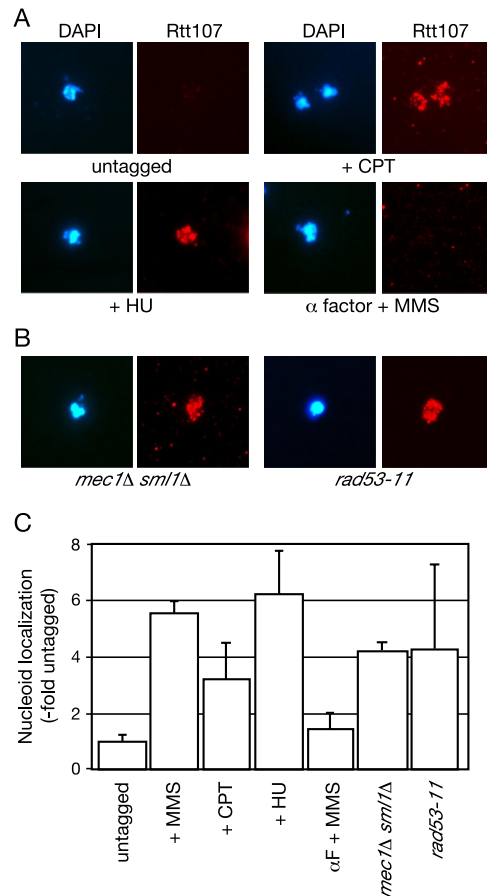
The interaction of Rtt107 with a number of DNA repair proteins (Ho *et al.*, 2002; Chin *et al.*, 2006; Roberts *et al.*, 2006; Zappulla *et al.*, 2006), and the role of Rtt107 in recovery from DNA damage (Rouse, 2004; Roberts *et al.*, 2006), suggested that Rtt107 might function as a scaffold to assemble repair proteins onto sites of DNA damage. We tested this hypothesis by asking whether Rtt107 is recruited to chromatin after DNA damage, by using chromatin spreads (Figure 1). Spreads were prepared by spheroplasting the cells, cross-linking them with formaldehyde, and lysing the spheroplasts on glass slides. Unbound proteins were washed away, and the resulting nucleoids were visualized by staining the DNA with DAPI. The association of Pds1, Orc2, and Rtt107 with the spread chromatin nucleoids was detected by indirect immunofluorescence, by using antibodies directed against the epitope tag fused to each protein (Figure 1, A and B). Pds1 is a soluble nuclear protein, and it was included as a negative control. Orc2 binds to replication origins throughout the cell cycle (Diffley *et al.*, 1994; Aparicio *et al.*, 1997), and it was included as a positive control. As expected, Pds1 was not detected on the spread chromatin, whereas Orc2 localization to the nucleoids was clear (Figure 1A). When spread chromatin was probed for bound Rtt107, no significant localization of Rtt107 to spread chromatin was evident in the absence of DNA damage (Figure 1B, -MMS). By



**Figure 1.** Rtt107 is recruited to chromatin in response to MMS. (A and B) Logarithmically growing cultures expressing the indicated epitope-tagged proteins were treated with 0% (–) or 0.03% (+) MMS for 1 h, before preparation of chromosome spreads. DNA was stained with DAPI (left), and the indicated epitope-tagged proteins were detected with  $\alpha$ -VSV (middle). The merged image is shown in the right panel. (C) VSV signal intensity minus background was quantified and expressed relative to the untagged control. The signal intensity was measured for at least 100 nucleoids in each experiment, and the average of two independent experiments is plotted, with error bars spanning 1 SD.

contrast, when cells were treated with MMS, which stalls DNA replication forks, localization of Rtt107 to chromatin was evident (Figure 1B, +MMS). We quantified the intensity of the Rtt107-VSV signal on 100 nucleoids for untagged and Rtt107-VSV strains, in the presence and absence of MMS (Figure 1C). The qualitative result in Figure 1B was borne out by this quantification, with MMS inducing an almost sixfold increase in Rtt107 localization to spread chromatin relative to the untagged control. Rtt107 localization in the absence of MMS was not significantly different from the background seen in the untagged control. Thus, DNA damage causes recruitment of Rtt107 to chromatin.

We next asked whether Rtt107 recruitment was specific for alkylation damage. We found that both HU and CPT caused a similar recruitment of Rtt107 to spread chromatin (Figure 2, A and B). Like MMS, HU and CPT cause replication forks to stall, although they do so by different mechanisms. This suggested that stalled replication forks, rather than DNA damage per se, caused recruitment of Rtt107 to



**Figure 2.** Rtt107 is recruited to chromatin in response to replication fork stalling. (A) Logarithmically growing cultures were treated with 15  $\mu$ g/ml camptothecin (+CPT) or 150 mM hydroxyurea (+HU) for 1 h, or they were treated with 8.3  $\mu$ g/ml  $\alpha$  factor for 2 h before addition of 8.3  $\mu$ g/ml  $\alpha$  factor and 0.03% MMS for 1 h ( $\alpha$  factor + MMS), before preparation of chromosome spreads. DNA was stained with DAPI (left) and VSV-tagged Rtt107 was detected with  $\alpha$ -VSV (right). (B) The role of checkpoint pathways in the localization of Rtt107 was analyzed by treating *rad53-11* or *mec1* $\Delta$  *smi1* $\Delta$  cells with 0.03% MMS for 1 h, before preparation of chromosome spreads. DNA was stained with DAPI (left) and VSV-tagged Rtt107 was detected with  $\alpha$ -VSV (right). (C) VSV signal intensity minus background was quantified and expressed relative to the untagged control. The signal intensity was measured for at least 100 nucleoids in each experiment, and the average of two independent experiments is plotted, with error bars spanning 1 SD. The analysis of MMS-treated cells from Figure 1C is included for comparison.

