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Loss of H3 K79 Trimethylation Leads to Suppression of Rtt107-dependent DNA Damage Sensitivity through the Translesion Synthesis Pathway*[S]

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Genomic integrity is maintained by the coordinated interaction of many DNA damage response pathways, including checkpoints, DNA repair processes, and cell cycle restart. In *Saccharomyces cerevisiae*, the BRCA1 C-terminal domain-containing protein Rtt107/Esc4 is required for restart of DNA replication after successful repair of DNA damage and for cellular resistance to DNA-damaging agents. Rtt107 and its interaction partner Slx4 are phosphorylated during the initial phase of DNA damage response by the checkpoint kinases Mec1 and Tel1. Because the natural chromatin template plays an important role during the DNA damage response, we tested whether chromatin modifications affected the requirement for Rtt107 and Slx4 during DNA damage repair. Here, we report that the sensitivity to DNA-damaging agents of *rtt107*Δ and *slx4*Δ mutants was rescued by inactivation of the chromatin regulatory pathway leading to H3 K79 trimethylation. Further analysis revealed that lack of Dot1, the H3 K79 methyltransferase, led to activation of the translesion synthesis pathway, thereby allowing the survival in the presence of DNA damage. The DNA damage-induced phosphorylation of Rtt107 and Slx4, which was mutually dependent, was not restored in the absence of Dot1. The antagonistic relationship between Rtt107 and Dot1 was specific for DNA damage-induced phenotypes, whereas the genomic instability caused by loss of Rtt107 was not rescued. These data revealed a multifaceted functional relationship between Rtt107 and Dot1 in the DNA damage response and maintenance of genome integrity.

Multiple mechanisms cooperate to maintain genome integrity, thus ensuring proper transmission of genetic information from one generation to the next. DNA damage is detected by sensors that activate the DNA damage checkpoint, which in turn elicits various cellular responses, including cell cycle arrest, DNA repair, apoptosis, and/or DNA damage-induced transcriptional programs (1, 2).

In *Saccharomyces cerevisiae*, the kinase proteins Mec1 and Tel1, the yeast homologues of mammalian ATR (ATM and Rad3-related) and ATM (ataxia-telangiectasia mutated), are crucial for transducing signals in the S phase checkpoint response (3, 4). The downstream signaling cascade leads to cell cycle arrest, replication fork stabilization, and DNA damage repair (5). Following successful DNA repair, the checkpoint must be deactivated to allow resumption of the cell cycle and restart of stalled replication forks. Although one of the main steps in this process is dephosphorylation of the effector kinase Rad53, checkpoint deactivation is further coordinated by many different proteins, including phosphatases, proteases, and helicases (6–9). In the event of irreparable DNA damage, tolerance mechanisms allow bypass of DNA lesions therefore enabling cells to survive (10). One of these pathways is the translesion synthesis (TLS)[6] pathway that uses special error-prone polymerases to allow replication past DNA lesions, resulting in an increased mutation frequency (11).

One of the downstream phosphorylation targets of Mec1 is Rtt107/Esc4, which is required for reinitiating replication after repair of alkylating DNA damage (12, 13). Accordingly, yeast lacking the nonessential *RTT107* gene or carrying an allele encoding for a nonphosphorylatable Rtt107 protein are hypersensitive to different DNA-damaging agents (12). These include the DNA-alkylating agent methyl methanesulfonate (MMS), the nucleotide reductase inhibitor hydroxyurea, and the topoisomerase I poison camptothecin (12–14). Moreover, *rtt107*Δ mutants have a chromosome instability phenotype and an increased incidence of Rad52 foci, indicative of homologous recombination occurring because of stalled DNA replication forks (15, 16). Aside from these roles in genome integrity, Rtt107 functions to repress the mobility of Ty1 transposons and to establish silent chromatin (17, 18).

Rtt107 contains several BRCA1 C-terminal homology domains, which often serve as phospho-binding modules to recruit signaling complexes and repair factors to DNA damage-induced lesions (12, 19). Consistent with a role as a scaffold for protein-protein interactions during the DNA damage response, Rtt107 interacts with a number of DNA repair and recombina-

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[6] The abbreviations used are: TLS, translesion synthesis; MMS, methyl methanesulfonate.

tion proteins (13, 20, 21). Of these, the interaction of Rtt107 with the structure-specific endonuclease Slx4 is best characterized and indicative of a close functional relationship between thetwoproteins.Slx4isrequiredforMec1-dependentphosphorylation of four (S/T)Q motifs in the C-terminal half of Rtt107 and, like Rtt107, facilitates resumption of DNA replication after DNA damage (12, 13).

In addition to the complex regulation of the DNA damage response by signaling cascades, chromatin structures in the cell also play many roles in regulating access to DNA during the repair process. One example of the emerging interface between chromatin and the DNA damage response pathways is the DNA damage-induced recruitment of Rtt107 to chromatin by the H3 K56 acetyltransferase Rtt109 and the cullin Rtt101 (20). There are many other chromatin modifications involved in the DNA damage response, such as the well studied H2A phosphorylation and H3 K79 methylation pathways (22). H2A S129 is phosphorylated by Mec1 in response to DNA damage, triggering the assembly of many repair proteins and chromatin modifiers acting at subsequent steps (22–25). To allow resumption of the cell cycle and DNA replication after successful completion of DNA repair, H2A S129 needs to be dephosphorylated by either Pph3 or Glc7, depending on the exact nature of the initial damage (26, 27). Dot1-mediated H3 K79 methylation, which is regulated by Bre1-mediated H2B K123 ubiquitination, is required for G_1 and S phase checkpoints (28–32). In part, this requirement is mediated through a functional linkage to the Rad9 adaptor protein (31, 33). Several lines of evidence suggest that Dot1 plays an additional role in DNA repair pathways, such as nucleotide excision repair, sister chromatid recombination, and repair of ionizing radiation damage (34–36). In contrast, Dot1 negatively regulates the error-prone TLS pathway through an unknown mechanism, thereby allowing bypass of DNA replication blocks (7). Aside from the function of Dot1 in DNA damage, it is also involved in gene silencing as well as differential H3 K79 methylation during the cell cycle (37, 38).

This study established a close connection between Rtt107 and the pathway resulting in a specific chromatin modification, H3 K79 trimethylation. Specifically, loss of H3 K79 trimethylation suppressed the DNA damage sensitivity of *rtt107*Δ and *slx4*Δ mutants. This suppression was not linked to restoration of Rtt107 or Slx4 phosphorylation but instead was dependent on the presence of a functional TLS pathway. Moreover, deletion of *DOT1* partially suppressed the cell cycle delay and the defect in resuming DNA replication of *rtt107*Δ mutants during the recovery from MMS-induced DNA damage. In contrast, deletion of *DOT1* rescued neither the chromosome instability phenotype nor the increased incidence of spontaneous Rad52 foci caused by loss of Rtt107.

