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Chl1 and Ctf4 are required for damage-induced recombinations

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Abstract

Deletion mutants of *CHL1* or *CTF4*, which are required for sister chromatid cohesion, showed higher sensitivity to the DNA damaging agents methyl methanesulfonate (MMS), hydroxyurea (HU), phleomycin, and camptothecin, similar to the phenotype of mutants of *RAD52*, which is essential for recombination repair. The levels of Chl1 and Ctf4 associated with chromatin increased considerably after exposure of the cells to MMS and phleomycin. Although the activation of DNA damage checkpoint did not affected in *chl1* and *ctf4* mutants, the repair of damaged chromosome was inefficient, suggesting that Chl1 and Ctf4 act in DNA repair. In addition, MMS-induced sister chromatid recombination in haploid cells, and, more importantly, MMS-induced recombination between homologous chromosomes in diploid cells were impaired in these mutants. Our results suggest that Chl1 and Ctf4 are directly involved in homologous recombination repair rather than acting indirectly *via* the establishment of sister chromatid cohesion.

Keywords: Chl1; Ctf4; Genome stability; Recombination; Repair; Sister chromatid cohesion

During the course of DNA replication, two identical copies of each chromosome, the sister chromatids, form and serve as templates for homologous recombination [1]. The sister chromatids are physically linked by cohesin, which holds them together until the metaphase-to-anaphase transition [2]. The cohesin complex of budding yeast, which consists of Smc1, Smc3, Scc1, and Scc3, forms a ring-like structure [3]. The cohesin complex holds sister chromatids together by trapping the sister DNA molecules within its ring.

DNA is often damaged by endogenous or exogenous stress, and repair of these damages is fundamental for cell survival and genomic stability. Cohesin mutants have decreased viability in response to DNA damage. Inactivation of cohesin in S or M phase reduces the efficiency of DNA repair [4], and cohesin is loaded on chromatin during G_1 phase and sister chromatid cohesion is established during S phase. In addition, cohesin is newly recruited around damaged DNA [5,6]. Thus, there seems to be overlap in the mechanism of sister chromatid cohesion and efficient DNA repair.

Mutations in *CTF4* and *CHL1* also cause a partial defect in the establishment of sister chromatid cohesion. *CHL1* and *CTF4* were originally identified by virtue of the decreased chromosome transmission fidelity or chromosome loss caused by their mutation. Mutations in *CHL1* and *CTF4* result in increased chromosome loss, sister chromatid nondisjunction, and mitotic recombination [7–9]. The human CHL1 protein (CHLR1) exhibits DNA helicase activity, binding both single- and double-stranded DNA [10]. Ctf4 protein binds DNA polymerase α *in vitro* [11]. *CHL1* and *CTF4* are required for efficient sister chromatid cohesion in cycling mitotic cells and for cohesion between sister centromeres during meiosis [7–9,12]. *ctf4* mutants are sensitive to MMS [13], although the mechanisms have not been addressed yet.

In this study, we investigated the role of budding yeast Chl1 and Ctf4 in DNA repair. We show that Chl1 and Ctf4 are loaded onto damaged chromatin and function not only in damage-induced sister chromatid recombina-

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tion but also in damage-induced recombination between homologous chromosomes, indicating that they are directly involved in the repair processes rather than having an indirect effect due to their function in establishing sister chromatid cohesion.

Materials and methods

Yeast strains. Construction of yeast strains are described in Appendix A (Supplemental Materials and methods). The yeast strains used in this study are listed in Supplementary Table S1.

Survival analysis following DNA damage. For survival analysis under continuous exposure to DNA damaging agents, 10-fold serial dilutions with distilled water of logarithmically growing cells (constructed as described above) were spotted onto YPAD plates or YPAD plates containing the indicated concentrations of methyl methanesulfonate (MMS), hydroxyurea (HU), phleomycin, or camptothecin. The plates were incubated for 3 days at 30 °C and then photographed.