chromatin. In agreement with this, Rtt107 was no longer associated with the nucleoids when cells arrested in G1 phase were treated with MMS (Figure 2, A and C,  $\alpha$  factor + MMS). These findings are consistent with a proposed role for Rtt107 at the site of stalled replication forks (Chin *et al.*, 2006; Roberts *et al.*, 2006). Although experimental evidence indicates that Rtt107 also functions during an unperturbed S phase (Rouse, 2004; Roberts *et al.*, 2006), we were unable to detect binding of Rtt107 to nucleoids in the absence of treatment with DNA-damaging agents. Perhaps the level of spontaneous replication fork stalling in the absence of DNA damage does not lead to sufficient Rtt107 recruitment to allow detection in our assay.

Rtt107 is a target of the checkpoint kinase Mec1 (Rouse, 2004). We examined the effect of the DNA damage check-



point on the localization of Rtt107. Rtt107 recruitment to chromatin in the presence of MMS was not dependent on the checkpoint kinases Rad53 or Mec1 (Figure 2, B and C), indicating that although Rtt107 is a target of Mec1 phosphorylation the localization of Rtt107 to chromatin in response to replication fork stalling was independent of checkpoint activation.

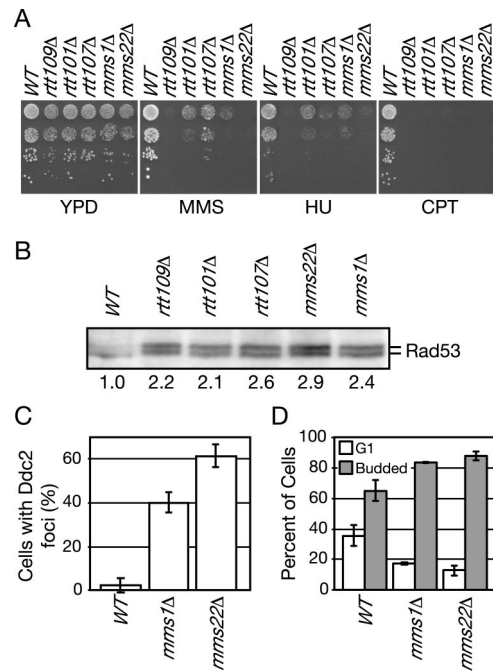
### Recruitment of Rtt107 to Chromatin Requires the Cullin Rtt101 and the Acetyltransferase Rtt109

To identify genes that act in the same pathway as *RTT107* and that might regulate the recruitment of Rtt107 to chromatin, we chose candidates based on two-dimensional hierarchical clustering of genetic interaction data in which *RTT107* clustered closely with *RTT109*, *RTT101*, *MMS22*, and *MMS1* (Collins *et al.*, 2007). These genes displayed the most similar genetic interaction profiles among a set of ~400 genes with roles in DNA replication, DNA repair, and other aspects of chromosome dynamics, suggesting that the products of these genes may form a functional pathway or complex (Collins *et al.*, 2007). Additional data link these genes, including the role of Rtt101 in promoting replication fork progression through DNA lesions (Luke *et al.*, 2006), which is similar to that of Rtt107, and protein-protein interactions between Mms22 and Rtt101 and Mms22 and Rtt107 in high-throughput analysis (Ho *et al.*, 2002). *SLX4* was also chosen as a candidate, because Rtt107 and Slx4 form a complex (Roberts *et al.*, 2006; Zappulla *et al.*, 2006). We tested whether additional phenotypes were shared by strains carrying deletions of these genes (Figure 3). All were sensitive to agents that stall DNA replication forks, MMS, HU, and CPT (Figure 3A). We also found that the checkpoint kinase Rad53 was hyperactive in *rtt109Δ*, *rtt107Δ*, *rtt101Δ*, *mms1Δ*, and *mms22Δ* during normal cell cycle progression, displaying levels of autophosphorylation 2–3 times that observed in wild-type cells (Figure 3B), suggesting the presence of elevated levels of spontaneous DNA damage. Consistent with this, DNA damage response foci formed by the checkpoint protein Ddc2 were elevated in *mms1Δ* and *mms22Δ*, by 8- and 12-fold relative to the wild type (Figure 3C), and *mms1Δ* and *mms22Δ* displayed a cell cycle delay in G2 phase (Figure 3D). Elevated levels of spontaneous DNA damage were noted previously for *rtt107Δ* (Roberts *et al.*, 2006), *rtt101Δ* (Luke *et al.*, 2006), and *rtt109Δ* (Han *et al.*, 2007a). Thus, in addition to having highly similar genetic interaction profiles (Collins *et al.*, 2007), these genes share a number of phenotypes relevant to maintenance of genome integrity, suggesting that they function in the same pathway.