EXPERIMENTAL PROCEDURES

Yeast Strains—All yeast strains used in this study are listed in supplemental Table 1 and were created using standard yeast genetic techniques (39). Complete gene deletions and integration of a triple FLAG tag at the 3′ end of genes (40) were achieved using one-step gene integration of PCR-amplified modules (41). Plasmid shuffling experiments were performed using 5-fluoroorotic acid as a counter-selecting agent for the *URA3* plasmid (pRS316, *HHT2-HHF2*) and shuffling in plasmids containing histone H3 K79 mutations (pRS314, *hht2-HHF2*) (42). Catalytically inactive Dot1 mutants were expressed from pRS315 plasmids (43), and a nonphosphorylatable mutant of Rtt107 (four Ser-Gln motifs substituted by Ala-Gln) was expressed from a plasmid (pRS315, *rtt107–4AQ*) that was a generous gift from Grant Brown and Tania Roberts (University of Toronto). BrdU-Incorporating (BrdU-Inc) wild-type and mutant strains containing constitutively expressed herpes simplex virus-thymidine kinase and human equilibrative nucleoside transporter (hENT1) were generated by genetic crosses with a previously published parental strain (44).

Sensitivity Measurements—Overnight cultures grown in YPD or SC-Leu at 30 °C were diluted to 0.3 A_{600}. The cells were 10-fold serially diluted and spotted onto solid YPD plates or SC-Leu plates with MMS (Sigma) at various concentrations. The plates were then incubated at 30 °C for 2–3 days.

Protein Extracts and Protein Blot Analysis—Overnight cultures were diluted to 0.3 A_{600}, grown in YPD to 0.8 A_{600}, and then treated with 0.03% MMS for 1 h. The FLAG-tagged Slx4 protein was extracted by an alkaline method using 0.2 M NaOH (45). Slx4-FLAG proteins were visualized using anti-FLAG M2 antibodies (Sigma) and SuperSignal-enhanced chemiluminescence (Pierce). The procedure for analytical scale immunoprecipitation of the FLAG-tagged Rtt107 protein was adapted from a previous report (46). 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 $NaPP_i$, 5 mM EGTA, 5 mM EDTA, 0.1 mM Na_3VO_4, 5 mM NaF, Complete™ protease inhibitor mixture) using acid-washed glass beads and mechanically disrupted using a bead beater (BioSpec Products). Rtt107-FLAG fusion proteins were captured using anti-FLAG M2-agarose beads (Sigma) and subsequently washed in TAP-IP buffer. Captured material was analyzed by protein blotting with anti-FLAG M2 (Sigma) and visualized using the Odyssey Infrared Imaging System (Licor).

Flow Cytometric Analysis and BrdU Incorporation Experiments—Cells were prepared under the same conditions for flow cytometric analysis and BrdU incorporation experiment. For the latter, we used wild-type and mutant strains containing the BrdU-Inc cassette (44) to allow for BrdU uptake in yeast. Briefly, cells were arrested in G_1 by addition of 2 μg/ml α-factor for 2 h at 30 °C in YPD, then washed with sterile 1× PBS, and resuspended in YPD containing 0.03% MMS for 1 h. MMS was removed by treating with 2% sodium thiosulfate and washing with sterile 1× PBS. The cells were resuspended in YPD and incubated at 30 °C during the recovery phase. For the BrdU incorporation experiment, 400 μg/ml of BrdU was added to the cultures during the recovery phase. Aliquots were removed at the indicated times and processed further for flow cytometric analysis or measurement of BrdU incorporation.

For flow cytometric analysis, cells were collected in 70% ethanol with 0.2 M Tris-HCl, pH 7.5, and prepared as described previously (47). Samples were analyzed using the BD FACSCalibur instrument and the Flow Jo software (Tree Star Inc., OR). For the BrdU incorporation experiments, cells were collected in buffer containing 100 mM EDTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, and 0.1% NaH_3. Total genomic DNA was

extracted by bead beating and use of the DNeasy kit (Qiagen) and sonicated using Bioruptor (Diagenode). The DNA concentration was adjusted to 20 ng/μl, then heat-denatured, and snap-cooled. 1 μg of DNA was spotted onto a nitrocellulose membrane (Bio-Rad) pre-soaked with 2× SSC using the Convertible Filtration Manifold System (Invitrogen) and subjected to ultraviolet cross-linking in a Stratalinker (Stratagene). Subsequently, the membrane was blocked with 5% milk in TTBS, probed with an anti-BrdU antibody (GE Healthcare), and visualized using the Odyssey Infrared Imaging System (Licor).

Quantitative Bimater Assay—The procedure for the bimater assay was modified from a previous method to allow quantification (16). Briefly, 12 independent colonies from each homozygous diploid strain were grown in YPD overnight at 30 °C and diluted to 2.0 A_{600}. Cells were plated on to solid YPD at appropriate dilutions to determine the total number of cells. Equal volumes of *MAT***a** mating tester cultures (10.0 A_{600}) and the homozygous diploid strain cultures (2.0 A_{600}) were plated onto solid media containing no amino acids and incubated at 30 °C for 3–4 days. Mating rates and 95% confidence intervals were calculated with the Ma-Sandri-Sarkar Maximum Likelihood Estimator method using the on-line Fluctuation Analysis Calculator (48).

Microscopy—Nuclear morphology was determined by treating cells as for flow cytometric analysis, except α-factor incubation was omitted, and SC-complete medium was used to minimize autofluorescence. Aliquots were removed at the indicated times and treated with 4% formaldehyde solution (Sigma) for 10 min. Cells were immobilized on a glass slide with a solution of 1 mg/ml polylysine (Sigma) and then stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma). Cells with medium to large buds were counted as being in G_2/M phase. Ambiguous cases where cells with separate nuclei were insufficiently spread were considered to be in G_1 phase.

To visualize Rad52-GFP foci, cells were grown at 30 °C in SC-complete medium to logarithmic phase and then immobilized on a glass slide with a solution of 1.0% agarose in double distilled H_2O. Multiple images were obtained at 0.3-μm intervals along the *z* axis, and Rad52-GFP foci were counted by inspection of all focal planes. At least 300 cells were counted for each time point. All imaging was done with the Zeiss Axioplan 2 fluorescence microscope using the Metamorph software. Statistical significance was assessed using Student's *t* test.

Measurement of Mutation Rates—Forward mutation rates were measured by mutations at the *CAN1* locus, which when mutated renders cells sensitive to canavanine (49). Cells from 12 independent colonies for each strain were grown in YPD to logarithmic phase; 0.005% MMS was added to half of each culture, and cells were further incubated at 30 °C for 20 h. Cells plated on SC-Arg were diluted 1:200,000, and cells plated on SC-Arg containing 50 μg/ml canavanine (Sigma) were diluted by a factor of 2. Plates were incubated at 30 °C for 2 days, and colonies were counted. The mutation rates and 95% confidence intervals were calculated with the Ma-Sandri-Sarkar Maximum Likelihood Estimator method using the on-line Fluctuation Analysis Calculator (48).

RESULTS

Elimination of H3 K79 Methylation Suppresses the Sensitivity of rtt107Δ and slx4Δ Mutants to the DNA-damaging Agent MMS—Rtt107 and its interaction partner Slx4 are required for yeast cells to survive exposure to DNA damage conditions, such as those caused by the alkylating agent MMS (13, 14). Given the importance of the natural chromatin template during the DNA damage response, and the existing link between Rtt107 and the histone acetyltransferase Rtt109 (20, 30, 50), we hypothesized that chromatin modifications might affect the requirement for Rtt107 and Slx4 during DNA damage repair. For this purpose, we created strains that, in addition to deletion of either *RTT107* or *SLX4*, lacked genes encoding several chromatin modifiers with roles in the DNA damage response to test whether their absence enhanced or suppressed the sensitivity of *rtt107*Δ or *slx4*Δ mutants to MMS. Although the majority of double mutants grew equally well as *rtt107*Δ or *slx4*Δ mutants, we found that deletion of *DOT1*, a nonessential gene encoding a histone methyltransferase catalyzing mono-, di-, and trimethylation of histone H3 K79, almost completely rescued the MMS sensitivity of *rtt107*Δ and *slx4*Δ mutants (Fig. 1*A*).

