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# $RFC^{Ctf18}$ and the Swi1-Swi3 Complex Function in Separate and Redundant Pathways Required for the Stabilization of Replication Forks to Facilitate Sister Chromatid Cohesion in *Schizosaccharomyces pombe*

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**Sister chromatid cohesion is established during S phase near the replication fork. However, how DNA replication is coordinated with chromosomal cohesion pathway is largely unknown. Here, we report studies of fission yeast Ctf18, a subunit of the $RFC^{Ctf18}$ replication factor C complex, and Chl1, a putative DNA helicase. We show that $RFC^{Ctf18}$ is essential in the absence of the Swi1–Swi3 replication fork protection complex required for the S phase stress response. Loss of Ctf18 leads to an increased sensitivity to S phase stressing agents, a decreased level of Cds1 kinase activity, and accumulation of DNA damage during S phase. Ctf18 associates with chromatin during S phase, and it is required for the proper resumption of replication after fork arrest. We also show that *chl1*Δ is synthetically lethal with *ctf18*Δ and that a dosage increase of $chl1^+$ rescues sensitivities of *swi1*Δ to S phase stressing agents, indicating that Chl1 is involved in the S phase stress response. Finally, we demonstrate that inactivation of Ctf18, Chl1, or Swi1-Swi3 leads to defective centromere cohesion, suggesting the role of these proteins in chromosome segregation. We propose that $RFC^{Ctf18}$ and the Swi1–Swi3 complex function in separate and redundant pathways essential for replication fork stabilization to facilitate sister chromatid cohesion in fission yeast.**

## INTRODUCTION

During the course of each cell cycle, the genome must be duplicated with a high degree of accuracy. However, environmental toxins or drugs cause DNA damage and impede the proper replication of chromosome DNA. To thwart this problem, eukaryotic cells are equipped with a DNA replication stress response pathway, termed the DNA replication checkpoint or S phase checkpoint (Boddy and Russell, 2001; Nyberg *et al*., 2002; Osborn *et al*., 2002). One of its major functions is to prevent the onset of mitosis. However, emerging evidence indicates that its most important activity is to stabilize replication forks by maintaining proper assembly of replisome components and DNA structures in replication competent states when forks stall (Lopes *et al*., 2001; Paciotti *et al*., 2001; Tercero and Diffley, 2001; Sogo *et al*., 2002; Tercero *et al*., 2003). In budding yeast treated with a DNA synthesis inhibitor, hydroxyurea (HU), failure to activate the replication checkpoint kinase Rad53 is associated with collapse and regression of replication forks and gross chromosomal rearrangements (Lopes *et al*., 2001; Tercero and Diffley, 2001; Kolodner *et al*., 2002; Sogo *et al*., 2002). In fission yeast, we have shown that Cds1, a Rad53 homologue, prevents fork collapse in response to HU (Noguchi *et al*., 2003), indicating that Cds1 is required for stabilization of replication forks in replication competent states. However, how Cds1 preserves stalled forks is largely unknown. Furthermore, the precise molecular mechanisms by which stalled forks activate the replication checkpoint are incompletely understood.

We have previously shown in fission yeast that the Swi1–Swi3 complex plays an important role in efficient activation of Cds1 (Noguchi *et al*., 2004). *swi1*Δ cells display replication fork collapse and a defect in recovery from replication fork arrest provoked by HU (Noguchi *et al*., 2003). Moreover, we have shown that Swi1-Swi3 travels with replication forks and is required to prevent accumulation of single-stranded DNA structures near the replication forks (Noguchi *et al*., 2004). Taken together, we have proposed that Swi1 and Swi3 form a "replication fork protection complex" (FPC) that is required for stabilization of stalled replication forks in a configuration that is recognized by replication checkpoint sensors (Noguchi *et al*., 2004).

The Swi1–Swi3 complex is evolutionarily conserved and is homologous to the Tof1–Csm3 complex in budding yeast and the Timeless–Tipin complex in humans (Gotter, 2003; Lee *et al*., 2004; Mayer *et al*., 2004; Noguchi *et al*., 2004). Tof1-

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Abbreviations used: CPT, camptothecin; FPC, fork protection complex; HU, hydroxyurea; MMS, methylmethane sulfonate; PCNA, proliferating cell nuclear antigen; PFGE, Pulsed field gel electrophoresis; RFC, replication factor C; TBZ, thiabendazole.

Csm3 has been shown to be part of the replisome or the replisome progression complex (RPC) and involved in Rad53 activation (Katou *et al*., 2003; Calzada *et al*., 2005; Nedelcheva *et al*., 2005; Gambus *et al*., 2006). In humans, Timeless-Tipin interacts with Chk1 and ATR to control activation of checkpoint kinase Chk1 (Chou and Elledge, 2006; Gotter *et al*., 2007; Unsal-Kacmaz *et al*., 2007; Yoshizawa-Sugata and Masai, 2007). Interestingly, in *Caenorhabditis elegans*, Tim-1, a Swi1 homologue, has been suggested to be involved in chromosome cohesion (Chan *et al*., 2003), which is essential for accurate chromosome segregation and holds replicated sister chromatids together until they are ready to be separated at anaphase. Consistently, *Saccharomyces cerevisiae csm3*Δ mutants seem to have a mild defect in meiotic chromosome segregation (Rabitsch *et al*., 2001), and recent studies have reported a partial sister chromatid cohesion defect in *tof1*Δ and *csm3*Δ cells (Mayer *et al*., 2004; Warren *et al*., 2004). These findings suggest that protection of stalled replication forks may be essential for proper establishment of chromosome cohesion. Moreover, in *S. cerevisiae*, it has also been reported that some proteins involved in the S phase checkpoint or DNA replication are essential in mutants that have defects in the chromosomal cohesion pathway (Mayer *et al*., 2004; Warren *et al*., 2004; Skibbens, 2005). One of them is Ctf18/Chl12, a protein related to the Rfc1 subunit of replication factor C. In budding yeast, Ctf18 associates with Rfc2, Rfc3, Rfc4, and Rfc5 to form an alternative $RFC^{Ctf18}$ complex and functions redundantly with Rad24 in the DNA replication checkpoint in budding yeast (Hanna *et al*., 2001; Mayer *et al*., 2001; Naiki *et al*., 2001). $RFC^{Ctf18}$ associates with two additional subunits, Dcc1 and Ctf8, to form a heptameric complex in budding yeast and humans, and it has been shown to have proliferating cell nuclear antigen (PCNA) loading and unloading activity and to play a role in sister chromatid cohesion (Hanna *et al*., 2001; Mayer *et al*., 2001; Naiki *et al*., 2001; Ohta *et al*., 2002; Bermudez *et al*., 2003; Merkle *et al*., 2003; Petronczki *et al*., 2004; Shiomi *et al*., 2004; Bylund and Burgers, 2005), although how $RFC^{Ctf18}$ controls the proper establishment of cohesion is unclear.