Recruitment of Rtt107 to chromatin was examined in the absence of each of these genes (Figure 4, A and B). Localization of Rtt107 required the acetyltransferase Rtt109 and the cullin Rtt101, but it was not dependent on Mms22, Mms1, or Slx4. Although localization of Rtt107 to chromatin in *slx4Δ* was clearly evident (Figure 4A), the fluorescence intensity was less than that observed in wild type (Figure 4B). Conversely, the signal intensity was higher in *mms1Δ* and *mms22Δ* (Figure 4B). The significance of this, if any, remains unclear. Rtt107 protein levels were not affected by deletion of any of these genes, indicating that the observed effects were not due to depletion of Rtt107 (Figure 4C). Thus, these data indicate that Rtt107 functions downstream of Rtt109 and Rtt101.

### Rtt101 Recruitment to Chromatin in the Presence of MMS

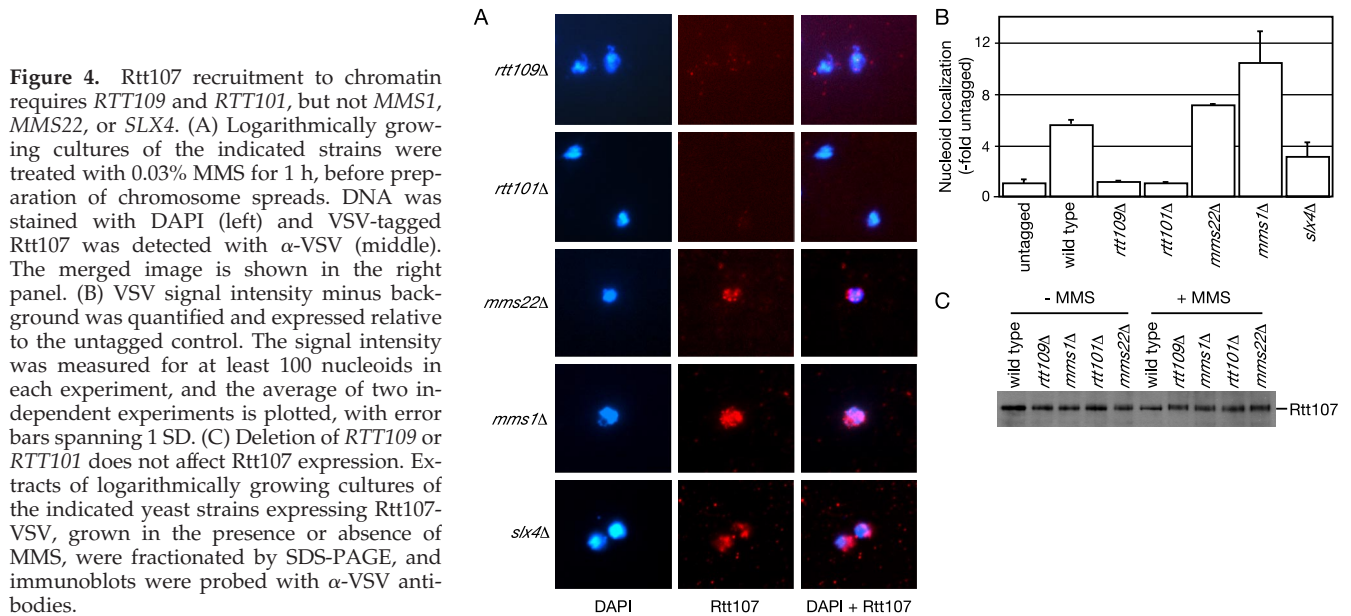
To order the function of the components of this putative pathway, we analyzed the association of Rtt101 with chro-



**Figure 3.** Deletion of *RTT107*, *RTT109*, *RTT101*, *MMS1*, or *MMS22* confers similar phenotypes. (A) Ten-fold serial dilutions of the indicated strains were spotted on media containing 0.03% MMS, 150 mM HU, 40  $\mu$ M CPT, or no drug (YPD), and strains were grown for 2–3 d at 30°C. (B) Spontaneous Rad53 activation was measured in logarithmically growing cells of the indicated strains by using an in situ kinase assay. An autoradiograph of Rad53 is shown, and the amount of autophosphorylation relative to the wild-type control is indicated. (C) The percentage of cells with spontaneous Ddc2 foci was measured for the indicated strains. At least 200 cells were counted in each of two experiments, and the average of the two experiments is plotted with error bars spanning 1 SD. (D) The percent of unbudded and budded cells was measured for the indicated strains. At least 200 cells were counted in each of two experiments, and the average of the two experiments is plotted with error bars spanning 1 SD.

matin in spreads (Figure 5). Like Rtt107, Rtt101 was recruited to chromatin in the presence (Figure 5A), but not the absence, of MMS (data not shown). Deletion of *RTT109* or *RTT107* abrogated the chromatin binding of Rtt101 (Figure 5, A and B). The levels of Rtt101 were not affected in the absence of *RTT109* or *RTT107*, indicating that the observed effect was not due to depletion of Rtt101 (Figure 5C). The simplest interpretation of these data is that Rtt109, Rtt101, and Rtt107 form a pathway that responds to stalled replication forks, with Rtt109 upstream of Rtt101 and Rtt107. Mms1, Mms22, and Slx4 were not detected on chromatin spreads (data not shown); so, their positions within the putative pathway could not be determined.