To determine whether this effect was dependent on the catalytic activity of Dot1, alleles encoding catalytically inactive Dot1 proteins were compared with the complete loss of Dot1 and with the presence of wild-type Dot1. The strains carrying *dot1G401R* and *dot1G401A* alleles, encoding for catalytically inactive forms of Dot1, suppressed the MMS sensitivity phenotype similar to the complete deletion (Fig. 1*B*). As expected, re-introducing wild-type *DOT1* in *rtt107*Δ*dot1*Δ double mutants restored MMS sensitivity to levels similar to that of *rtt107*Δ single mutants. These data suggested that eliminating the catalytic activity of Dot1 enabled cells lacking Rtt107 to survive otherwise detrimental conditions during exposure to MMS. The same results were obtained for *slx4*Δ mutants, except that *slx4*Δ mutants were less sensitive to MMS than *rtt107*Δ mutants (supplemental Fig. S1*A*).

The only known target of Dot1 methyltransferase activity to date is the Lys-79 residue located in the core of histone H3, but formally it is possible that Dot1, similar to other chromatin modifiers, has other enzymatic targets not yet identified. To examine whether the suppression of the MMS sensitivity of *rtt107*Δ mutants by loss of Dot1 was due to lack of H3 K79 methylation, strains with mutated forms of H3 K79, which cannot be methylated, were tested for their ability to survive chronic MMS exposure in the absence of Rtt107. Changing lysine 79 to either alanine or arginine rescued the DNA damage sensitivity of the *rtt107*Δ mutants, analogous to the *DOT1* deletion (Fig. 1*C*). Therefore, we concluded that the reversal of the MMS sensitivity of *rtt107*Δ mutants was due to the loss of Dot1-mediated H3 K79 methylation. Similarly, *H3 K79A* and *H3 K79R* mutants also suppressed the MMS sensitivity of the *slx4*Δ strain (supplemental Fig. S1*B*).

Methylation of H3 K79 by Dot1 is regulated through cross-talk with another histone modification, mono-ubiquitination of H2B K123, which is catalyzed by the Bre1/Rad6 enzyme complex (28, 29, 32, 51–53). Thus, we wanted to test whether upstream regulators of H3 K79 methylation would have a sim-

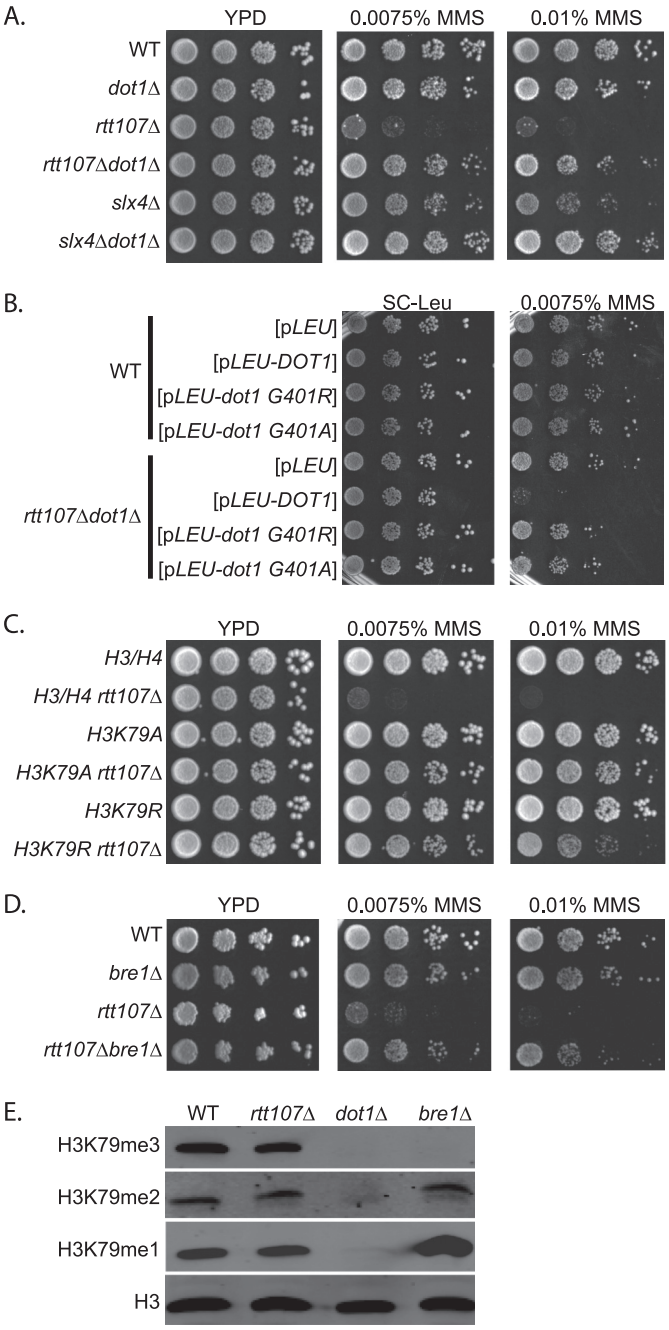

FIGURE 1. **Abrogation of H3 K79 trimethylation suppressed the MMS sensitivity of strains lacking Rtt107 or Slx4.** 10-Fold serial dilutions of the indicated strains were plated onto media containing 0.0075 or 0.01% MMS. *A,* loss of Dot1 suppressed MMS sensitivity of *rtt107*Δ and *slx4*Δ mutants. *B,* loss of Dot1 catalytic activity; *C,* H3 K79A, K79R; or *D,* loss of Bre1 suppressed MMS sensitivity of *rtt107*Δ mutants. *E,* Bre1 affected mainly H3 K79 trimethylation and not di- or monomethylation. Whole cell extracts of indicated strains were analyzed by protein blotting with anti-H3 K79 tri-, di-, or monomethyl antibodies. Antibodies against H3 were used as a loading control.

ilar effect on the MMS sensitivity of *rtt107*Δ and *slx4*Δ mutants. Indeed, deletion of *BRE1* also rescued the MMS sensitivity of the strains lacking Rtt107 or Slx4 (Fig. 1*D* and supplemental Fig. S1*C*).

