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Rmi1 functions in S phase-mediated cohesion establishment *via* a pathway involving the Ctf18–RFC complex and Mrc1

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ABSTRACT

Saccharomyces cerevisiae RecQ helicase (Sgs1) combines with DNA topoisomerase III (Top3) and RecQ-mediated genome instability 1 (Rmi1) to form an evolutionarily conserved complex that is required for processing homologous recombination intermediates and restarting collapsed replication forks. It was previously reported that Rmi1 contributes to sister chromatid cohesion; however, the underlying molecular mechanism has been unclear. In the present study, Rmi1 was found to be enriched at the region close to an early-firing replication origin when replication forks were arrested near their origins in the presence of hydroxyurea. Genetic analyses revealed that Rmi1 promoted sister chromatid cohesion in a process that was distinct from both the cohesion establishment pathway involving Ctf4, Csm3, and Chl1 and the pathway involving the acetylation of Smc3. On the other hand, Rmi1 seemed to function in a pathway involving the Ctf18–RFC complex and Mrc1, which were previously predicted to regulate leading-strand DNA replication.

1. Introduction

Sgs1 of *Saccharomyces cerevisiae* is a DNA helicase of the RecQ family, which is involved in maintaining the integrity of the genome. A human homolog of Sgs1, BLM, is the gene product responsible for Bloom syndrome [1], a rare autosomal recessive disorder characterized by severe growth retardation, immunodeficiency, reduced fertility, and a predisposition to various cancer types [2]. BLM associates with DNA topoisomerase IIIα (TOPOIIIα), RecQ-mediated genome instability 1 (RMI1, also known as BLAP75), and RMI2 to form a complex that functions coordinately to process a diverse array of DNA structures [3]. Sgs1 also interacts with Top3 and Rmi1. The Sgs1–Top3–Rmi1 complex has been suggested to function in damage-induced recombination, the resolution of recombination intermediates, the facilitation of end resection, and the restarting of stalled replication forks [4–8]. In addition, we previously found that Rmi1 and Top3 contribute to sister chromatid cohesion [9].

Sister chromatid cohesion, the mechanism that holds the two copies of sister chromatids together from the moment of duplication to the onset of anaphase, ensures that chromosome segregation occurs accurately during mitosis [10]. This process is mediated by the cohesin ring complex that, in budding yeast, consists of Smc1, Smc3, Scc1, and Scc3 (Fig. 1A and B) [10]. The cohesin ring complex is loaded onto chromatin by the Scc2/Scc4 complex in G_1 phase [10]. Establishment of sister chromatid cohesion requires an additional essential protein called Eco1, an acetyltransferase that acetylates Smc3 during S phase (Fig. 1A and B) [10]. Acetylated Smc3 counteracts the antiestablishment activity of Wap1, which interacts with cohesin complex [10,11]. Sister chromatid cohesion is maintained through the action of Pds5 until the onset of anaphase, when the cohesin rings are cleaved by separase, which leads to the separation of the sister chromatids [10]. In addition to the cohesin complex and Eco1, an increasing number of proteins have been found to contribute to the establishment of sister chromatid cohesion [12–15]. Genetic analyses have revealed the existence of two distinct cohesion establishment pathways: one pathway includes the Ctf18–Ctf8–Dcc1 complex and Mrc1, whereas the other includes Ctf4, Tof1–Csm3, and Chl1 (Fig. 1A) [16].

Ctf18, Ctf8, and Dcc1 form an alternative replication factor C (RFC) complex with Rfc2-Rfc5 (the Ctf18–RFC complex), catalyze loading and unloading of the sliding clamp PCNA on DNA *in vitro*, and recruit PCNA to replication forks in the cell in early S phase [17,18]. The Ctf18–RFC complex interacts with DNA polymerase ε

Abbreviations: HU, hydroxyurea; ChIP, chromatin immunoprecipitation; WT, wild type.