Detection of Rad53 modification. To detect modification of Rad53-13Myc in response to DNA damage, logarithmically growing cells were exposed to 0.1% MMS. After 2 h, the cells were harvested for immunoblotting analysis. The proteins were detected by immunoblotting with an anti-Myc antibody (9E10).

Assay for sister chromatid recombination (SCR) frequency and recombination frequency between homologous chromosomes carrying heteroalleles. The strains constructed for detecting unequal SCR were described previously [14]. Logarithmically growing cells were inoculated onto SC-His plates and YPAD plates containing various concentrations of MMS to evaluate the incidence of sister chromatid recombination and colony forming units, respectively. For measuring hetroallelic recombination, diploid strains with the MR101 background were constructed such that recombination between the heteroalleles *his1-1* and *his1-7* could be detected by the restoration of histidine prototrophy, as described previously [15].

Pulsed-field gel electrophoresis. Logarithmically growing cells were exposed to 0.1% MMS for 1 h at 30 °C, washed to remove the MMS, and then cultured at 30 °C in YPAD medium for the indicated periods of time. Agarose plugs containing chromosomal DNA were prepared as described previously [14].

Cell fractionation. Whole cell extracts (WCE) and chromatin pellets (ChP) were prepared as described previously [16]. WCE were centrifuged through a sucrose cushion, and the resulting pellet (chromatin-containing fraction; ChP) was examined by immunoblot analysis for the presence of Chl1-13Myc or Ctf4-13Myc. Histone H3 was used as a loading control for protein levels in the WCE and ChP. The proteins were detected by immunoblotting with an anti-Myc antibody (9E10) or anti-histone H3 antibody (Abcam).

Cohesion assay. The *CHL1* and *CTF4* genes were deleted from the strain YPH1477, which expresses a Tet repressor-GFP fusion protein and contains a Tet operator repeat integrated 35 kb from the centromere of chromosome V [17]. Cells were grown logarithmically in YPAD medium and them arrested in G2/M phase by incubating in the presence of 15 μg/ml nocodazole for 3 h at 30 °C. Cells were treated with 0.1% MMS and then fixed by incubation with an equal volume of 4% paraformaldehyde for 30 min, washed once with SK (1 M sorbitol, 0.05 M K_2PO_4), and resuspended in 50 μl SK for cohesion assessment.

Results and discussion

chl1, ctf4, and rad52 cells are sensitive to a spectrum of DNA damage

Cohesin is involved in the linkage of sister chromatid around damaged DNA [5,6], suggesting that sister chromatid cohesion is important for DNA repair. Chl1 and Ctf4 are not included in the cohesin complex but are related to sister chromatid cohesion. To elucidate the repair function of cohesion in more detail, we investigated the relationship of Chl1 and Ctf4 to DNA repair. We first examined the sensitivity of *chl1* and *ctf4* cells to the DNA-damaging agents methyl methanesulfonate (MMS), hydroxyurea (HU), phleomycin, and camptothecin. *ctf4* cells were highly sensitive like as *rad52* cells and *chl1* cells were moderately sensitive to these DNA damaging agents, as compared to wild-type cells (Fig. 1A).

Chl1 and Ctf4 associate with damaged chromatin

Sister chromatid cohesion is established during S phase and not in G_2 phase [2], but DNA damage activates a pathway that leads to loading of cohesin onto the damaged sites and to *de novo* establishment of sister chromatid cohesion even in G_2/M phase [5,6]. To examine whether Chl1 and Ctf4 bind to damaged DNA, we analyzed the association of Chl1 and Ctf4 with chromatin after the induction of DNA damage by MMS. The amount of Chl1 and Ctf4 in the chromatin-containing fraction increased notably after exposure of cells to MMS (Fig. 1B). Moreover, the association of Chl1 and Ctf4 with chromatin was observed even in G_2/M phase-arrested cells after the induction of DNA damage (Supplementary Fig. S1).

chl1 and ctf4 mutant cells have defects in DNA repair

Sensitivity of *chl1* and *ctf4* cells to DNA damaging agents suggests that these mutants have defects in DNA damage checkpoints or DNA repair. To test the former possibility, we examined the phosphorylation of Rad53, which is a hallmark of the activation of the damage checkpoint [18]. The phosphorylated form of Rad53 appeared in *chl1* and *ctf4* cells as well as in wild-type cells upon exposure to MMS (Fig. 1C), suggesting that Chl1 and Ctf4 are not essential for activation of the DNA damage checkpoint.