To learn more about the biochemical nature underlying the observed effects, we assessed the total levels of mono-, di-, or trimethylated H3 K79 in whole cell extracts. Interestingly, although Dot1 broadly catalyzes mono-, di-, and trimethylation

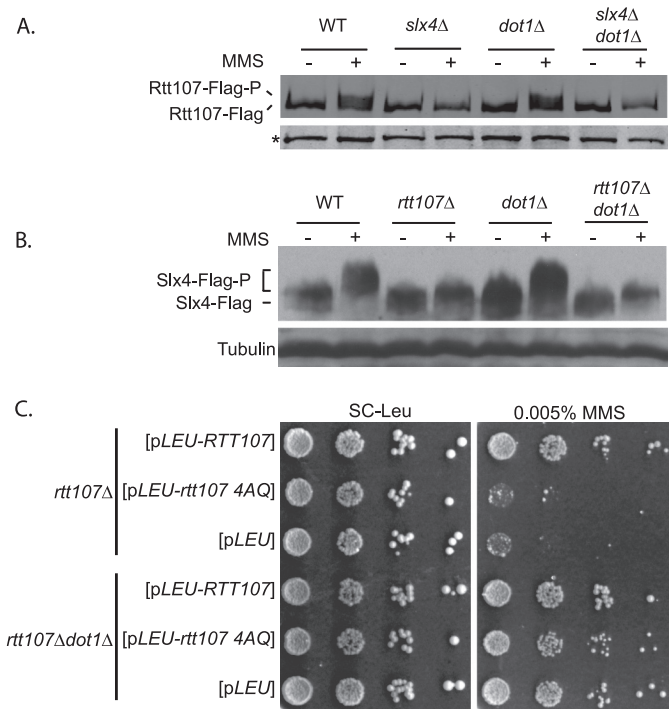

FIGURE 2. **Suppression of *rtt107*Δ MMS sensitivity by deletion of *DOT1* was not dependent on the phosphorylation of Slx4 and vice versa.** *A,* cells expressing Rtt107-FLAG were untreated or treated with 0.03% MMS for 1 h. Analytical scale immunoprecipitations of Rtt107-FLAG were performed and analyzed by protein blotting with anti-FLAG antibodies. The reduced mobility of Rtt107-FLAG indicated phosphorylation of the protein. Background bands (*) were used as a loading control. *B,* cells expressing Slx4-FLAG were treated as described in *A*. Whole cell extracts were analyzed by protein blotting with anti-FLAG antibodies, with reduced mobility of Slx4-FLAG being indicative of phosphorylation. Antibodies against tubulin were used as a loading control. *C,* deletion of *DOT1* suppressed the MMS sensitivity of the mutants expressing the nonphosphorylatable Rtt107–4AQ. 10-fold serial dilutions were plated onto SC-Leu containing 0.005% MMS.

of H3 K79 (43, 54, 55), Bre1 primarily affected Lys-79 trimethylation (Fig. 1*E*). These results suggested that specifically a lack of H3 K79 trimethylation caused the suppression of the MMS sensitivity of *rtt107*Δ and *slx4*Δ mutants.

Deletion of DOT1 Suppresses DNA Damage Sensitivity in the Absence of MMS-induced Phosphorylation of Rtt107 or Slx4—In response to DNA damage induced by various agents, Rtt107 and Slx4 are phosphorylated on several Ser/Thr residues by the checkpoint kinase Mec1 (12, 13). Phosphorylation of Rtt107 is essential for its function in the DNA damage response and depends on Slx4 (12, 13). It was in principle possible that, in *slx4*Δ*dot1*Δ double mutants, an alternative pathway directed Rtt107 phosphorylation in the absence of Slx4, thereby enabling cells to survive the otherwise detrimental MMS-induced DNA damage. To test this possibility, Rtt107 phosphorylation was measured in strains lacking Slx4, Dot1, or both simultaneously. As expected, exposure to MMS induced phosphorylation of Rtt107 in wild-type strains but not in strains lacking Slx4 (Fig. 2*A*) (13). Although deletion of *DOT1* suppressed the MMS sensitivity of *slx4*Δ mutants, it did not overcome the requirement of Slx4 for Rtt107 phosphorylation (Fig. 2*A*). Loss of Dot1 had no effect on Rtt107 phosphorylation in response to MMS when Slx4 was present (Fig. 2*A*). Therefore, the suppression by *dot1*Δ did not involve a restoration of Rtt107 phosphorylation

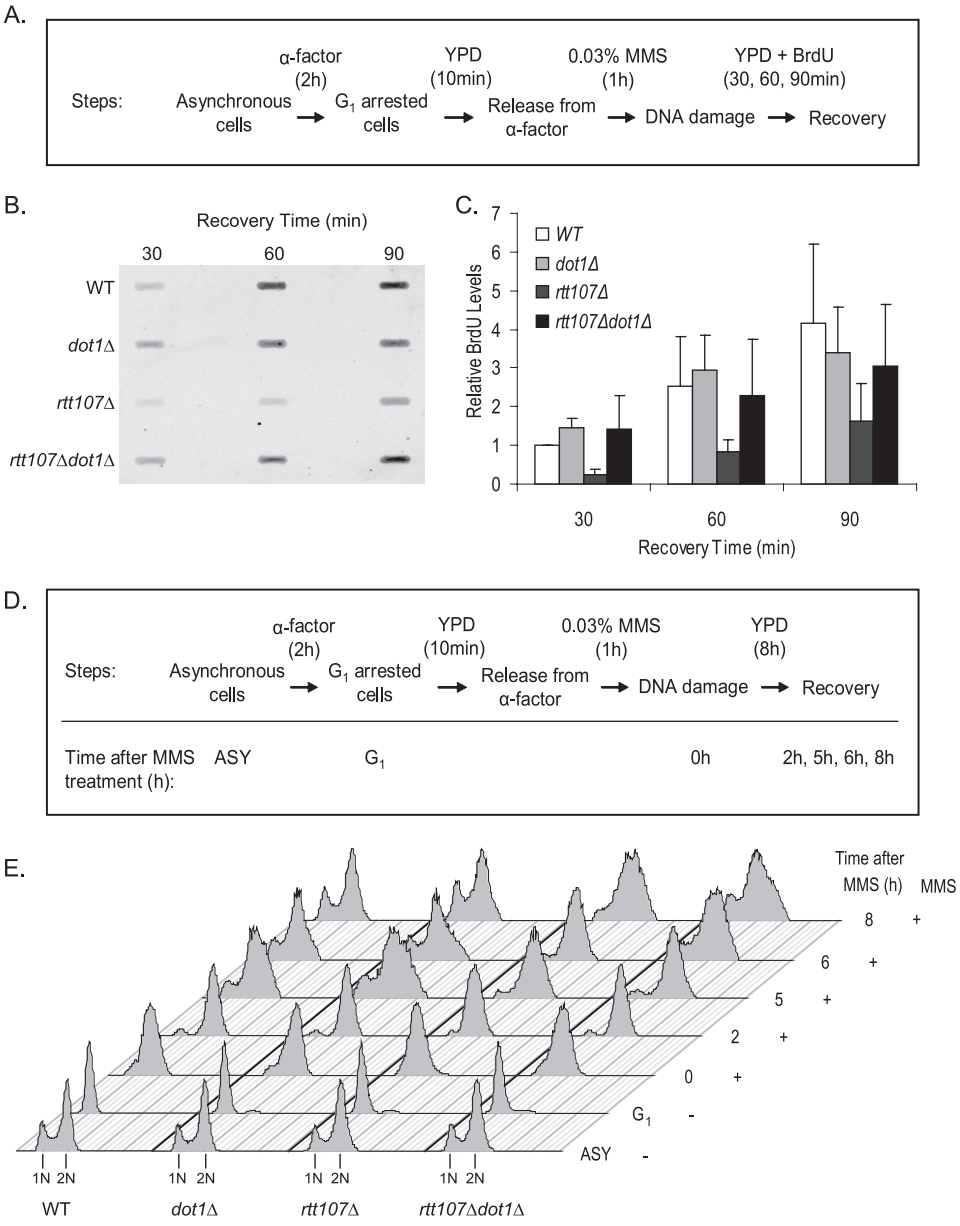

FIGURE 3. **Requirement of Rtt107 for resumption of DNA replication and cell cycle after DNA damage was partially suppressed by deletion of *DOT1*.** *A,* diagram of experimental strategy used for BrdU incorporation experiment. *B,* BrdU incorporation into nascent DNA indicated *rtt107*Δ*dot1*Δ mutants more efficiently resumed DNA replication after DNA damage than *rtt107*Δ mutants. *C,* quantification of newly replicated DNA as measured by BrdU signals. All values are relative to wild type at 30 min. *Error bars* represent standard deviations of values from three independent experiments. *D,* diagram of experimental strategy used for FACS analysis. *E,* FACS analysis showed *rtt107*Δ*dot1*Δ mutants recovered from DNA damage earlier than *rtt107*Δ mutants. *ASY*, asynchronous cells.