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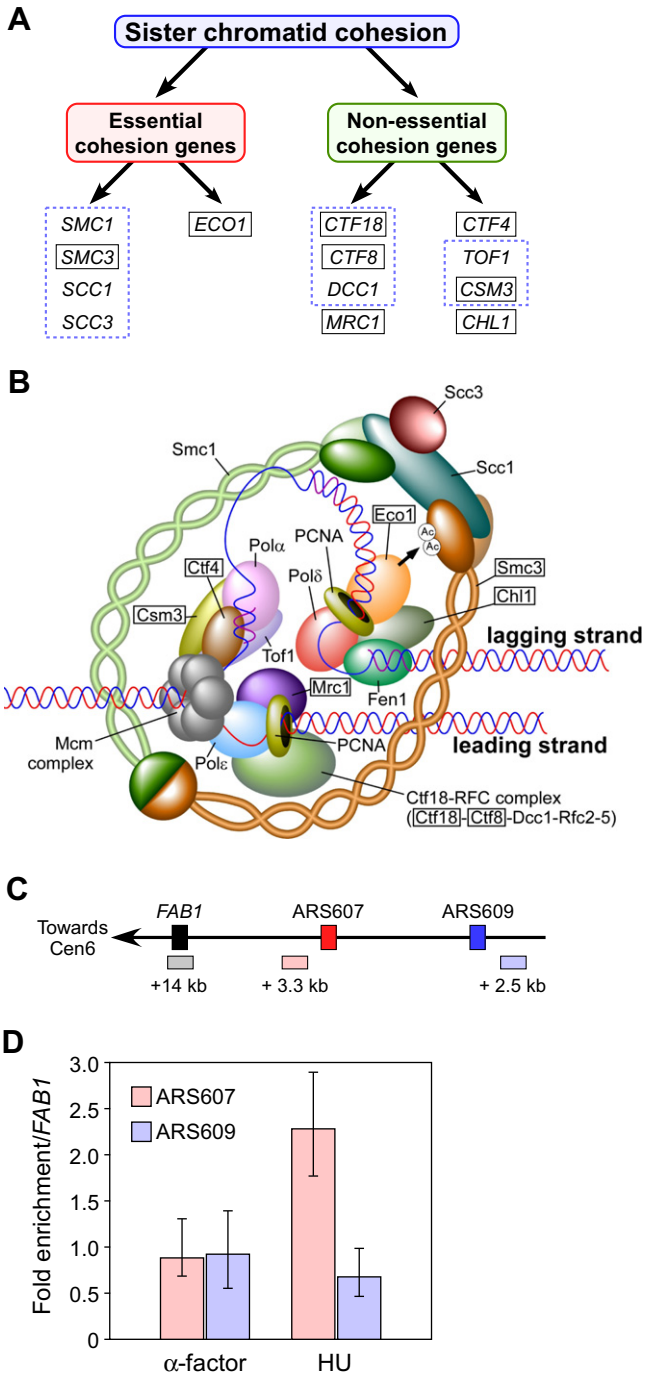

Fig. 1. Rmi1 exists near replication forks. (A) Genes involved in sister chromatid cohesion. Individual gene roles are described in the text. Enclosed genes were characterized in this study. Genes enclosed by the dashed line encode proteins to form a complex. (B) Replisome and the cohesin ring complex. Enclosed proteins were characterized in this study. (C) Schematic representation of the regions amplified by quantitative real-time PCR. Primer pairs spanning regions +3.3 kb from the early-firing replication origin ARS607 (active in HU arrest) and +2.5 kb from the late-firing replication origin ARS609 (inactive in HU arrest) were used to analyze the ChIP samples [18]. *FAB1* (+14 kb from ARS607) was used as a control locus. (D) Association of Rmi1 with replication fork. ChIP analysis was conducted with wild-type cells bearing a genomic copy of Rmi1–13Myc. Cells were synchronized in G_1 phase with α-factor and released into 0.2 M HU. ChIP samples were taken after incubation for 0 and 60 min in 0.2 M HU and analyzed by quantitative real-time PCR for loci close to ARS607 and ARS609. The Rmi1–13Myc signal intensity is plotted as fold enrichment over the control locus *FAB1*. Error bars represent the standard deviations of three independent experiments.