There is emerging evidence showing a strong connection between DNA replication and sister chromatid cohesion (Skibbens, 2005). However, how DNA replication proteins actually facilitate the cohesion process is largely unknown. In this report, we describe the genetic interaction between Swi1-Swi3, $RFC^{Ctf18}$, and Chl1 in fission yeast. We show that these proteins are involved in the protection of stalled replication forks and proper sister chromatid cohesion. Our studies suggest that the fork stabilization mechanism plays a crucial role in regulating establishment of sister chromatid cohesion in fission yeast.

## MATERIALS AND METHODS

### *General Techniques*

The methods used for genetic and biochemical analyses of fission yeast have been described previously (Moreno *et al*., 1991; Alfa *et al*., 1993). Immunoblotting and UV sensitivity assay were performed as described in our previous study (Noguchi *et al*., 2004). Microscopic analyses of yellow fluorescent protein (YFP) and green fluorescent protein (GFP) were performed using an Olympus PROVIS AX70 microscope equipped with a SPOT RT camera model 2.3.1 (Diagnostic Instruments, Sterling Heights, MI). Images were acquired with OpenLab software (Improvision, Lexington, MA).

### *Gene Cloning, Plasmids, Primers, and Schizosaccharomyces pombe Strain Construction*

The *S. pombe* strains used in this study were constructed using standard techniques (Alfa *et al*., 1993), and their genotypes are listed in Supplemental Table S1. The 3.6-kb *swi1*$^+$ genomic fragment was amplified by EXtaq polymerase (TaKaRa, Ohtsu, Japan) and introduced into the SmaI site of pUC28, resulting in pUC28-Swi1. The *swi1*$^+$ SacI–XbaI fragment was excised and transferred into the SacI/XbaI site of pDblet (Brun *et al*., 1995), resulting in pDblet-Swi1. Finally, the 2.86-kb *ade6*$^+$ fragment was introduced into pDblet-swi1, resulting in pDblet-Swi1-Ade6. *ctf18-5FLAG* (*ctf18-5FLAG-Kan*$^r$) and *ctf18-TAP* (*ctf18-TAP-Kan*$^r$) were generated by a one-step polymerase chain reaction (PCR) method (Bähler *et al*., 1998) by using primers P532 and P533 to construct a 5xFLAG and a TAP tag at the C terminus of *ctf18*, respectively. *chk1*Δ (*chk1*::*Kan*$^r$) was generated by a two-step PCR method (Krawchuk and Wahls, 1999) by using primers P534, P535, P538, and P539 to replace the *chk1*$^+$ open reading frame with the *Kan*$^r$ gene. *ctf18*Δ (*ctf18*::*Kan*$^r$) was generated by a two-step PCR method (Krawchuk and Wahls, 1999) by using primers P545, P547, P548, and P574 to replace the *ctf18*$^+$ open reading frame with the *Kan*$^r$ gene. *chl1*Δ (*chl1*::*Kan*$^r$) was generated by a two-step PCR method (Krawchuk and Wahls, 1999) by using primers P525, P526, P529, and P530 to replace the *chl1*$^+$ open reading frame with the *Kan*$^r$ gene. *chl1*Δ (*chl1*::*hph*) was generated from the *chl1*::*Kan*$^r$ strain by a one-step marker switch method as described previously (Sato *et al*., 2005). *ctf18*Δ (*ctf18*::*his3*$^+$) was generated by replacing the *ctf18*$^+$ open reading frame with the *his3*$^+$ fragment that was amplified using primers Ctf18-KO1 and Ctf18-KO2. *rad3*Δ (*rad3*::*Kan*$^r$) was generated by transforming a *rad3*::*ura4*$^+$ strain (Bentley *et al*., 1996) with the *Kan*$^r$ fragment amplified using primers UraKan-T1 and UraKan-B1. pFA6a-KanMX6 (Bähler *et al*., 1998), pFA6a-5FLAG-KanMX6 (Noguchi *et al*., 2004), and pFA6a-TAP-KanMX6 (Saitoh *et al*., 2002) were used as the templates for the PCR-base gene deletion and tagging. The primer sequences used in these procedures are listed in Supplemental Table S2. Mutations and epitope-tagged genes have been described previously for *cdc25-22* (Fantes, 1979), *swi1*Δ (*swi1*::*Kan*$^r$) (Noguchi *et al*., 2003), *swi3*Δ (*swi3*::*Kan*$^r$) (Noguchi *et al*., 2004), *cds1*Δ (*cds1*::*ura4*$^+$) (Boddy *et al*., 1998), *chk1*Δ (*chk1*::*ura4*$^+$) (al-Khodairy *et al*., 1994), *rad3*Δ (*rad3*::*ura4*$^+$) (Bentley *et al*., 1996), *rad22-YFP* (*rad22-YFP-Kan*$^r$) (Noguchi *et al*., 2003), *rad21-K1* (*rad21-K1-ura4*$^+$) (Tatebayashi *et al*., 1998) *rqh1*Δ (*rqh1*::*ura4*$^+$) (Stewart *et al*., 1997), *nda3-KM311* (Hiraoka *et al*., 1984), and *lys1*$^+$-lacO repeat *his7*$^+$*-dis1promoter-GFP-LacI-NLS* (Ding *et al*., 2004).