Because Rtt107 was required for the chromatin binding of Rtt101 and vice versa, Rtt101 and Rtt107 might be recruited as a complex in response to replication fork stalling. To examine this possibility, epitope-tagged Rtt101 was immunoprecipitated, and the bound fraction was assayed for the presence of Rtt107 by immunoblotting. We found that Rtt101 specifically coimmunoprecipitated Rtt107 (Figure 5D). The interaction between Rtt101 and Rtt107 was independent of DNA damage (data not shown). These data are consistent with Rtt101 and Rtt107 being recruited to chromatin as a complex.

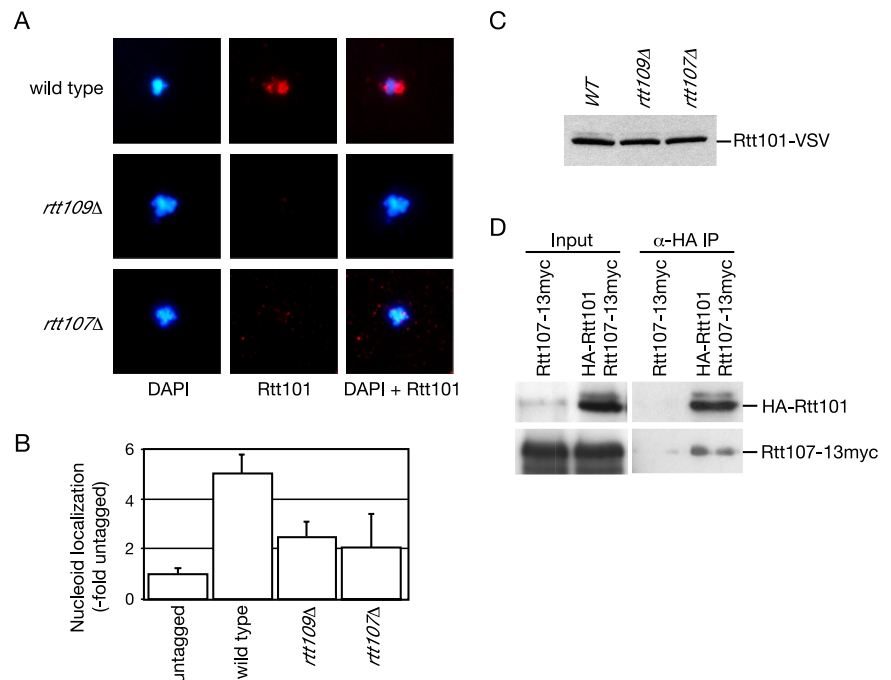


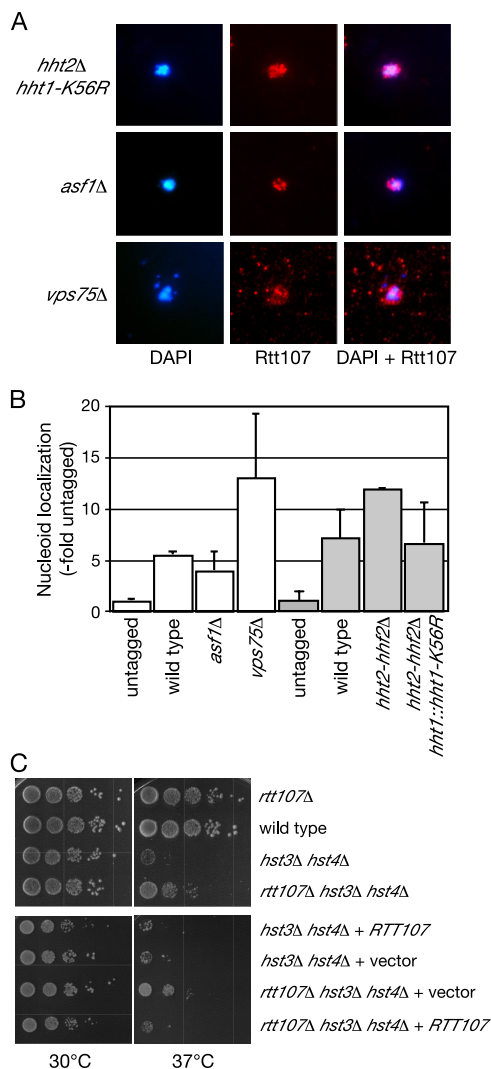
#### Rtt107 Recruitment to Chromatin Does Not Require Histone H3 Lysine 56 Acetylation

Because the histone acetyltransferase Rtt109 was required for recruitment of Rtt107 to chromatin in the presence of replication fork stalling, we tested whether acetylation of known Rtt109 target histone H3 lysine 56 (Collins *et al.*, 2007; Han *et al.*, 2007a; Driscoll *et al.*, 2007) is important for the association of Rtt107 with DNA. Recruitment of Rtt107 to chromatin in the absence of H3-K56 acetylation was monitored by performing chromosome spread analysis of Rtt107-VSV in the absence of Asf1, a histone chaperone required for K56 acetylation (Recht *et al.*, 2006), and in a strain expressing only mutant histone H3 that cannot be acetylated on K56