[19], which is responsible for leading-strand DNA replication [20], and suppresses its activity *in vitro* [19]. Mrc1, which is reported to promote normal replication fork progression [21], also interacts with DNA polymerase ε (Fig. 1B) [22]. On the other hand, Ctf4 directly interacts with DNA polymerase α [23], which is involved in the synthesis of Okazaki fragments during lagging strand replication. The Tim–Tipin complex, which is a vertebrate counterpart of the Tof1–Csm3 complex, interacts with the human Ctf4 counterpart AND1. The Tim/Tipin/AND1 complex promotes the binding of DNA polymerase α to chromatin [24]. Furthermore, budding yeast Chl1 aids in the establishment of sister chromatid cohesion [25]. ChlR1, the human ortholog of Chl1, associates with hFen1 [26], which is known to process Okazaki fragments [27]. Thus, two genetically distinct cohesion establishment pathways, one involving the Ctf18–RFC complex (including Ctf18, Ctf8, and Dcc1) and Mrc1, and the other involving Ctf4, the Tof1–Csm3 complex, and Chl1, seem to deal with the replisome, mainly in the context of leading and lagging strand DNA replication, respectively (Fig. 1B).

We previously found that the deletion of *RMI1* or *TOP3* caused a moderate defect of sister chromatid cohesion, and deletion of *SGS1* partially suppressed cohesion defects in *top3* and *rmi1* mutants [9]. Based on these findings, we proposed the existence of a new pathway involving Sgs1–Top3–Rmi1 that led to the proper establishment of sister chromatid cohesion [9]. To understand the pathway involving Sgs1–Top3–Rmi1 better, we focused on Rmi1. Because the relationship between Rmi1 and the cohesion establishment factors (Eco1, Ctf18, Ctf8, Mrc1, Ctf4, Csm3, and Chl1) has remained elusive, in the present study, we sought to determine whether Rmi1 functions in known pathways involving these proteins or in a novel, as-yet-unreported pathway.

2. Methods

2.1. Yeast strains and culture medium

All of the strains used in this study are listed in Supplementary Tables 1 and 2. Mutants were constructed by standard yeast genetic methods. Yeast strains were cultured in yeast extract/peptone/dextrose (YPD) medium with adenine.

2.2. Spot assay

Log-phase cells grown in YPD were harvested, washed once in distilled water, counted, and diluted. Ten-fold serial dilutions of cells (10^5, 10^4, 10^3, and 10^2 cells) were spotted onto YPD plates. The plates were incubated at the indicated temperature for 3 days, and the cells were photographed.

2.3. Sister chromatid cohesion assay

Tet repressor-GFP/Tet operator repeat strains were arrested in G_2/M phase with 15 μg/mL nocodazole or in G_1 phase with 5 μg/mL α-factor for 5 h at 30 °C. The cells were fixed with 3.7% formaldehyde for 15 min, washed once with SK solution (1 M sorbitol, 0.05 M K_2PO_4), and resuspended in 50 μL of SK solution. Cohesion assessment was performed with the Carl Zeiss Axiovert 200 M microscope.

2.4. Chromatin immunoprecipitation and quantitative PCR

Chromatin immunoprecipitation (ChIP) was performed essentially as described previously [28]. Briefly, cells were harvested and incubated in 1% formaldehyde for 15 min to crosslink proteins

to DNA, and the reaction was quenched by incubating cells in 125 mM glycine for 5 min. Cells were lysed with glass beads, and extracts were sonicated to shear DNA to an average size of 0.5 kb. Extracts were divided into two aliquots of input DNA and immunoprecipitation (IP) DNA, at a ratio of 1:20. Immunoprecipitation was carried out with a monoclonal anti-Myc antibody (9E10) (Santa Cruz Biotechnology, Inc.), and immune complexes were captured by Dynabeads Protein G (Dynal Biotech) for 4 h at 4 °C. After a series of washes, proteins were released from the beads by incubation for 6 h at 65 °C and treated with Proteinase K. The DNA was purified, and precipitated DNA was quantified with real-time PCR with SYBR green (TaKaRa Thermal Cycle Dice Real Time System). The sequence information of the primers used is available upon request.