We next monitored the formation and subsequent repair of DNA damage after exposure to MMS in *chl1* and *ctf4* cells by pulsed-field gel electrophoresis (PFGE). Logarithmically growing cells were treated with MMS and then cultured in MMS-free medium. Chromosomal DNA was isolated from these cells and subjected to PFGE (Fig. 1D). Distinct chromosomal DNA bands were absent just after exposure to MMS in *chl1* and *ctf4* cells as well as wild-type cells and, concurrently, a low-molecular-weight DNA smear appeared. Restoration of chromosome-sized DNA bands was observed in wild-type cells after culture in MMS-free medium. However, restoration of chromosome-sized DNA was impaired in *chl1* and *ctf4* cells. Since strand breaks reportedly occur in MMS-treated DNA during heat treatment of PFGE plugs, the amount of fragmented DNA does not necessarily reflect the level of DNA double strand breaks [19]. However, the appearance

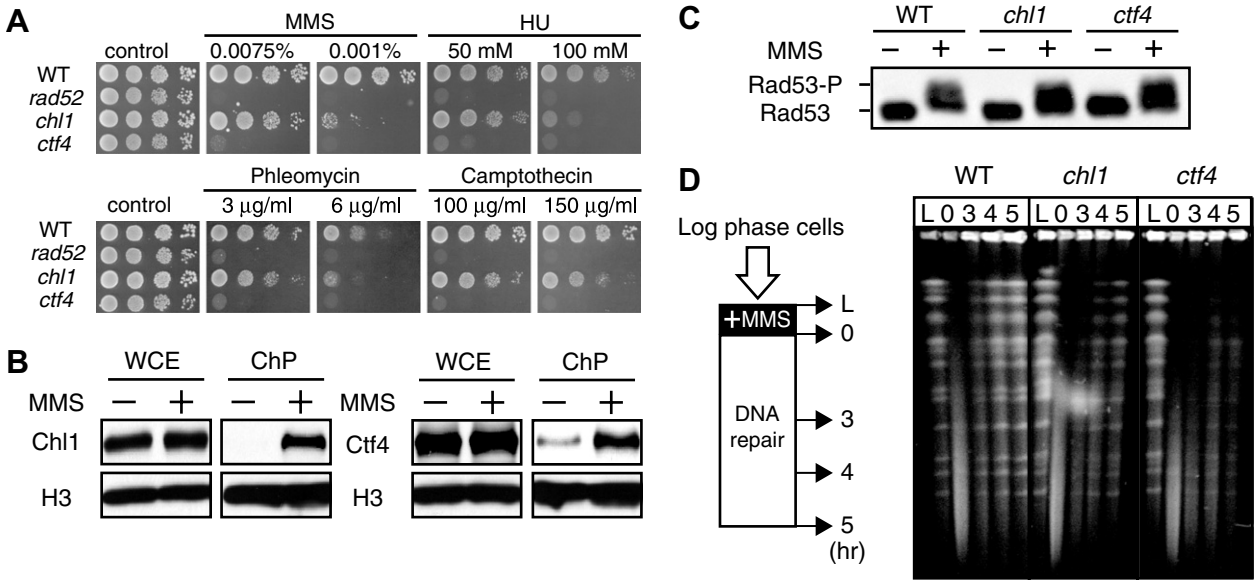

Fig. 1. Chl1 and Ctf4 in DNA repair. (A) Sensitivity to various DNA damaging agents. Wild-type (SCRL), *rad52* (YHO602), *chl1* (YHO603) and *ctf4* (YHO604) cells were inoculated on YPAD plates containing the indicated concentrations of methyl methanesulfonate (MMS), hydroxyurea (HU), phleomycin, or camptothecin. The plates were incubated for 3 days at 30 °C and then photographed. (B) Chromatin binding of Chl1 and Ctf4 after exposure to MMS. Logarithmically growing cells expressing Chl1-13Myc (YHO509) and Ctf4-13Myc (YHO510) were exposed to 0.1% MMS for 2 h. Aliquots of the cultures were taken before (−) and after (+) exposure to MMS. Whole cell extracts (WCE) and chromatin pellets (ChP) were prepared and analyzed by immunoblotting for the presence of the indicated proteins. Histone H3 was used as a loading control for protein levels. The Myc-tagged proteins and histone H3 were detected using an anti-Myc monoclonal antibody and a specific anti-histone H3 antibody, respectively. (C) Modification of Rad53 in response to DNA damage. Wild-type (YHO302), *chl1* (YHO306) and *ctf4* (YHO307) cells expressing Rad53-13Myc were grown to logarithmic phase in YPAD medium and then exposed to 0.1% MMS for 2 h at 30 °C. Aliquots of the cultures were taken before (−) and after (+) exposure to MMS for 2 h. Proteins prepared from these cells were