### *Isolation of the ctf18*$^+$ *and chl1*$^+$ *Genes*

The *S. pombe ade6* mutants form red colonies due to accumulation of an adenine-intermediate-derived pigment. *swi1*Δ *ade6-210* cells were transformed with a plasmid pDblet-Swi1-Ade6 that contains *swi1*$^+$, *ura4*$^+$, and *ade6*$^+$ genes. Transformants formed white colonies in the absence of uracil, whereas they formed red or red-white sectored colonies on YES agar medium because of plasmid loss. These cells ($2 \times 10^8$ cells) were washed in water, resuspended in sodium phosphate buffer, and treated with 3% ethyl methanesulfonate for 95 min at 30°C. The mutagenized cells were washed once in water and twice with 5% sodium thiosulfate and plated on YEA agar medium to allow cells to lose the plasmid pDblet-Swi1-Ade6. Strains that formed white colonies were further tested for sensitivity to 5-fluoroorotic acid (5-FOA). Only one strain, which we designated Y660, seemed to be dependent on the *swi1*$^+$ (pDblet-Swi1-Ade6) plasmid for viability. Y660 was backcrossed twice with a *swi1*Δ strain, and we confirmed that Y660 contained a single mutation that is synthetically lethal with *swi1*Δ. To identify the mutated gene in Y660, we transformed this strain with *S. pombe* genomic library cloned into a pARS2004LEU2 vector. Transformed cells were plated on agar medium lacking leucine, incubated for 2 d at 30°C and replica-plated on agar medium containing 5-FOA followed by another 5-d incubation. Library derived genomic DNAs cloned into pARS2004LEU2 were then isolated from 5-FOA–resistant clones. Among four 5-FOA–resistant clones, two contained the *swi1*$^+$ gene, other two contained the *ctf18*$^+$ and *SPAC3G6.11*$^+$ genes, respectively. As described in *Results*, *SPAC3G6.11* seemed to be a homologue of *CHL1* of budding yeast and *Chl1* of humans.

### *Precipitation of Tandem Affinity Purification (TAP)-tagged Protein*

Cells expressing the TAP and Myc fusion proteins at their own genomic loci were cultured in YES medium until an optical density of 1.2 at 600 nm was reached, and cells were collected. Cells were lysed with glass beads in lysis buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 50 mM NaF, 1 mM $Na_3VO_4$, 5 mM EDTA, 5 mM *N*-methylmaleimide, 1 $\mu$M microcyctin, 0.1 $\mu$M okadaic acid, 0.2 mM *p*-4-amidoinophenyl-methane sulfonyl fluoride hydrochloride monohydrate [*p*-APMSF] and Roche Complete EDTA-free protease inhibitor cocktail [Roche Diagnostics, Basel, Switzerland]) by using a FastPrep cell disrupter (Qbiogene, Irvine, CA) for two cycles of 20 s at speed 6, with a 1-min interval on ice between the two cycles. Protein extracts were clarified by centrifugation at 13,000 rpm in an Eppendorf microcentrifuge 5415D for 10 min at 4°C, mixed with immunoglobulin G-Sepharose beads (GE Healthcare, Piscataway, NJ) and incubated for 2 h at 4°C. The Sepharose beads were collected and washed three times in lysis buffer A. Proteins associated with the beads were analyzed by immunoblotting. TAP and Myc fusion proteins were probed with the anti-c-Myc 9E10 monoclonal antibody (Covance, Berkeley, CA) and peroxidase anti-peroxidase (Sigma-Aldrich, St. Louis, MO), respectively.

### *Cds1 Kinase Assay*

Cds1 kinase assay was performed essentially as described previously (Lindsay *et al*., 1998). Exponentially growing cells were washed in STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, and 1 mM $NaN_3$) and lysis buffer B (50 mM Tris, pH 7.5, 80 mM β-glycerophosphate, 250 mM NaCl, 15 mM nitrophenylphosphate, 50 mM NaF, 5 mM EDTA, 1 mM dithiothreitol [DTT], and 0.1% NP-40 supplemented with protease inhibitor cocktail [Complete EDTA-free protease inhibitor cocktail; Roche Diagnostics], and *p*-APMSF). Protein extract was prepared as described in the previous section and incubated at 4°C for 90 min with 20 μl of protein A agarose (Sigma-Aldrich) preincubated with the Cds1 antibody. The protein A-agarose beads were washed three times each with lysis buffer B and kinase buffer (10 mM HEPES, pH 7.5, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM DTT). The immunocomplex containing Cds1 bound on protein A-agarose beads was incubated with 10 μl of 2X kinase buffer, 5 μCi of [γ-$^{32}$P]ATP, 0.2 μl 10 mM ATP, and 0.5 μl of 10 mg/ml myelin basic protein (MBP; Cds1 substrate) at 30°C for 15 min. The reaction was stopped by the addition of 25 μl of 2X SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and subsequent boiling for 5 min. MBP was separated on 15% polyacrylamide gels and detected by Coomassie Brilliant Blue staining. The gel was dried, and radioactivity incorporated in MBP was detected with a Storm 840 machine (GE Healthcare). After imaging, the radioactivity levels (cpm) of MBP were determined in an LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA).

### *Detection of Rad22-YFP DNA Repair Foci by Fluorescent Microscopy*

Cells expressing Rad22-YFP from its own promoter were grown at 25°C in YES liquid medium until mid-log phase. We have used 25°C to obtain stronger YFP signal. Cells were concentrated by centrifugation and kept on ice before microscopic analysis. Rad22-YFP localization was analyzed and imaged as described in General Techniques. Quantification of Rad22-YFP foci has been performed at least four times, and at least 200 cells were counted for each strain in each experiment. The cell cycle position of cells containing Rad22-YFP foci was estimated by analyzing cell length, nuclei number and position, and the presence of a division plate.