3. Results and discussion

3.1. Rmi1 exists near or in replication forks

The proteins involved in the establishment of sister chromatid cohesion, such as Eco1, Ctf18, Ctf8, Mrc1, Ctf4, and Csm3, reportedly exist close to replication forks, at least when cells are arrested in early S phase by hydroxyurea (HU) (Fig. 1B) [18]. In the establishment of cohesion, our previous results suggested that the Top3–Rmi1 complex functions downstream of Sgs1 [9], which was also shown to exist in replication forks [7]. Before investigating the role of Rmi1 in sister chromatid cohesion, we first asked whether Rmi1 exists near or in replication forks like the other proteins listed above.

We monitored the existence of Rmi1 in the region close to the replication origins in HU-treated cells by ChIP. Cells carrying a genomic copy of Rmi1–13Myc were arrested in G_1 phase with α-factor and then released from G_1 arrest into medium containing 0.2 M HU, to arrest the cells in early S phase. Samples prepared from both G_1 and early S phase-arrested cells were analyzed for the association of Rmi1 with DNA close to the early-firing origin, ARS607, and the late-firing origin, ARS609 (Fig. 1C).

In G_1 phase-arrested cells, the level of Rmi1 close to ARS607 was comparable to that found in the region close to ARS609 (Fig. 1D). However, in early S phase-arrested cells, Rmi1 was enriched in the region close to ARS607 relative to the region close to ARS609 (Fig. 1D). This finding suggests that Rmi1 possibly acts together with these factors (Eco1, Ctf18, Ctf8, Mrc1, Ctf4, and Csm3) at the replication forks. Therefore, we next examined whether Rmi1 functions in known pathways involving these proteins or in a novel pathway that has not yet been addressed.