subjected to SDS–PAGE. The Rad53 protein was detected by Western blotting using an anti-Myc antibody. (D) Defect in DNA repair in *chl1* and *ctf4* cells monitored by PFGE. Logarithmically growing wild-type (MR101), *chl1* (YHO216) and *ctf4* (YHO217) cells were exposed to 0.1% MMS for 1 h, and then cultured in MMS-free medium for the indicated periods of time. The cells were harvested, and their DNA was analyzed by PFGE as described in the Materials and methods. Lane L, untreated cells; lane 0, cells treated with MMS for 1 h; lanes 3–5, cells treated with MMS for 1 h, then cultured in MMS-free medium for 3, 4, and 5 h, respectively.

of chromosome-sized DNA bands reflects the repair of DNA breaks. Thus, we may conclude that MMS-induced lesions are not efficiently repaired in *chl1* and *ctf4* cells.

Chl1 and Ctf4 are required for MMS-induced sister chromatid recombination

The responses of *chl1* and *ctf4* to a spectrum of DNA-damaging agents were similar to those of *rad52* (Fig. 1A) but not y*ku70* (data not shown). Rad52 and Yku70 are a main component for homologous recombination and non-homologous end joining (NHEJ), respectively. As expected, using an assay system measuring NHEJ activity at *MAT* locus [20] (Fig. 2A, panel a), we observed no severe defect in NHEJ in *chl1* and *ctf4* cells (Fig. 2A, panel b). Thus, Chl1 and Ctf4 are not important for the repair by NHEJ. Next, we examined whether Chl1 and Ctf4 are involved in recombination repair. We measured the frequency of unequal sister chromatid recombination (uSCR) in haploid cells (Fig. 2B, panel a). The frequency of uSCR in wild-type cells increased with increasing concentrations of MMS. Although *chl1* cells showed an increased frequency of spontaneous uSCR the recombination frequency did not increase in the both mutants (Fig. 2B, panel b). These results suggest that Chl1 and Ctf4 are involved in the recombination between sister chromatids that is induced by exposure to MMS. Since sister chromatid recombination is affected by status of sister chromatid cohesion, we examined whether the defect of sister chromatid cohesion observed in *chl1* and *ctf4* cells is exaggerated upon exposure to MMS. As shown in Fig. 2C, no difference was observed in the level of cohesion defect in presence and absence of MMS in these mutants.