### *Pulsed Field Gel Electrophoresis (PFGE)*

Exponentially growing cells were treated with 12 mM HU for 4 h at 30°C, and then they were washed and released into fresh media. Cells were collected at the indicated times at a concentration of $2.5 \times 10^8$ and washed in 20 ml of CSE (20 mM citric acid, 20 mM sodium phosphate [$Na_2HPO_4 \cdot 7H_2O$], adjusted to pH 5.6, 1.2 M sorbitol, and 40 mM EDTA, pH 8.0). Cell pellets were suspended in 1 ml of CSE + 1 mg/ml Zymolase 100T and incubated at 37°C for 2 h. Cells were resuspended to a concentration of $8 \times 10^8$ cells/ml in 300 μl of TSE (10 mM Tris-HCl, pH 7.5, 0.9 M sorbitol, and 45 mM EDTA, pH 8.0). Cell suspension was warmed to 42°C and mixed with 300 μl of 1.1% low melting temperature agarose in TSE. Aliquots were dispensed into plug molds and allowed to solidify at 4°C, and then they were suspended in 3 ml of Tris-EDTA-SDS (0.25 M EDTA, pH 8.0, 50 mM Tris-HCl, pH 7.5, and 1% SDS) and incubated at 55°C for 90 min. Plugs were then incubated at 55°C for 48 h in 3 ml of NDS (10 mM Tris-HCl, 0.5 M EDTA, pH 8.0, pH adjusted to 9.5, and 1% lauryl sarcosine) supplemented with 1 mg/ml proteinase K (Invitrogen, Carlsbad, CA). Plugs were equilibrated in 1 ml of TE and stored in 5 ml of 0.5 M EDTA at 4°C. To analyze chromosome DNA embedded in plugs, the plugs were equilibrated in TE and run on 0.8% Megabase agarose gel (Bio-Rad, Hercules, CA) in 1X TAE by using a CHEF-DR II system (Bio-Rad) at the following settings: block 1, 2 V/cm, initial and final switch time of 1800 s; 14°C; and pump speed, 70, for 72 h. Gels were stained with 0.5 μg/ml ethidium bromide in $H_2O$ for 30 min and destained with water for 1–2 h.

### *Chromatin Immunoprecipitation (ChIP) Assay*

ChIP assay was performed essentially as described previously (Noguchi and Noguchi, 2007). Briefly, *S. pombe* cells ($5 \times 10^8$) were fixed in 1% formaldehyde for 20 min at room temperature, and then they were quenched in 125 mM glycine for 5 min. Cells were washed in Tris-buffered saline and disrupted in lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with protease inhibitors {0.2 mM *p*-APMSF and Roche protease inhibitor cocktail). The broken cells were sonicated six times for 20 s each with a Misonix Sonicator 3000 until chromatin DNA was sheared into 500- to 700-base pair fragments. Cell lysate was clarified by two rounds of maximum speed centrifugation in an Eppendorf 5415C microcentrifuge at 4°C. Immunoprecipitations were performed in these cell extracts using anti-FLAG M2 agarose (Sigma-Aldrich). PCR amplification conditions and the specific primers used in these studies have also been described previously (Ogawa *et al*., 1999).

### *Chromosome Cohesion Assay*

We used a strain harboring bacterial LacO tandem repeat sequences inserted in the vicinity of the centromere on chromosome I (Ding *et al*., 2004). This strain is engineered to express the LacI repressor fused to GFP-nuclear localization signal (NLS), which is recruited to LacO repeat sequences, allowing us to visualize the centromere I (Ding *et al*., 2004). Cohesion assays were performed using the following three conditions. 1) Cells were grown to exponential phase in liquid YES medium at 25°C, synchronized in early S phase in the presence of 10 mM HU for 2 h, and released into medium containing 100 μg/ml thiabendazole (TBZ). At the indicated time, GFP foci were monitored and imaged as described in General Techniques. 2) *cdc25-22* temperature-sensitive cells were grown to mid-log phase at 25°C and synchronized at the G2-M transition at 33°C for 3 h. Cells were then released at 25°C into medium containing 100 μg/ml TBZ. At the indicated time, GFP foci were monitored and imaged. 3) *nda3-KM311* cold-sensitive cells were grown to mid-log phase at 30°C and shifted to a restrictive temperature, 20°C. At the indicated time, GFP foci were monitored and imaged. Quantification of GFP foci has been performed at least three times, and at least 200 cells were counted for each strain in each experiment.

## RESULTS

### *Synthetic Lethal Genetic Interactions Involving RFC$^{Ctf18}$, Chl1, and Swi1-Swi3*

To further understand the role of the Swi1–Swi3 replication fork protection complex in genomic integrity, we carried out a genetic screen to isolate mutations that are synthetically lethal with deletion of *swi1*$^+$ (see *Materials and Methods*). This screen identified one mutant strain that was inviable in a *swi1*Δ background. Therefore, we designated the mutation carried by the isolated strain *lws1-1* (*l*ethal *w*ith *s*wi1 deletion). To isolate dosage suppressors of *lws1-1 swi1*Δ lethality, *lws1-1 swi1*Δ double mutant cells harboring a *swi1*$^+$-*ura4*$^+$ plasmid was transformed with an *S. pombe* genomic library. We sought clones that could grow in the presence of 5-fluoroorotic acid, a compound that forces the loss of a *swi1*$^+$-*ura4*$^+$ plasmid via counterselection of *ura4*$^+$ cells. This screen then identified plasmids containing *swi1*$^+$, *SPAC3G6.11*$^+$, or *ctf18*$^+$ as dosage suppressors of the lethality of *lws1-1 swi1*Δ. Our genetic analyses found that the *lws1-1* was not allelic to either of *SPAC3G6.11*$^+$ or *ctf18*$^+$. Identify of *lws1-1* is currently under investigation. Because *SPAC3G6.11* had not been characterized in *S. pombe*, we performed a BLAST search by using *SPAC3G6.11* open reading frame as the query protein sequence and found that SPAC3G6.11 is highly homologous to the DEAD box DNA helicase Chl1 in humans (E-value of $1 \times 10^{-143}$) (Amann *et al*., 1996) and budding yeast (E-value of $2 \times 10^{-126}$) (Gerring *et al*., 1990). Therefore, we named the *SPAC3G6.11* gene as *S. pombe chl1*$^+$.