in the absence of Slx4, arguing against an alternative pathway for Rtt107 phosphorylation. Consistent with the physical interaction and close functional relationship between Rtt107 and Slx4, we found that MMS-induced phosphorylation of Slx4 was dependent on Rtt107 but not on Dot1 (Fig. 2*B*). Analogous to the results obtained for Rtt107, eliminating *DOT1* did not restore Slx4 phosphorylation in the absence of Rtt107 (Fig. 2*B*).

To test whether the suppression by deletion of *DOT1* was linked to the phosphorylation of Rtt107 at specific Ser-Gln sites, we utilized mutants expressing the nonphosphorylatable form of Rtt107. Using a plasmid expressing *rtt107–4AQ* in cells lacking Dot1, we tested the MMS sensitivity of the double mutants. Consistent with the importance of Rtt107 phosphorylation, deletion of *DOT1* suppressed the MMS sensitivity of the *rtt107 4AQ* mutants (Fig. 2*C*). Taken together, these results indicated that MMS sensitivity of mutants lacking Rtt107 phosphorylation was suppressed by deletion of *DOT1*.

Requirement of Rtt107 for Resumption of Cell Cycle after S Phase Damage Is Partially Suppressed by Lack of Dot1—To further understand the molecular mechanism leading to the suppression of *rtt107*Δ MMS sensitivity, we tested whether loss of Dot1 could compensate for the requirement of Rtt107 during the restart of DNA replication (12, 13). Cells arrested in G_1 were released into S phase in the presence of MMS for 1 h, and restart of DNA replication was directly measured by BrdU incorporation into nascent genomic DNA (Fig. 3*A*). Because BrdU was added after MMS treatment, it serves as a quantitative indicator of newly replicated DNA during the recovery process. As expected, BrdU levels increased in wild-type cells during the course of the experiment, indicating successful resumption of DNA replication (Fig. 3*B*). In contrast, BrdU levels in *rtt107*Δ mutants were consistently lower than the wild-type at each time point. Although the levels of BrdU incorporation in *dot1*Δ mutants increased similar to the wild-type cells, *rtt107*Δ*dot1*Δ mutants incorporated BrdU at intermediate levels between wild-type and *rtt107*Δ mutants (Fig. 3*C*). This result suggested that loss of Dot1 could partly suppress the defect of *rtt107*Δ mutants in resuming DNA replication.

Next, we used FACS analysis to test whether loss of Dot1 would have a similar effect on the resumption of cell cycle progression after DNA damage (Fig. 3*D*). At the end of the MMS treatment (0 h), wild-type and *rtt107*Δ mutants were initially arrested in S phase due to activation of the DNA damage checkpoint (12, 13), although *dot1*Δ mutants had proceeded through S phase as judged by the shift of the signal to 2N, consistent with a requirement for Dot1 in the DNA damage checkpoint as described previously (Fig. 3*E*) (30, 31). 2 h after removal of MMS, *rtt107*Δ mutants were still in S phase, and all other strains had progressed to G_2/M. Further differences between

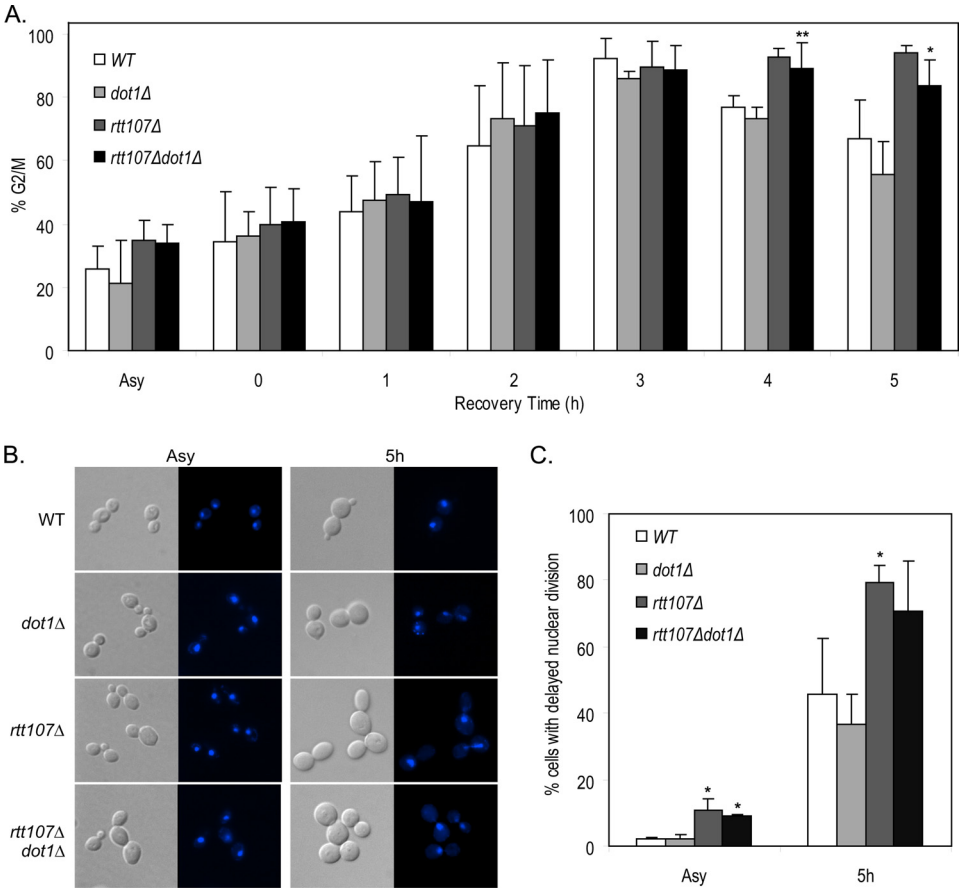

FIGURE 4. **Nuclear division delay of *rtt107*Δ mutants was not suppressed by deletion of *DOT1* in the absence and presence of MMS.** Cells were treated with 0.03% MMS for 1 h, washed, and then resuspended in complete media for DNA damage recovery and stained with DAPI at the indicated time points. *A,* increased percentage of cells in G_2/M of *rtt107*Δ mutants after DNA damage was not suppressed by deletion of *DOT1*. The percentage of cells with medium to large buds (% G_2/M) was calculated by dividing the number of cells with medium to large buds by the total number of cells. *B,* representative differential interference contrast images are shown on the *left* and the corresponding DAPI images on the *right*. *C,* increased percentage of cells exhibiting nuclear division delay in *rtt107*Δ mutants was not suppressed by loss of Dot1. The percentage of cells with delayed nuclear division was calculated by dividing the number of large budded cells with unsegregated nuclei by the total number of cells. In both *A* and *C*, at least 200 cells were counted in three independent experiments. *Error bars* represent standard deviations of the values. *, $p < 0.05$; **, $p < 0.005$ when compared with the wild-type strain in the same time point. *ASY*, asynchronous cells.