(H3-K56R) (Figure 6, A and B). Rtt107 localization to chromatin was not affected by the absence of H3-K56 acetylation, indicating that this modification was not required for the recruitment of Rtt107 to chromatin. Rtt109 copurifies with a histone chaperone, Vps75, which is not required for acetylation of H3-K56 (Collins *et al.*, 2007; Han *et al.*, 2007c; Selth and Svejstrup, 2007; Tsubota *et al.*, 2007), suggesting that Rtt109 might have additional acetylation targets. Rtt107 was still localized to chromatin in *vps75* $\Delta$  cells (Figure 6, A and B), indicating that Vps75 was not required for the recruitment of Rtt107 to chromatin. Thus, although the recruitment of Rtt107 to chromatin in response to stalled replication forks required the acetyltransferase Rtt109, it did

**Figure 5.** Recruitment of Rtt101 to chromatin requires *RTT109* and *RTT107*. (A) Logarithmically growing cultures were treated with 0.03% MMS for 1 h, before preparation of chromosome spreads. DNA was stained with DAPI (left), and VSV-tagged Rtt101 was detected with  $\alpha$ -VSV (middle). The merged image is shown in the right panel. (B) VSV signal intensity minus background was quantified and expressed relative to the untagged control. The signal intensity was measured for at least 100 nucleoids in each experiment, and the average of two independent experiments is plotted, with error bars spanning 1 SD. (C) Deletion of *RTT109* or *RTT107* does not affect Rtt101 expression. Extracts of logarithmically growing cultures of the indicated yeast strains expressing Rtt101-VSV, grown in the presence or absence of MMS, were fractionated by SDS-PAGE, and immunoblots were probed with  $\alpha$ -VSV antibodies. (D) Rtt101 forms a complex with Rtt107. Extracts from cells expressing the indicated tagged proteins were immunoprecipitated with anti-HA antibody. The input and immunoprecipitate fractions were analyzed on immunoblots with anti-HA antibodies to detect HA-Rtt101 and with anti-myc antibodies to detect Rtt107-13myc.





**Figure 6.** Rtt107 recruitment to chromatin does not require acetylation of H3-K56. (A) Logarithmically growing cultures of the indicated strains were treated with 0.03% MMS for 1 h, before preparation of chromosome spreads. DNA was stained with DAPI (left) and VSV-tagged Rtt107 was detected with  $\alpha$ -VSV (middle). The merged image is shown in the right panel. (B) VSV signal intensity minus background was quantified and expressed relative to the untagged control. The signal intensity was measured for at least 100 nucleoids in each experiment, and the average of two independent experiments is plotted, with error bars spanning 1 SD. (C) Deletion of *RTT107* suppresses the temperature sensitivity of *hst3Δ hst4Δ*. Ten-fold serial dilutions of cultures of the indicated strains were spotted onto YPD (top) or SD-Leu (bottom) and incubated at 30°C or 37°C for 3 d.

not require the acetylation of the known Rtt109 target histone H3-K56.

#### *rtt107Δ* Suppresses the Temperature Sensitivity of *hst3Δ hst4Δ*

Hst3 and Hst4 are the deacetylases that act in opposition to Rtt109 by deacetylating histone H3-K56 (Celic *et al.*, 2006; Maas *et al.*, 2006; Miller *et al.*, 2006). *hst3Δ hst4Δ* mutant cells are temperature sensitive at 37°C, and they are sensitive to DNA damage (Celic *et al.*, 2006; Maas *et al.*, 2006). Several lines of evidence suggest that these phenotypes are largely