To understand the relationship among the genes identified in our screen, we performed tetrad analyses to examine genetic interactions of these genes. As shown in Figure 1A, none of the *swi1*Δ *ctf18*Δ and *chl1*Δ *ctf18*Δ double mutant strains grew after tetrad analyses, whereas *swi1*Δ *chl1*Δ double mutants showed a normal growth comparable to *swi1*Δ mutants (Figure 6B and Table 1). These data established that *ctf18*Δ is synthetically lethal with *swi1*Δ or *chl1*Δ. We have noticed that spore viability is somewhat low in *swi1*Δ or *swi3*Δ crosses (Figure 1A). This might be due to improper chromosome segregation during meiosis, because *C. elegans* Tim-1, a homologue of Swi1, has been suggested to play an important role in proper chromosome segregation by regulating both mitotic and meiotic sister chromatid cohesion (Chan *et al*., 2003). We also found that *swi3*Δ showed similar genetic interaction with *chl1*Δ and *ctf18*Δ (Figure 1A and Table 1), indicating that RFC$^{Ctf18}$ plays an essential role for cell survival in the absence of the Swi1–Swi3 complex. Because Swi1-Swi3 is required for the stabilization of replication forks and activation of the replication checkpoint (Noguchi *et al*., 2004), we have hypothesized that RFC$^{Ctf18}$ is also involved in these cellular mechanisms in fission yeast. Therefore, in this report, we investigated this possibility.

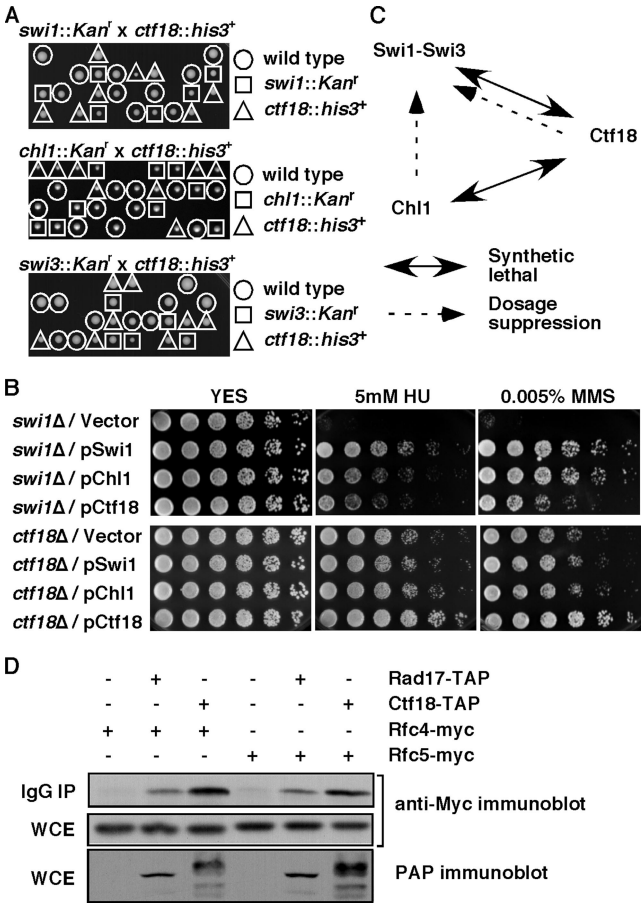


**Figure 1.** Genetic interaction involving Swi1-Swi3, RFC$^{Ctf18}$, and Chl1. (A) None of the viable spores from *swi1*::*Kan*$^r$ x *ctf18*::*his3*$^+$, *chl1*::*Kan*$^r$ × *ctf18*::*his3*$^+$, and *swi3*::*Kan*$^r$ × *ctf18*::*his3*$^+$ crosses were able to grow on YES medium containing G-418 and EMM2, Edinburgh minimal media lacking histidine, indicating that *swi1*Δ *ctf18*Δ, *chl1*Δ *ctf18*Δ, and *swi3*Δ *ctf18*Δ cells are inviable. Genotypes of viable spores are shown. Representative images of >40 tetrad dissections from each cross are shown. (B) Damage sensitivities of *swi1*Δ were suppressed by an increased gene dosage of *chl1*$^+$ or *ctf18*$^+$. *swi1*Δ or *ctf18*Δ cells were transformed with the indicated plasmid and plated on YES medium containing no drug (YES), 5 mM HU, or 0.005% MMS. Fivefold serial dilution of cells were plated and incubated for 2–3 d at 32°C. Representative images of repeat experiments are shown. (C) Summary of genetic interaction involving Swi1-Swi3, RFC$^{Ctf18}$, and Chl1. (D) Ctf18 associates with Rfc4 and Rfc5. Protein extracts were prepared from cells expressing the indicated fusion proteins. Ctf18-TAP was precipitated and probed with anti-myc antibodies. Rfc4-Myc and Rfc5-Myc are shown to be equally expressed in each cell line (whole-cell extract, WCE). As a positive control, Rad17-TAP is shown to associate with Rfc4 and Rfc5. IgG IP, precipitated fraction.