the mutants were observed as strains continued to recover from DNA damage. For example, at 5 h a substantial fraction of cells in the wild-type and *dot1*Δ mutant had undergone cell division as judged by the appearance of a G_1 peak and S phase fraction, whereas *rtt107*Δ mutants had predominantly a 2N peak, suggesting that they were still residing in G_2/M. These differences persisted until 8 h after recovery when the G_1 peak and S phase fraction first appeared in the *rtt107*Δ mutants. The *rtt107*Δ *dot1*Δ double mutants had an intermediate phenotype, as the G_1 peak and S phase fraction became visible at 6 h, which was earlier than *rtt107*Δ mutants but later than wild-type or *dot1*Δ mutants (Fig. 3*E*). Consistent with this, the delayed appearance of intact chromosomes during recovery in the *rtt107*Δ mutants was partially rescued by concurrent loss of Dot1 as visualized by pulsed-field gel electrophoresis (supplemental Fig. S2).

The defect of *rtt107*Δ mutants in completing the G_2/M phase of the cell cycle during recovery from transient DNA damage can also be observed by examining nuclear morphology (13). In wild-type and *dot1*Δ mutants, the percentage of cells in G_2/M increased after exposure to MMS, reached a peak at 3 h of recovery, and started to decrease as the cells completed mitosis (Fig. 4, *A* and *B*). As expected, the percentage of *rtt107*Δ mutants in G_2/M also reached a peak at 3 h, but the increased level lasted up to 5 h after MMS treatment. As judged by the percentage of G_2/M cells, the kinetics of recovery from DNA damage in *rtt107*Δ*dot1*Δ double mutants was slower than wild-type and *dot1*Δ mutants but faster than *rtt107*Δ mutants, although this did not reach statistical significance (Fig. 4*A*). Another known phenotype of *rtt107*Δ mutants is the delay of nuclear division, as judged by the higher percentage of large budded cells with elongated nuclei spanning the bud neck. Consistent with previous reports, nuclear division was delayed in *rtt107*Δ mutants when compared with wild type 5 h after exposure to MMS (Fig. 4*C*). In contrast, *dot1*Δ mutants did not show any delay of nuclear division. A similar phenotype was also observed in asynchronous cultures not exposed to MMS. In both conditions, deletion of *DOT1* did not rescue the defect caused by loss of Rtt107.

*TLS Pathway Is Required for Suppression of the MMS Sensitivity of rtt107*Δ *Mutants by Deletion of DOT1*—Next, we sought to determine the pathway by which deletion of *DOT1* suppressed the requirement for *RTT107* during MMS exposure. In addition to the suppression of the DNA damage sensitivity of *rtt107*Δ and *slx4*Δ mutants reported here, lack of Dot1 suppresses the MMS sensitivity of strains lacking a variety of repair proteins, and this effect is dependent on the TLS polymerases ζ and Rev1 (7). To address whether the suppression of *rtt107*Δ DNA damage sensitivity by loss of Dot1 was similarly dependent on the TLS pathway, we constructed triple mutants lacking two main components of the TLS pathway as follows: Rev3 (catalytic subunit of polymerase ζ) or Rev1 (dC-transferase) (56). Lack of Dot1 did not suppress the MMS sensitivity of *rtt107*Δ mutants in the absence of Rev3 (Fig. 5*A*). Similarly, Rev1 was necessary for the reversal of *rtt107*Δ sensitivity by deletion of *DOT1*, suggesting that the *dot1*Δ suppression was dependent on the TLS pathway in general and not specifically on Rev3 (Fig. 5*B*). Very low concentrations of MMS were used in this assay due to the extreme MMS sensitivity of the triple mutants. It is interesting to note that both *dot1*Δ*rev3*Δ and *rtt107*Δ*rev3*Δ double

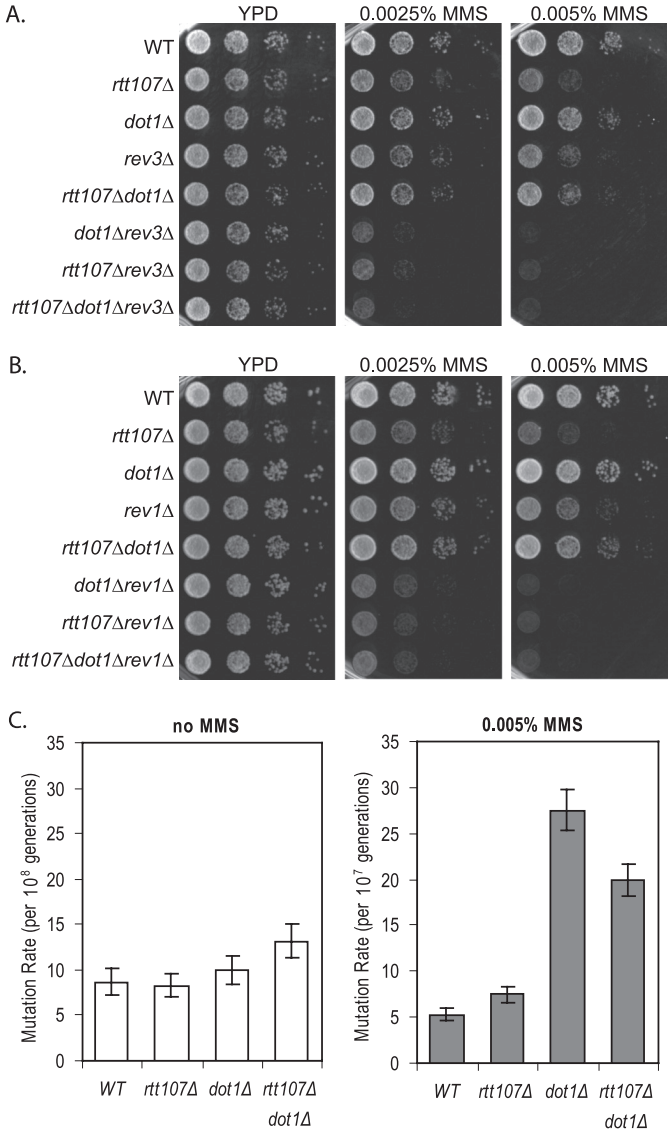

FIGURE 5. **Suppression of the *rtt107*Δ MMS sensitivity by deletion of *DOT1* was dependent on the TLS pathway.** Deletion of *REV3* or *REV1* in *rtt107*Δ*dot1*Δ mutants resulted in loss of the suppression. *rev3*Δ (*A*) or *rev1*Δ mutants (*B*) in combination with the indicated deletions of *RTT107* and/or *DOT1* were plated in 10-fold serial dilutions onto YPD containing 0.0025 or 0.005% MMS. *C,* loss of Dot1 resulted in increased mutation rates in presence of MMS. Mutation rates of indicated strains with and without 0.005% MMS were determined using 12 independent colonies. Mutation rates and 95% confidence intervals were calculated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator method.