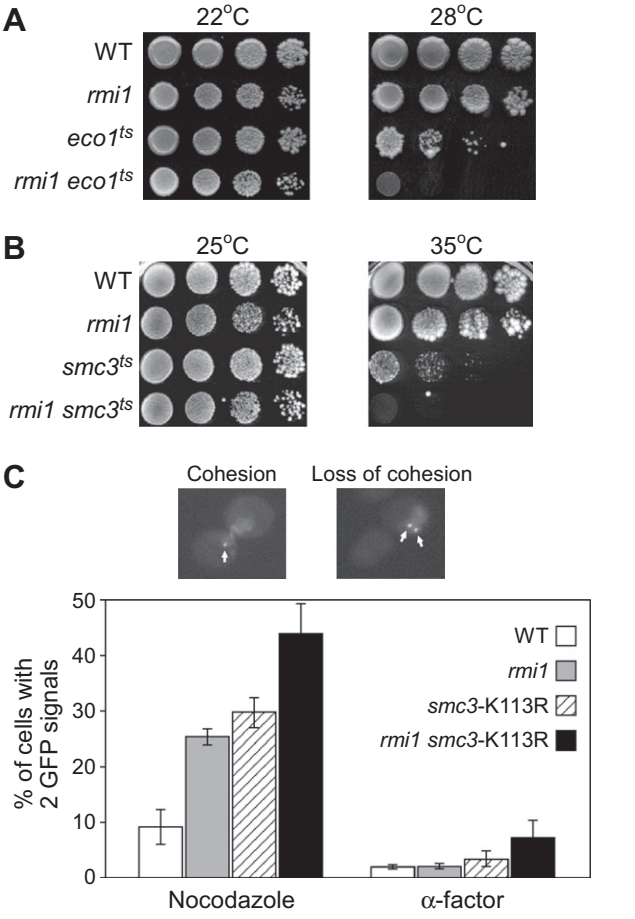

Fig. 2. Rmi1 contributes to cohesion establishment in a process other than Smc3 acetylation. (A) *RMI1* genetically interacts with *ECO1*. Ten-fold serial dilutions of log-phase cultures of wild-type (WT), *rmi1*, *eco1^{ts}*, and *rmi1 eco1^{ts}* cells were spotted onto growth plates, incubated at 22 or 28 °C for 3 days, and photographed. (B) *RMI1* genetically interacts with *SMC3*. Ten-fold serial dilutions of log-phase cultures of WT, *rmi1*, *smc3^{ts}*, and *rmi1 smc3^{ts}* cells were spotted onto growth plates, incubated at 25 or 35 °C for 3 days, and photographed. (C) The *rmi1* cells exhibit additive cohesion defects when combined with *smc3-K113R*. Upper panel: micrographs of *rmi1 smc3-K113R* cells in which the sister chromatid locus was visualized at Tet repressor-GFP/Tet operator repeats. The presence of one GFP signal focus indicates that the sister chromatids are properly adhered to each other, whereas two distinct GFP foci indicate that the sister chromatids are prematurely separated. Lower panel: wild-type, *rmi1*, *smc3-K113R*, and *rmi1 smc3-K113R* cells were arrested in M phase with nocodazole or in G_1 phase with α-factor, and then fixed with formaldehyde. One hundred cells of each strain were scored for the number of cells with two GFP signal foci. Error bars represent the standard deviations of three independent experiments.

3.2. Role of Rmi1 is genetically distinct from Eco1-mediated Smc3 acetylation

Eco1 acetylates the head domain of Smc3, a subunit of cohesin (Fig. 1B) [29]. To examine whether Rmi1 functions in this known pathway for the establishment of cohesion (Eco1-mediated Smc3 acetylation), we evaluated the genetic interactions between *RMI1* and *ECO1* and between *RMI1* and *SMC3* (Fig. 1A). In the case of Eco1, the *rmi1*, *eco1^{ts}*, and *rmi1 eco1^{ts}* cells grew similarly well at 22 °C. In contrast to the single mutants, *rmi1 eco1^{ts}* cells were synthetic lethal at 28 °C, the semipermissive temperature for *eco1^{ts}* cells (Fig. 2A). In the case of Smc3, yeast strains harboring mutations in *RMI1*, *SMC3*, or both were spotted onto YPAD plates and incubated at either 25 or 35 °C. The *rmi1 smc3^{ts}* cells showed synthetic lethality at 35 °C, the semipermissive temperature for *smc3^{ts}* cells (Fig. 2B). These results suggest that Rmi1 functions in a distinct process involving Eco1 and Smc3.

To confirm that Rmi1 functions in a process other than the acetylation of Smc3, we utilized a yeast mutant harboring nonacetylable Smc3. Yeast Smc3 molecules are acetylated *in vivo* at two conserved lysine (K) residues, i.e., K112 and K113, by Eco1 during S phase [29]. Preventing Smc3 acetylation by mutating lysine 113 to arginine (K113R) caused the cells to display pronounced defects in sister chromatid cohesion [29]. We performed cohesion analysis (Fig. 2C) in *rmi1*, *smc3-K113R*, and *rmi1 smc3-K113R* cells. Cells were arrested in M phase with nocodazole and in G_1 phase with α-factor and fixed, and scored for the number of cells with two GFP signal foci. As reported [9,28], two GFP signal foci appeared frequently in M-phase cells carrying either the *rmi1* or the *smc3-K113R* mutation (~25–30%; Fig. 2C). In the presence of both mutations, cohesion defects were further increased (~45%; Fig. 2C). Therefore, Rmi1 seems to function in a process other than the acetylation of Smc3 during the establishment of sister chromatid cohesion.

3.3. Rmi1 promotes sister chromatid cohesion via a pathway involving Ctf18, Ctf8, and Mrc1

Nonessential cohesion proteins contributing to the establishment of sister chromatid cohesion have been classified into two cohesion pathways, one pathway including Ctf18, Ctf8, and Mrc1, and one pathway including Ctf4, Csm3, and Chl1 (Fig. 1A) [16]. To examine whether Rmi1 functions in these two known pathways, the relationship between Rmi1 and Ctf18, Ctf8, or Mrc1 in the establishment of cohesion was tested. Deletion of *RMI1*, *CTF18*, *CTF8*, or *MRC1* caused cohesion defects in approximately 25–30% of cells exhibiting two GFP signal foci (Fig. 3A–C), as reported. Intriguingly, the *rmi1* cells showed similar levels of cohesion defects even when the *CTF18*, *CTF8*, or *MRC1* gene was further mutated. These results indicate that Rmi1 contributes to the same cohesion pathway in which Ctf18, Ctf8, and Mrc1 function.