**Table 1.** Synthetic genetic interaction described in this study

| Genotype | Phenotype | Source |
|---|---|---|
| *swi1*Δ *ctf18*Δ | Lethal | This study |
| *swi3*Δ *ctf18*Δ | Lethal | This study |
| *swi1*Δ *chl1*Δ | No apparent phenotypic enhancement | This study |
| *swi3*Δ *chl1*Δ | No apparent phenotypic enhancement | This study |
| *chl1*Δ *ctf18*Δ | Lethal | This study |
| *swi1*Δ *rad21-K1* | Lethal | This study |
| *ctf18*Δ *rad21-K1* | Phenotypic enhancement | This study |
| *chl1*Δ *rad21-K1* | Phenotypic enhancement | This study |
| *swi1*Δ *rqh1*Δ | Severe growth defect | Noguchi *et al.* (2003) |
| *swi3*Δ *rqh1*Δ | Lethal | This study |
| *ctf18*Δ *rqh1*Δ | Phenotypic enhancement | This study |
| *ctf18*Δ *cds1*Δ | No apparent increase in UV and HU sensitivity | This study |
| | Phenotypic enhancement in MMS and CPT sensitivity | This study |
| *ctf18*Δ *chk1*Δ | Phenotypic enhancement in UV and HU sensitivity | This study |
| | Phenotypic enhancement in MMS and CPT sensitivity | This study |
| *ctf18*Δ *rad3*Δ | No apparent increase in UV and HU sensitivity | This study |
| | Phenotypic enhancement in MMS and CPT sensitivity | This study |
| *chl1*Δ *cds1*Δ | No apparent increase in MMS, CPT, and TBZ sensitivity | This study |
| *chl1*Δ *chk1*Δ | Phenotypic enhancement in MMS and CPT sensitivity | This study |
| *chl1*Δ *rad3*Δ | Phenotypic enhancement in MMS, CPT, and TBZ sensitivity | This study |

Inactivation of Swi1 is known to render cells sensitive to HU and methylmethane sulfonate (MMS) (Noguchi *et al.*, 2003; Sommariva *et al.*, 2005). HU depletes the dNTP pool and inhibits DNA synthesis, whereas MMS introduces alkylation to template DNA and also causes an arrest of replication fork progression. To further characterize genetic interactions between the three genes, we examine whether HU and MMS sensitivity of *swi1*Δ is suppressed by a multicopy vector carrying *swi1*$^+$, *ctf18*$^+$, or *chl1*$^+$ (Figure 1B). MMS sensitivity of *swi1*Δ cells was greatly suppressed by a *chl1*$^+$ plasmid and significantly by a *ctf18*$^+$ plasmid. In fact, the suppression conferred by the *chl1*$^+$ plasmid was comparable to the suppression by the *swi1*$^+$ plasmid. In contrast, HU sensitivity of *swi1*Δ cells was significantly suppressed both by *chl1*$^+$ and *ctf18*$^+$, but it was not as dramatic as the suppression by *swi1*$^+$ plasmid. These results suggest that RFC$^{Ctf18}$ and Chl1 are both involved in mechanisms that are important for proper cellular responses to S phase stress. We have also examined whether *ctf18*Δ phenotypes can be suppressed by an increased dosage of *swi1*$^+$ or *chl1*$^+$ (Figure 1B). As described later, *ctf18*Δ cells showed weak sensitivity to HU and MMS. Unlike the case for *swi1*Δ cells, HU and MMS sensitivity of *ctf18*Δ cells were not significantly suppressed by *swi1*$^+$ or *chl1*$^+$ dosage increases (Figure 1B). Results of our genetic interaction analyses are summarized in Figure 1C, and Tables 1 and Table 2. Because *chl1*Δ is synthetically lethal with *ctf18*Δ but not with *swi1*Δ, and Chl1 overexpression can suppress MMS and HU sensitivity of *swi1*Δ but not *ctf18*Δ, our data are consistent with the notion that Chl1 is closely involved in the S phase stress response pathway regulated by Swi1. In contrast, the role of Ctf18 in the S phase stress response may represent a mechanism that is independent of but partially redundant with the mechanisms regulated by the Swi1–Swi3 complex.

### *Ctf18 Associates with Rfc4 and Rfc5 in S. pombe Cell Extract*

Ctf18 is related to the replication factor C subunit Rfc1 and is known to form the RFC$^{Ctf18}$ complex together with Rfc2, Rfc3, Rfc4, Rfc5, Ctf8, and Dcc1 in budding yeast. RFC$^{Ctf18}$ has been shown to have a role in PCNA loading or unloading in budding yeast and humans and play an important

**Table 2.** Dosage suppression described in this study

| Mutant | Multicopy plasmid | Degree of suppression[a] HU | MMS | TBZ |
|---|---|---|---|---|
| *swi1*Δ | Vector | − | − | − |
| *swi1*Δ | pSwi1 | ++ | ++ | ++ |
| *swi1*Δ | pChl1 | + | ++ | + |
| *swi1*Δ | pCtf18 | + | + | + |
| *ctf18*Δ | Vector | − | − | − |
| *ctf18*Δ | pSwi1 | − | − | − |
| *ctf18*Δ | pChl1 | − | − | ++ |
| *ctf18*Δ | pCtf18 | ++ | ++ | ++ |

[a] ++, strong suppression; +, partial suppression; and −, no suppression.

role during sister chromatid cohesion and the replication checkpoint (Hanna *et al*., 2001; Mayer *et al*., 2001; Naiki *et al*., 2001; Ohta *et al*., 2002; Bermudez *et al*., 2003; Merkle *et al*., 2003; Petronczki *et al*., 2004; Shiomi *et al*., 2004; Bylund and Burgers, 2005). In *S. pombe*, *ctf18*$^+$, *ctf8*$^+$, or *dcc1*$^+$ has been shown to be essential in the absence of functional RFC$^{Rfc1}$ (Kim *et al*., 2005), also suggesting the role of RFC$^{Ctf18}$ in PCNA loading. However, how RFC$^{Ctf18}$ controls these mechanisms is poorly understood. To address whether Ctf18 forms a similar complex in *S. pombe*, we generated an *S. pombe* strain that expresses the Ctf18–TAP fusion protein at endogenous levels from the *ctf18*$^+$ promoter. This strain is engineered to express Rfc4-13myc or Rfc5-13myc from their genomic loci to determine the interaction between Ctf18 and Rfc subunits. These tagged alleles had no apparent effect on cell viability or growth. Ctf18-TAP was precipitated from *S. pombe* cell extracts by using immunoglobulin (Ig)G-Sepharose, and precipitates were then probed with anti-myc antibody. As shown in Figure 1D, both Rfc4-13myc and Rfc5-13myc copurified with Ctf18-TAP, indicating that RFC$^{Ctf18}$ complex exists in *S. pombe*. Another alternative RFC complex involving Rad17 served as a positive control for coprecipitation with Rfc4 and Rfc5 (Figure 1D). Although Rfc2 and Rfc3 were not tested in this current report, it is likely that these proteins also bind Ctf18 to form the RFC$^{Ctf18}$ complex.