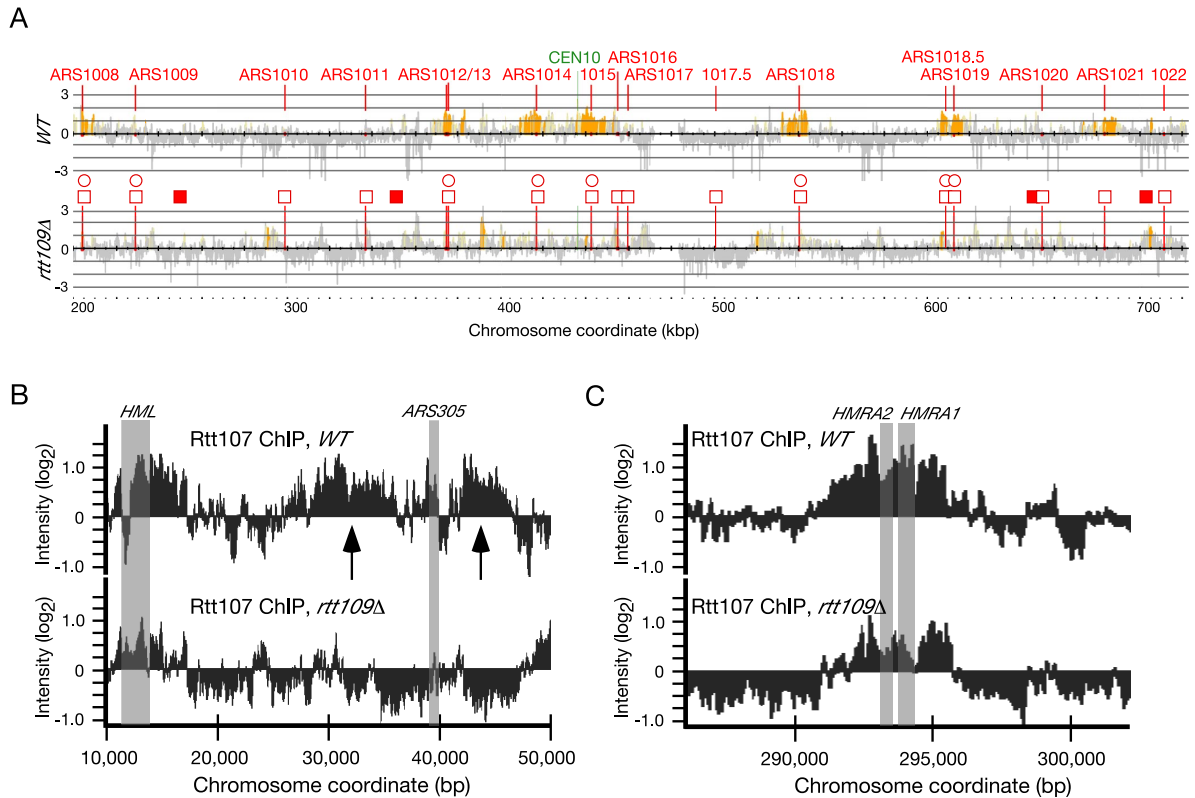
due to hyperacetylation of H3-K56 (Celic *et al.*, 2006; Maas *et al.*, 2006). It remains formally possible, however, that hyperacetylation of additional targets contributes to these phenotypes, so we tested whether deletion of *RTT107* suppresses the temperature sensitivity of *hst3Δ hst4Δ* cells. As shown in Figure 6C, *rtt107Δ* partially suppressed the temperature sensitivity of *hst3Δ hst4Δ*, whereas *rtt107Δ* had no effect on cell growth in a wild-type background. The temperature sensitivity of *rtt107Δ hst3Δ hst4Δ* was restored when Rtt107 was expressed from a plasmid, indicating that the partial suppression of the temperature sensitivity of *hst3Δ hst4Δ* cells was due to deletion of *RTT107*. These data are consistent with hyperacetylation of some target in the absence of Hst3 and Hst4 activating a pathway that contains Rtt107, resulting in temperature and DNA damage sensitivity. Because we also found that Rtt107 recruitment is independent of H3-K56 acetylation, it is likely that Rtt109 acetylation of a distinct target is required for Rtt107 recruitment, although it remains formally possible that the role of Rtt109 in recruiting Rtt107 is independent of acetylation.

#### *Rtt107* Binds Regions Flanking Early Replication Origins When Replication Forks Stall

Although Rtt107 is hypothesized to act at stalled replication forks, and we have found that Rtt107 binds to chromatin when replication forks stall, the chromosomal sites of Rtt107 action have not been identified. We used a chromatin immunoprecipitation strategy, with tiling microarrays, to determine whether Rtt107 is bound to specific regions of chromosomes when replication forks are stalled by nucleotide depletion. Cells were arrested in G1 phase and then released synchronously into S phase in the presence of HU to stall replication forks close to early-firing replication origins. After cross-linking with formaldehyde, Rtt107–DNA complexes were isolated and the bound DNA was hybridized to a whole-genome tiling microarray. When compared with whole genomic DNA from the unbound fraction a distinctive pattern of enrichment at particular genome sites was observed. Figure 7A shows peaks of enrichment along the length of chromosome X, with significant enrichment indicated by yellow bars. Peaks of enrichment coincided with the positions of replication origins known to “fire” in HU (Feng *et al.*, 2006), indicated by open circles. Enrichment of sequences flanking late firing origins (marked by open squares only), which are dormant in HU, was not observed. This pattern of enrichment of sites adjacent to early-firing replication origins was evident on all chromosomes, and it was reminiscent of the pattern displayed by replication fork proteins such as Cdc45 (Katou *et al.*, 2003) and Ctf4 (Lengronne *et al.*, 2006). Enrichment of origin proximal regions was not observed when Rtt107 was immunoprecipitated from cells that had not been treated with HU (data not shown). The replication origin proximal pattern of Rtt107 binding, and the similarity to the pattern of the known replication fork components support the conclusion that Rtt107 is bound at stalled replication forks after treatment of cells with HU.