Chl1 and Ctf4 are required for MMS-induced recombination between homologous chromosomes

Homologous recombination occurs not only between sister chromatids but also between homologous chromosomes in diploid cells. We therefore investigated the frequency of recombination between homologous chromosomes carrying the heteroalleles *his1-1*/*his1-7* in diploid cells (Fig. 3A). Heteroallelic recombination spontaneously increased in *chl1* and *ctf4* cells compared with wild-type cells (Fig. 3B) as reported previously [7–9]. The frequency of heteroallelic recombination in wild-type cells increased with increasing MMS concentration (Fig. 3C). In contrast, the induction of recombination was greatly

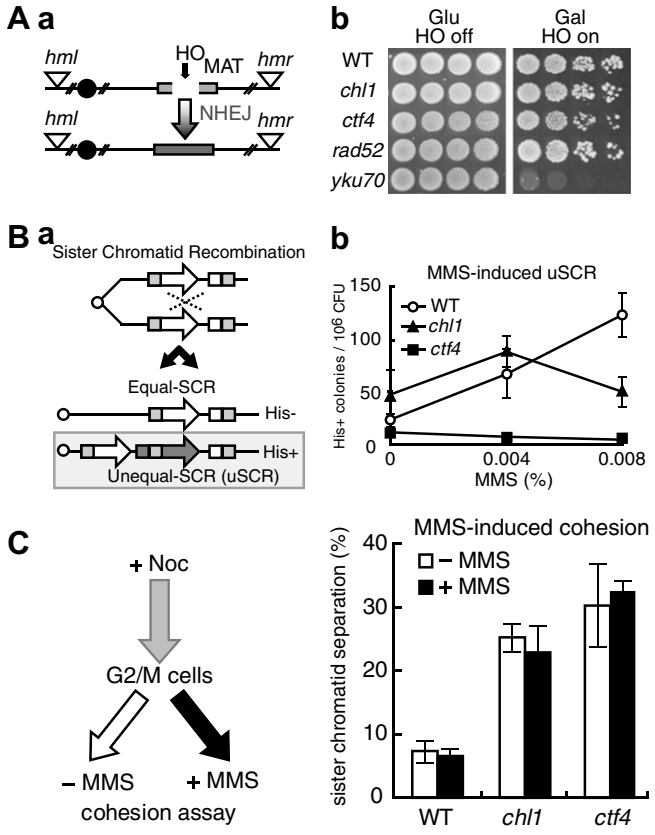

Fig. 2. Chl1 and Ctf4 are required for MMS-induced sister chromatid recombination but not for the NHEJ DSB repair. (A) (a) Schematic representation of the process and outcome of NHEJ at the galactose-inducible HO endonuclease specific DSB site on chromosome III in yeast strain JKM179. (b) Viability of wild type (JKM179), *chl1* (YHO507), *ctf4* (YHO508), *rad52* (YHO506), and *yku70* (YHO505) mutants in the background of an HR-repair defective yeast strain grown on either glucose (HO off), or galactose (HO on). A galactose-inducible HO endonuclease was integrated at the *ADE3* locus of a haploid yeast strain that was defective in HR-mediated DSB repair. Upon switching the cells to galactose, HO endonuclease is expressed, inducing a single DSB at the *MAT* locus. Functional DSB repair can occur only through the NHEJ pathway in this strain, since the *HM* donor loci, *HML* and *HMR*, are deleted. (B) (a) Schematic representation of the process and outcome of unequal sister chromatid recombination. (b) MMS-induced sister chromatid recombination (SCR). Wild-type (SCRL), *chl1* (YHO603) and *ctf4* (YHO604) cells were inoculated onto SC plates lacking His plates containing the indicated concentrations of MMS, then incubated at 30 °C for 3 days. The frequency of SCR is presented as the number of His^+ colonies per 10^6 colony forming units (CFU). To measure recombination frequency, at least four cultures were taken for each point. The data represents the means and standard deviation. (C) Effect of MMS on sister chromatid cohesion. Wild-type (YPH1477), *chl1* (YHO701) and *ctf4* (YHO702) cells were grown logarithmically in YPAD medium and incubated in the presence of 15 μg/ml nocodazole (Noc) for 3 h at 30 °C to arrest them in G2/M phase. The cultures were divided into 2, one with 0.1% MMS and the other without MMS, and incubated for 2 h at 30 °C. Two independent strains were scored in two independent experiments for sister chromatid cohesion defects. At least 100 cells were scored for each strain. The average percentage of cells with two GFP signals indicating sister chromatid separation is given along with the standard deviation.

impaired in *chl1* and *ctf4* cells. Thus, Chl1 and Ctf4 are required for MMS-induced recombination between homologous chromosomes as well as sister chromatids.