### *Ctf18 Is Required for Survival after UV- or HU-induced Fork Arrest and for Proper Activation of Replication Checkpoint Kinase Cds1*

Ctf18 is shown to have some redundant roles with Rad24 in the DNA replication checkpoint in budding yeast (Naiki *et al*., 2001). Therefore, the genetic interactions involving RFC$^{Ctf18}$ and Swi1-Swi3 suggest that RFC$^{Ctf18}$ plays an important role, not only in the activation of the replication checkpoint, but also in the stabilization of replication forks in fission yeast. To address this possibility, we first examined whether Ctf18 is involved in tolerance of fork arrest. To induce replication fork arrest, we used UV irradiation, which creates DNA lesions that arrest forks (Friedberg *et al*., 1995), and hydroxyurea to inhibit ribonucleotide reductase and deplete dNTP pools (Boddy and Russell, 2001; Nyberg *et al*., 2002; Osborn *et al*., 2002). *ctf18*Δ cells were weakly sensitive to both UV and HU (Figure 2, A and B), suggesting that Ctf18 plays a role in cellular tolerance of fork arrest.

Next, we performed epistasis analysis between *ctf18*Δ and deletion mutations of checkpoint kinases Chk1 and Cds1 in

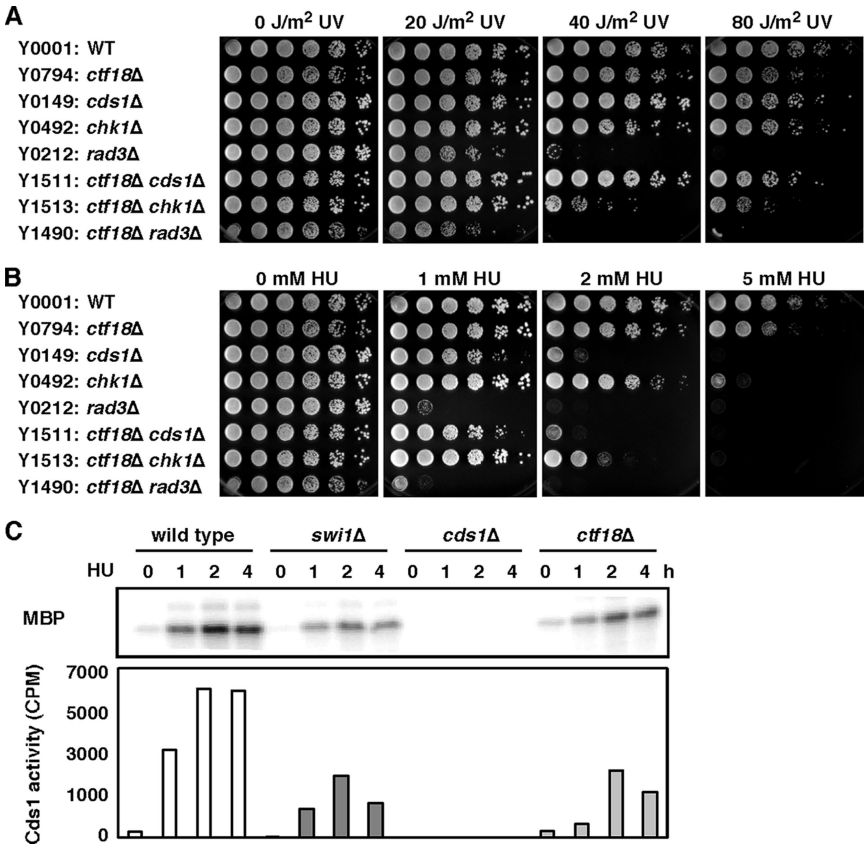


**Figure 2.** Ctf18 is involved in the Cds1-dependent replication checkpoint. (A and B) Synergistic interaction of *ctf18*Δ and *chk1*Δ in UV and HU survival assays indicates that Ctf18 is required for cellular tolerance to fork arrest. For UV survival assays, fivefold serial dilution of cells were plated on YES agar medium and exposed to the indicated doses of UV. Agar plates were then incubated for 2–3 d at 32°C. For HU sensitivity assays, fivefold serial dilution of cells were incubated on YES agar medium supplemented with the indicated amounts of HU for 2–4 d at 32°C. Representative images of repeat experiments are shown. (C) Cds1 activation is strongly reduced in *ctf18*Δ cells. Cells of the indicated genotypes were incubated in YES liquid medium supplemented with 12 mM HU for 0, 1, 2, and 4 h at 30°C. Kinase activity of immunoprecipitated Cds1 was measured using MBP as a substrate. The radiolabeled MBP was detected after gel electrophoresis (top). The radioactivity levels (cpm) of MBP were then determined in a liquid scintillation counter (bottom). Representative results from repeat experiments are shown.