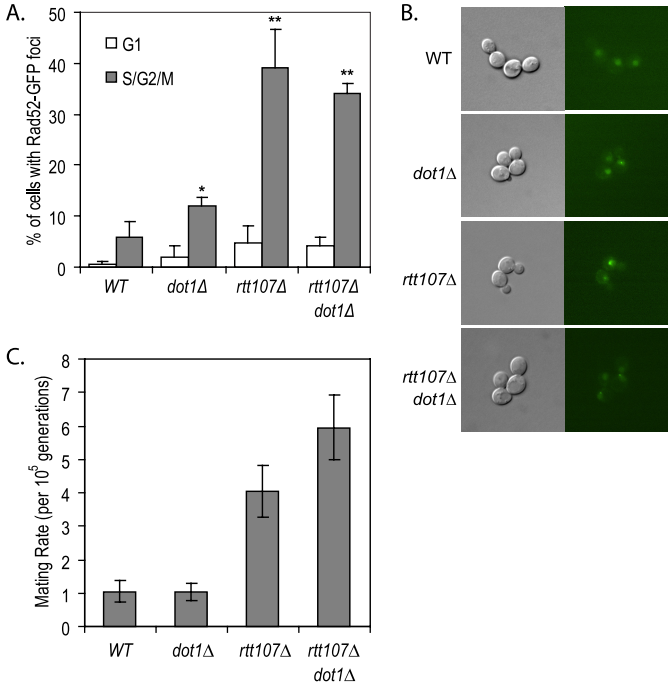

FIGURE 6. **Genomic instability of the *rtt107*Δ mutants was not suppressed by deletion of *DOT1*.** *A,* increased number of Rad52-GFP foci in *rtt107*Δ mutants was not suppressed in the absence of Dot1. The percentage of cells in G_1 or $S/G_2/M$ phase containing Rad52-GFP foci was calculated by dividing the number of cells in G_1 or $S/G_2/M$ phase containing Rad52-GFP foci by the total number of cells in G_1 or $S/G_2/M$, respectively. At least 200 cells were counted in three independent experiments. *Error bars* represent standard deviations of the values. *, $p < 0.05$; **, $p < 0.005$ when compared with the wild-type strain for the same cell cycle phase. *B,* representative differential interference contrast images are shown on the *left* and the corresponding GFP images on the *right*. *C,* deletion of *DOT1* did not suppress the increased loss of heterozygosity in *rtt107*Δ mutants. Mating rates of homozygous diploids of indicated strains were determined by using 12 independent colonies. Mating rates and 95% confidence intervals were calculated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator method.

mutants were very sensitive to MMS, and a similar phenotype was also observed for *dot1*Δ*rev1*Δ and *rtt107*Δ*rev1*Δ mutants. This indicated that a functional TLS pathway became more important for DNA damage resistance in the absence of Dot1 or Rtt107.

The TLS pathway is error-prone, and its activation would therefore be expected to cause an increased mutation rate. Using the *CAN1* forward mutagenesis assay, we observed a 4–5-fold increased mutation rate in the presence of 0.005% MMS in both *dot1*Δ and *dot1*Δ*rtt107*Δ mutants (Fig. 5*C*). Together, these results suggested that deletion of *DOT1* led to activation of the TLS pathway, thereby allowing the survival of *rtt107*Δ mutants.

Rtt107 Has Functions in Maintaining Genomic Integrity That Are Independent of Dot1 Activity—The role of Rtt107 in the maintenance of genome stability is not restricted to its specific function of restarting the cell cycle during S phase after DNA damage. During normal cell cycle progression, cells lacking Rtt107 have increased numbers of Rad52 and Ddc2 foci, indicative of spontaneous DNA damage and/or replication fork stalling (13, 15). Consistent with this, we observed that the percentage of cells with Rad52-GFP foci in $S/G_2/M$ phase was ~7-fold higher in *rtt107*Δ mutants than in wild type (Fig. 6, *A* and *B*). Deletion of *DOT1* in the *rtt107*Δ background did not significantly alter the number of cells containing Rad52-GFP foci. However, consistent with published data, we observed a 2-fold increase in the number of cells with Rad52-GFP foci in *dot1*Δ mutants, suggesting that *RTT107* was epistatic to *DOT1* in suppressing spontaneous DNA damage and/or replication fork stalling (7).

Further indicative of a broader role of Rtt107 in genome stability is the chromosome instability phenotype of *rtt107*Δ mutants (16). Compared with wild-type cells, *rtt107*Δ homozygous diploid mutants have a higher loss of heterozygosity at the *MAT***a** and *MAT*α loci, which is due to either enhanced mitotic recombination between homologous chromosomes, chromosome loss, rearrangement, or gene conversion (16). Using a quan-

titative version of the original Bimater screen used to define *RTT107* as a chromosome instability gene (57), we tested whether this phenotype was suppressed by loss of Dot1. Consistent with increased loss of heterozygosity, strains lacking Rtt107 had a 4-fold increase in mating rate when compared with wild-type or *dot1*Δ mutants (Fig. 6*C*). Deletion of *DOT1* did not rescue the chromosome instability phenotype of *rtt107*Δ mutants, but rather it resulted in a further increase in the mating rate to 6-fold as compared with wild type. Hence, it appeared that rather than rescuing the requirement for Rtt107 in preventing loss of heterozygosity, Dot1 cooperated with Rtt107 in this process.

DISCUSSION

In this study, we uncover a close functional relationship between chromatin and the cellular processes regulated by the BRCA1 C-terminal domain-containing protein Rtt107 and its interaction partner Slx4. Loss of Dot1, likely mediated by loss of histone H3 K79 trimethylation, suppressed the DNA damage sensitivity of *rtt107*Δ mutants through a mechanism that was dependent on the presence of a functional TLS pathway. The DNA damage-induced phosphorylation of Rtt107 and Slx4, which was mutually dependent, was not restored in the absence of Dot1. Furthermore, deletion of *DOT1* partially reversed the cell cycle progression and replication fork restart defect caused by the lack of Rtt107. In contrast, other genomic instability defects of *rtt107*Δ mutants were worsened or unaffected by loss of *DOT1*. Together, these data point to a complex functional relationship between Rtt107 and Dot1 in both the DNA damage response and preservation of genome integrity.