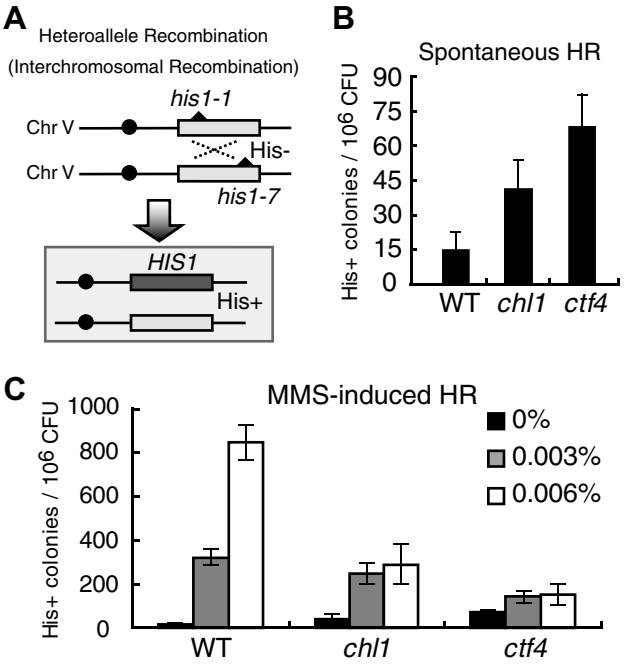

Fig. 3. Requirement of Chl1 and Ctf4 for MMS-induced recombination between homologous chromosomes. (A) Schematic representation of recombination between homologous chromosomes carrying heteroalleles at the *HIS1* locus. (B) Spontaneous recombination frequency. (C) MMS-induced recombination frequency. Wild-type (MR101), *chl1* (YHO216), and *ctf4* (YHO217) cells were inoculated onto SC plates lacking His and containing the indicated concentrations of MMS, then incubated at 30 °C for 3 days. The frequency of HR is presented as the number of His^+ colonies per 10^6 colony forming units (CFU). To measure recombination frequency, at least four cultures were taken for each point. The data represents the means and standard deviation.

How do Chl1 and Ctf4 contribute to recombination repair?

The data presented here seem to suggest that Chl1 and Ctf4 function in some steps of recombination not exerting their function in establishment of cohesion. Chl1 is a protein belonging to the DEAH helicase family that possesses DNA-dependent ATPase and DNA-helicase activities [10]. Thus we speculate that Chl1 functions as a helicase to promote some steps of recombination. This issue must be addressed in future studies.

Ctf4 binds polymerase α *in vitro* and *in vivo* [11,13], interacts genetically with Dna2, a helicase involved in lagging strand DNA synthesis [13], and is included in replisome progression complexes (RPCs) [21]. Interestingly, it has been suggested that the DNA polymerases involved in DNA replication, α, δ, and ε, function redundantly in recombination repair [22]. Taking into consideration that Ctf4 interacts with DNA polymerase α, it is conceivable that Ctf4 plays a role in affecting DNA polymerase α activity for efficient or proper DNA synthesis during DNA recombination.

Finally, inappropriate or inefficient recombination drives tumor formation and underlies certain premature aging diseases in humans. Since both Chl1 and Ctf4 are

evolutionarily conserved proteins, it is conceivable that Chl1 and Ctf4 might function as caretaker proteins to prevent chromosomal instability, and mutations in human CHL1 and CTF4 could lead to the chromosomal instability known as a hallmark of most cancers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.185.

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