3.4. Rmi1 functions in a pathway distinct from the pathway involving Ctf4, Csm3, and Chl1

The roles of Ctf4, Csm3, and Chl1 in the establishment of sister chromatid cohesion are genetically different from those of Ctf18, Ctf8, and Mrc1 (Fig. 1A). Because Rmi1 belongs to the pathway involving Ctf18, Ctf8, and Mrc1 (Fig. 3), Rmi1 is predicted to follow a pathway that is genetically different from the one involving Ctf4, Csm3, and Chl1. As expected, the frequencies of the two GFP signal foci in M phase-arrested *rmi1 ctf4*, *rmi1 csm3*, or *rmi1 chl1* double mutant cells were substantially higher than those in the corresponding single mutants (Fig. 4A–C), suggesting that Rmi1 functions in a cohesion pathway that is distinct from that involving Ctf4, Csm3, and Chl1. Considering all of the data in Figs. 2–4, we conclude that Rmi1 is clearly demonstrated to be a member of the Ctf18–RFC complex and Mrc1 cohesion establishment pathway.

3.5. Possible roles of Rmi1 in sister chromatid cohesion

Biochemical data indicate that Rmi1 and Top3 function as a complex and cooperate with Sgs1 [5]. We previously showed that Rmi1 and Top3 function downstream of Sgs1 to establish sister chromatid cohesion [9]. How do these proteins contribute to the establishment of sister chromatid cohesion?

Sister chromatid cohesion is established during S phase, presumably through the passage of the replication fork. One of the most commonly accepted models for the establishment of sister chromatid cohesion is that one cohesin ring entraps two sister

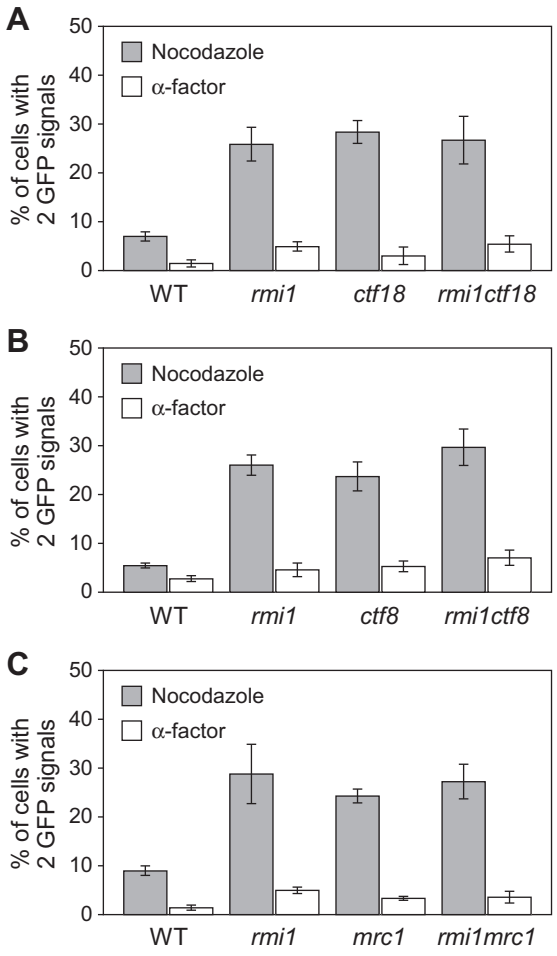

Fig. 3. Rmi1 functions in the cohesion pathway involving Ctf18, Ctf8, and Mrc1. (A–C) The *rmi1* cells exhibit no increase in cohesion defects when combined with a mutation in *CTF18* (A), *CTF8* (B), or *MRC1* (C). Cohesion assays were performed with the indicated strains and the same conditions described in Fig. 2C. For each strain, 100 cells were scored for the number of cells with two GFP signal foci. Error bars represent the standard deviations of three independent experiments.