Because we had observed that the recruitment of Rtt107 to chromatin required *RTT109*, we tested whether the binding of Rtt107 to origin proximal regions upon fork stalling had a similar requirement. When a chromatin immunoprecipitation of Rtt107 was performed using *rtt109Δ* cells, the enrichment at replication origin proximal regions was absent, as shown for chromosome X (Figure 7A). Interestingly, we also observed binding of Rtt107 to the silent mating loci *HML* and *HMR* on chromosome III (Figure 7, B and C), consistent with a proposed role for Rtt107 in transcriptional silencing





**Figure 7.** Rtt107 localizes to origin-proximal regions after replication fork arrest. (A) ChIP-on-chip analysis was performed following synchronous arrest of wild-type (top) or *rtt109Δ* (bottom panel) cells in early S phase by releasing  $\alpha$  factor-arrested cells into 200 mM HU for 2 h at 23°C. After cross-linking and DNA fragmentation, Rtt107 was precipitated, and enrichment of DNA fragments in the Rtt107-bound fraction relative to the unbound fraction is shown along chromosome X. The signal intensity ratio on a log<sub>2</sub> scale is shown on the y-axis and the position along the chromosome is shown on the x-axis. Positive signal represents occupancy by Rtt107, and regions where the positive signal is statistically significant (Katou *et al.*, 2006) are shown in yellow. Annotated, confirmed replication origins (Nieduszynski *et al.*, 2007) are indicated by open squares. Potential origins marked by MCM protein binding (Wyrick *et al.*, 2001; Xu *et al.*, 2006) are indicated by closed squares. Origins that are active in HU as measured by accumulation of ssDNA (Feng *et al.*, 2006) are indicated by open circles. (B) Distribution of Rtt107 near *HML* and *ARS305* in wild type and *rtt109Δ*. Data from the ChIP-chip experiment in A was analyzed across the indicated chromosome coordinates of chromosome III. Arrows indicate two regions of Rtt107 binding flanking *ARS305*. The positions of *HML* and *ARS305* are indicated by gray bars. (C) Distribution of Rtt107 near *HMR* in wild type and *rtt109Δ*. Data from the ChIP-chip experiment in A was analyzed across the indicated chromosome coordinates of Chromosome III. The position of *HMR* is indicated by gray bars.

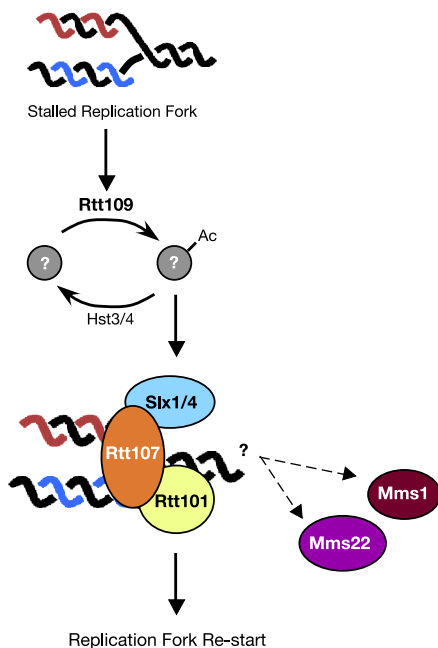
(Zappulla *et al.*, 2006). In this analysis, the enrichment of sequences flanking *ARS305* was evident in the Rtt107-bound fraction (Figure 7B, top, arrows). This enrichment was entirely absent when Rtt107 was immunoprecipitated from *rtt109Δ* cells (Figure 7B, bottom). In contrast to the binding of Rtt107 to *ARS305* flanking regions, the binding of Rtt107 to the silent mating loci remained, albeit at lower levels, when *rtt109* was deleted (Figure 7, B and C, bottom). Binding to the silent loci, then, serves as a positive control for Rtt107 activity in the *rtt109Δ* experiment. Thus, the binding of Rtt107 to stalled replication forks required Rtt109, as did recruitment of Rtt107 to chromatin.

## DISCUSSION

We find that Rtt107 functions as part of a DNA damage response pathway, composed of Rtt109, Rtt101, and Rtt107, which preserves genome integrity when replication forks stall. Rtt107 is recruited to chromatin when cells are treated with DNA-damaging agents that cause replication forks to arrest. This damage-dependent chromatin binding does not require checkpoint activation or complex formation between Rtt107 and the DNA repair protein Slx4. Rtt107 recruitment

depends on the acetyltransferase Rtt109 and the cullin Rtt101, which, like Rtt107, is recruited to chromatin in a DNA damage- and Rtt109-dependent manner. Finally, we find that Rtt107 is bound at or near stalled replication forks *in vivo*. Together, these results indicate that Rtt109, Rtt101, and Rtt107 form a pathway that recruits Rtt107 complexes to damaged or stalled replication forks (Figure 8). Furthermore, our data indicate that Rtt109 sits at the top of the pathway, likely recruiting an Rtt101/Rtt107 complex to stalled replication forks.

One striking feature of our study is that it indicates that Rtt109 function is not exclusively dependent on acetylation of histone H3-K56. Several lines of evidence support this assertion. First, *RTT109* but not *ASF1*, *VPS75*, or *K56* was required for chromatin binding of Rtt107. Second, the temperature sensitivity of *hst3Δ hst4Δ* was partially suppressed by deletion of *RTT107*, indicating that Rtt107 functions downstream of an acetylation event. Third, the *rtt109Δ* mutant was more damage sensitive than *rtt107Δ*, consistent with Rtt109 having a damage resistance role not shared by Rtt107, presumably histone H3-K56 acetylation. Finally, although K56 acetylation was not required for Rtt107 recruitment, Rtt109 was. Because our data indicate that Rtt107 acts



**Figure 8.** Model of the Rtt107 replication fork restart pathway. See text for details.

downstream of an acetylation event, we propose that the role of Rtt109 in recruiting Rtt107 to stalled replication forks involves the acetylation of an unidentified target. However, until the active site residues of Rtt109 are identified with certainty, it remains possible that the role of Rtt109 in recruiting Rtt107 does not involve acetylation.