We propose a model to explain the inhibitory effect of H3 K79 trimethylation on growth during DNA damage conditions in yeast cells lacking Rtt107 or Slx4 (Fig. 7). Bre1-mediated H2B K123 ubiquitination is required for Dot1 to catalyze H3 K79 trimethylation, which in turn prevents *rtt107*Δ and *slx4*Δ mutants from surviving DNA damage conditions. This effect likely is mediated through inhibition of the TLS pathway by H3 K79 trimethylation, either directly or indirectly through a nexus to the Dot1-mediated DNA damage checkpoint. We favor a direct mechanism that could involve binding to the H3 K79 trimethylation mark by a protein that inhibits TLS. Alternatively, H3 K79 trimethylation might directly create a chromatin conformation that in some way is refractory to TLS. An indirect enhancement of TLS might be caused by the compromised DNA damage checkpoint due to loss of Dot1, allowing *rtt107*Δ mutants to survive DNA damage conditions. However, currently there is no evidence linking the roles of Dot1 in the TLS and DNA damage checkpoint. Moreover, UV exposure of DNA damage checkpoint-deficient mutants does not result in an increased mutation rate, thereby disfavoring a link between the DNA damage checkpoint and TLS (58). Finally, it is formally possible that the suppression is an indirect effect of an altered transcriptional response caused by lack of Dot1, Bre1, or H3 K79 methylation, which could involve reduced expression of an unknown inhibitor of the TLS. In any case, given our finding that the suppression of *rtt107*Δ phenotypes was linked to loss of trimethylation of H3 K79, it is tempting to speculate that specific genomic regions might be more prone to mediate this effect than others. This is supported by a genome-wide analysis showing that regions containing H3 K79 trimethylation are distinct from those containing H3 K79 dimethylation (38). Further studies are required to elucidate the precise mechanism whereby Dot1-mediated H3 K79 trimethylation inhibits the TLS pathway.

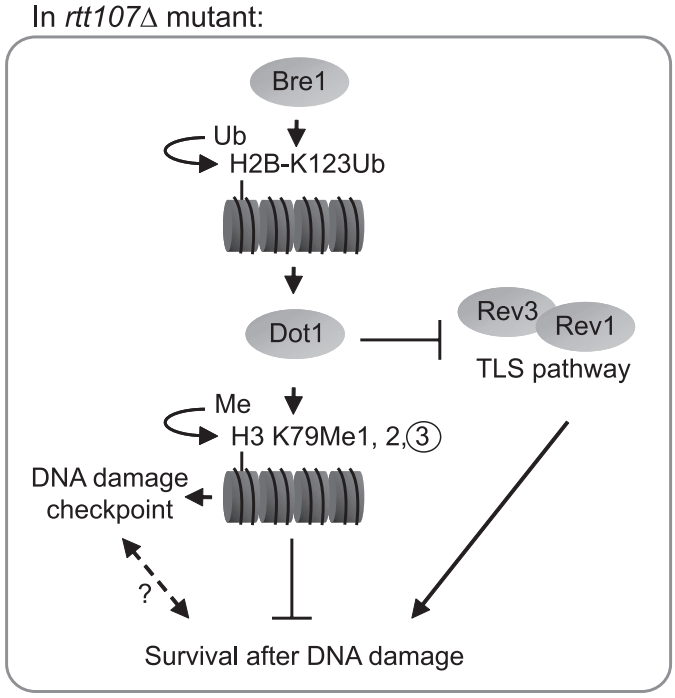

FIGURE 7. **Model for repressive effect of chromatin modifications on DNA damage survival in *rtt107*Δ mutants.** During DNA damage response, Bre1-mediated H2B K123Ub and by extension, Dot1-mediated H3 K79Me3, are required for checkpoint function. In *rtt107*Δ mutants, the presence of H3 K79Me3 is inhibitory to the yeast survival in DNA damage conditions. Loss of Dot1 increases the activity of the TLS pathway that bypasses the requirement of Rtt107 for cell survival. *Ub,* ubiquitin.

Our data showed that Mec1-mediated phosphorylation of Rtt107 was dependent on Slx4, consistent with earlier reports (13). We also showed that MMS-induced phosphorylation of Slx4 was dependent on Rtt107, suggesting a mutual requirement of these two proteins for their respective phosphorylation. Interestingly, neither Rtt107 nor Slx4 was phosphorylated in *slx4*Δ or *rtt107*Δ mutants, respectively, when *DOT1* was also deleted. Furthermore, the MMS sensitivity of a strain containing a nonphosphorylatable form of Rtt107 was rescued by deletion of *DOT1*. Together, this biochemical and genetic evidence suggested that DNA damage-dependent phosphorylation of Rtt107 is essential for resistance to MMS only when Dot1 is present to methylate H3 K79. Although it is not clear what mechanistic change is triggered by Rtt107 phosphorylation, it is likely to involve a DNA damage-induced protein-protein interaction. Whatever the mechanism might be, it is clear that Rtt107 phosphorylation in the DNA damage response becomes dispensable when H3 K79 trimethylation is inhibited.

The suppression of *rtt107*Δ by deletion of *DOT1* was restricted to situations of induced DNA damage, suggesting that the functional interaction between Rtt107 and Dot1 was context-dependent. Confirming published data from a high throughput screen for regulators of Rad52 foci formation, we found that loss of Rtt107 caused a significant increase in the

number of Rad52 foci-positive cells (15). In contrast to the suppression of MMS sensitivity of *rtt107*Δ mutants, this phenotype was not rescued by loss of Dot1. This suggested that Rtt107 had a role in preventing spontaneous DNA damage, likely caused by stalled DNA replication forks, which was not negatively regulated by H3 K79 methylation. Furthermore, our work uncovered additional evidence for a complex relationship between Dot1 and Rtt107 in the maintenance of genomic integrity. Rtt107 was required for chromosome stability, as determined by a genetic assay (16). Although loss of Dot1 alone did not affect chromosome stability, it enhanced the defect caused by loss of Rtt107. This suggested that in the absence of Rtt107, Dot1 plays a minor role in maintenance of chromosome stability. Taken together, these data point to multiple activities of Rtt107, where only those induced by external DNA-damaging agents were suppressed by deletion of *DOT1*. Presumably, both the increased number of Rad52 foci and the chromosome instability in *rtt107*Δ mutants were not suppressed by deletion of *DOT1* because the TLS pathway is unlikely to be activated in these conditions.

The suppression of DNA damage sensitivity by loss of Dot1 reported here is interesting in light of other findings suggesting certain chromatin modifications act as negative regulators of DNA replication, recombination, and repair. For example, the H3 K36 histone methyltransferase Set2 and the ATP-dependent chromatin remodeler Chd1 exert an inhibitory effect on DNA replication, as deletions of these genes suppress the hydroxyurea sensitivity of mutations in several genes involved in DNA replication (59). In addition, deletion of *CHD1* can suppress the lethality normally caused by disruption of the gene encoding either Mec1 or Rad53 DNA damage checkpoint kinases (59). The UV sensitivity and G_2/M checkpoint defects of *rad9*Δ and *mec1-21* mutants can be suppressed by loss of genes encoding components of the Rpd3/Sin3 histone deacetylase (60). Rpd3 and the aforementioned Set2 also repress meiotic recombination at the *HIS4* meiotic recombination hot spot (61). However, not all chromatin modifications involved in DNA metabolism exert a negative effect, as mutations in the genes encoding members of the histone acetyltransferase complex NuA4, the ATP-dependent chromatin remodelers RSC, Swi/Snf, SWR1-C, and INO80 cause sensitivity to MMS, as does loss of the histone variant H2A.Z or the Mec1-dependent phosphorylation targets in H2A (62, 63). Together, all these data suggest a complex and differentiated role for the chromatin template in DNA repair, recombination, and replication. Our work revealed that the role of Dot1 in the DNA damage response is multifaceted and extends to regulation of the TLS pathway and maintenance of chromosome stability, although the mechanisms are still unclear. The challenge of future research will be to uncover the intricate network between chromatin modifiers and DNA damage response effectors.

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