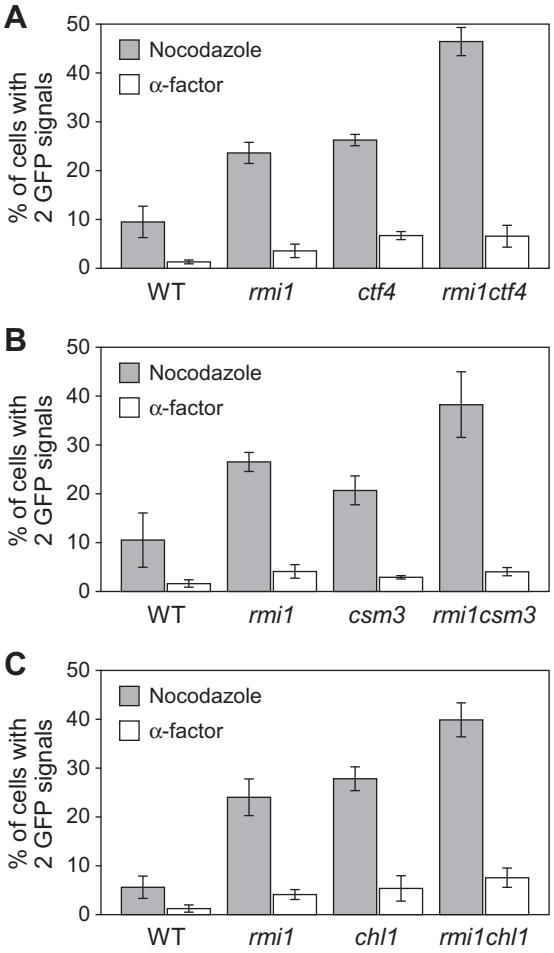

Fig. 4. Rmi1 functions in parallel with the cohesion pathway involving Ctf4, Csm3, and Chl1. (A–C) The *rmi1* cells exhibit additive cohesion defects when combined with a mutation in *CTF4* (A), *CSM3* (B), or *CHL1* (C). Cohesion assays were performed with the indicated strains. Cells were arrested in M phase with nocodazole or in G_1 phase with α-factor, and then fixed with formaldehyde. For each strain, 100 cells were scored for the number of cells with two GFP signal foci. Error bars represent the standard deviations of three independent experiments.

DNA molecules [10]. Because the Eco1-mediated acetylation of Smc3 (a subunit of cohesin) counteracts the inhibitory activity of Wap1 for the establishment of sister chromatid cohesion, acetylated Smc3 is required for maintaining the established status of sister chromatid cohesion [11]. The results of the present study suggest that this Eco1-Smc3 pathway operates independently of Rmi1 function (Fig. 2).

Although the cohesin ring is large enough to encircle the two sister DNA molecules associated with histones, it does not seem to be large enough to allow passage of the replisome, which performs both leading and lagging strand DNA replication [30]. When the replication fork encounters cohesin, leading and lagging strand DNA replication should temporarily be uncoupled by modulation of the replisome, such that the replication fork is able to pass through the cohesin ring. In this context, the two cohesion establishment pathways, one involving Ctf18–RFC complex and Mrc1 and the other involving Ctf4, Csm3, and Chl1, are expected to modulate leading and lagging strand DNA replication, respectively (Fig. 1B). Because Rmi1 acts in the pathway involving Ctf18, Ctf8, and Mrc1 (Fig. 3), but not in the pathway involving Ctf4, Csm3, and Chl1 (Fig. 4), Rmi1 seems to have some roles related to leading strand DNA replication during the establishment of sister chromatid cohesion. Interestingly, a large-scale genetic network analysis also identified similarities between the genetic interaction profiles of Rmi1 and Ctf18 [31], suggesting that our results are consistent with the hypothesis that these two proteins function in similar cellular processes.

Because Sgs1 translocates in the 3′–5′ direction in single-stranded DNA where it binds [32], Sgs1 may potentially modulate the template of leading-strand DNA replication. Sgs1 and Top3 are reported to be involved in template switching and are thought to contribute to replisome stability and recovery from arrested replication forks [33]. Because Rmi1 functions as a complex with Top3, Rmi1 likely also functions in the above processes. However, the exact mechanism of action of Rmi1 with respect to the establishment of sister chromatid cohesion remains elusive and will be the subject of future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.124.

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