We envisage two possible models for Rtt109 function in regulating the recruitment of Rtt107 to stalled replication forks. In the first model, Rtt109 acetylation of a protein target provides a binding site for an Rtt101–Rtt107 complex at stalled replication forks. Candidates for this putative target include replication fork proteins and Rtt101 and Rtt107 themselves. In the second model, Rtt109 is required to maintain the integrity of stalled replication forks rather than directly regulating recruitment of Rtt101 and Rtt107. In the absence of Rtt109, replication proteins that normally provide a binding site for Rtt101–Rtt107 dissociate from the stalled replication fork, thereby abrogating Rtt107 recruitment. Although evidence is emerging that loss of Rtt109 affects replisome stability (Han *et al.*, 2007b), loss of Asf1 has similar effects on replisome stability (Franco *et al.*, 2005), yet it does not eliminate Rtt107 recruitment. Irrespective of the mechanism of Rtt109 action, our data indicate that it is required, directly or indirectly, for recruitment of Rtt101 and Rtt107 to chromatin when replication forks stall, and for the binding of Rtt107 to stalled replication forks. The role of Rtt101 in the recruitment of Rtt107 to stalled replication forks seems to be direct, because Rtt101 and Rtt107 were found in complex together and chromatin binding by Rtt101 and Rtt107 was mutually dependent.

The roles of Mms1 and Mms22 in this pathway remain mysterious. The *mms1Δ* and *mms22Δ* mutants share a number of phenotypes with *rtt107Δ*, including sensitivity to drugs that stall replication forks and elevated levels of spontaneous DNA damage, and they have similar genetic interaction profiles (Collins *et al.*, 2007). However, neither *MMS1* nor *MMS22* were required for the recruitment of Rtt107 to stalled replication forks, suggesting that they may act down-

stream of Rtt107 or in a pathway parallel to Rtt107. Because high-throughput protein–protein interaction data and yeast two-hybrid analysis indicate that Mms22 interacts with both Rtt101 and Rtt107 (Ho *et al.*, 2002; Zappulla *et al.*, 2006), and DNA combing analysis indicates that *mms1Δ* and *mms22Δ* have defective replication fork recovery after stalling (Peter, unpublished data), we favor the possibility that Mms1 and Mms22 are also recruited to stalled replication forks, likely by an Rtt101–Rtt107 complex, where they carry out some as yet unidentified downstream function (Figure 8).

The most likely function of Rtt107 at stalled DNA replication forks is the assembly of DNA repair proteins to facilitate the efficient resumption of DNA synthesis. Rtt107 is found in complex with a number of DNA repair proteins, including Slx4, Rad55, and Rad57 (Chin *et al.*, 2006; Roberts *et al.*, 2006; Zappulla *et al.*, 2006). The Rtt107–Slx4 complex is particularly relevant to the resumption of DNA synthesis after fork stalling, because mutants in *RTT107* and *SLX4* display replication defects after treatment with agents that stall DNA replication forks (Chang *et al.*, 2002; Rouse, 2004; Chin *et al.*, 2006; Roberts *et al.*, 2006; Flott *et al.*, 2007). DNA combing analysis has further indicated that unreplicated gaps remain after fork stalling in *slx4Δ* and *rtt101Δ* (Flott *et al.*, 2007; Luke *et al.*, 2006), and *rtt107Δ* displays an anaphase arrest morphology (Roberts *et al.*, 2006) consistent with the presence of unreplicated regions (Torres-Rosell *et al.*, 2007). We found that recruitment of Rtt107 to stalled replication forks did not require Slx4; yet, Rtt107 and Slx4 are found in complex (Roberts *et al.*, 2006), suggesting that Rtt107 brings Slx4 to sites of replication fork stalling (Figure 8). Considerable evidence suggests that the Slx1–Slx4 nuclease is active on replication fork structures and that it could be a critical element in the restart of stalled forks (Kaliraman and Brill, 2002; Fricke and Brill, 2003; Coulon *et al.*, 2004, 2006; Flott and Rouse, 2005; Roberts *et al.*, 2006). In addition to Slx4, we suspect that Rtt107 assembles other repair proteins onto stalled replication forks, particularly because *rtt107Δ* mutants are considerably more sensitive to MMS and HU than *slx4Δ* mutants (Chang *et al.*, 2002; Chin *et al.*, 2006; Roberts *et al.*, 2006; Zappulla *et al.*, 2006), and *rtt107Δ slx4Δ* double mutants are slightly more MMS sensitive than single mutants (Roberts *et al.*, 2006). Thus, we propose that the action of Rtt109 and Rtt101 leads to recruitment of Rtt107 to stalled replication forks where Rtt107 acts as a scaffold for the assembly of DNA repair enzymes such as Slx4 to facilitate the bypass or removal of the replication fork block, ultimately resulting in the restart of stalled replication forks (Figure 8).

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