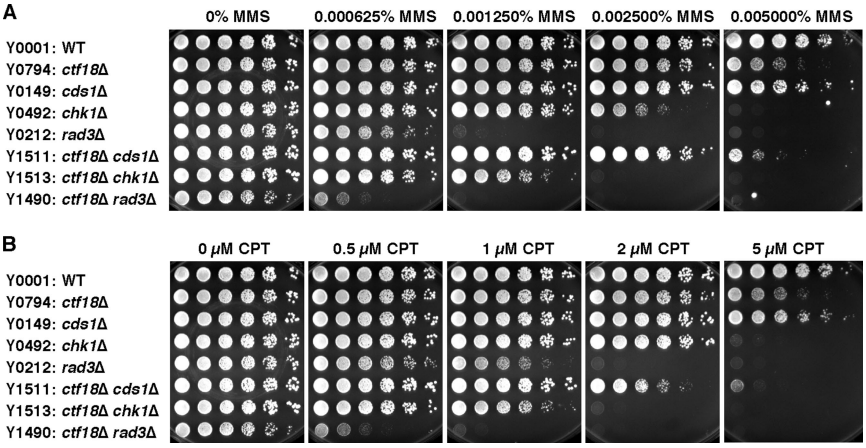


**Figure 3.** Ctf18 constitutes a checkpoint-independent S phase DNA damage response pathway. (A and B) Fivefold serial dilution of cells were incubated on YES agar medium supplemented with the indicated amounts of MMS or CPT for 2–4 d at 32°C. Representative images of repeat experiments are shown.

a UV-survival assay. The *ctf18*Δ *chk1*Δ cells were substantially more sensitive than either single mutant (Figure 2A and Table 1). In contrast, there was no significant genetic interaction between *ctf18*Δ and *cds1*Δ in UV-survival assays (Figure 2A and Table 1). We also examined the effect of inactivating Ctf18 in *chk1*Δ and *cds1*Δ backgrounds in an HU-survival assay. Again, HU sensitivity of *ctf18*Δ cells was further enhanced by *chk1* deletion (Figure 2B and Table 1), the effector kinase of the G2-M DNA damage checkpoint. However, there was no significant genetic interaction between *ctf18*Δ and *cds1*Δ in HU survival assay (Figure 2B and Table 1). Therefore, these results suggest that UV- or HU-induced stalled fork structures accumulated in *ctf18*Δ mutants are converted to different fork forms that activate the Chk1-dependent G2-M DNA damage checkpoint pathway. Similar results have been reported for inactivation of Swi1 and Swi3, components of the replication fork protection complex (Noguchi *et al*., 2003; Noguchi *et al*., 2004).

Cds1 and Chk1 define redundant pathways of checkpoint activation in response to fork arrest (O'Connell *et al*., 2000; Rhind and Russell, 2000a, b; Boddy and Russell, 2001; Nyberg *et al*., 2002). Because both Cds1 and Chk1 pathways are controlled by the Rad3 kinase, we examined genetic interaction between *ctf18*Δ and *rad3*Δ in UV- and HU-survival assays. As shown in Figure 2, A and B, *rad3*Δ and *ctf18*Δ *rad3*Δ cells were similarly sensitive to UV and HU. Thus, the strong genetic interaction in HU- or UV-survival assays involving *ctf18*Δ and *chk1*Δ (Figure 2, A and B, and Table 1) suggests that Ctf18 is involved in activation of the replication checkpoint enforced by Cds1. To test this possibility, we measured Cds1 kinase activity in wild-type and *ctf18*Δ cells treated with HU. As shown in Figure 2C, HU treatment induced a robust activation of the Cds1 kinase. However, Cds1 activation was strongly decreased in *ctf18*Δ cells (Figure 2C). A similar defect in Cds1 activation was observed in *swi1*Δ cells (Figure 2C) (Noguchi *et al*., 2003), supporting the idea that Ctf18 is involved in the activation of the Cds1 replication checkpoint kinase. The residual Cds1 activation observed in *swi1*Δ or *ctf18*Δ cells might be due to a redundant requirement of Swi1 and Ctf18 in the activation of Cds1. However, we were unable to test this possibility directly because *swi1*Δ *ctf18*Δ cells are not viable (Figure 1A).

### *Ctf18 Is a Component of Checkpoint-independent S Phase Stress Response Pathways to Alkylation Damage and Replication Fork Breakage*

To understand the role of Ctf18 in the S phase DNA damage response, we examined the sensitivity of *ctf18*Δ cells to MMS, which lead to alkylation damage that interferes with DNA replication. Cells repair these damages by cell cycle checkpoint, postreplication repair, recombination repair, and base excision repair pathways (Xiao *et al*., 1996; Chang *et al*., 2002). *ctf18*Δ cells showed significant sensitivity to 0.005% MMS (Figure 3A). As is the case for HU- and UV-sensitivity assays, this sensitivity was further enhanced by Chk1 inactivation (Figure 3A and Table 1), suggesting that *ctf18*Δ cells accumulate abnormal DNA structures that must be repaired by the Chk1-dependent G2-M checkpoint. Unlike the situation upon UV exposure, there was a reproducible synergistic interaction between *ctf18*Δ and *cds1*Δ at 0.005% MMS (Figure 3A). This genetic interaction suggests that Ctf18 has a Cds1-independent role in tolerance of alkylation damage. In addition, our analyses showed that *ctf18*Δ *rad3*Δ cells were much more sensitive to MMS than either single mutant (Figure 3A and Table 1). *rad3*Δ mutants are defective for the activation of both Cds1 and Chk1, thereby lacking checkpoint-dependent cell cycle arrest in response to replication block or DNA damage (O'Connell *et al*., 2000; Rhind and Russell, 2000b; Boddy and Russell, 2001; Nyberg *et al*., 2002). Therefore, the synergistic interaction between *ctf18*Δ and *rad3*Δ in MMS sensitivity assays suggests that Ctf18 has an important function that is independent of cell cycle checkpoints, and this function may contribute to the recovery from alkylation damage that causes stalled replication forks and other lesions.

To further examine the role of Ctf18 in the S phase DNA damage response, we introduced replication fork breakage during S phase by exposure to camptothecin (CPT), a drug that traps topoisomerase I on DNA (Pommier, 2006). *ctf18*Δ cells showed significant sensitivity to CPT (Figure 3B), suggesting that Ctf18 has an important role in the tolerance of DNA damage during S phase. As with MMS, *ctf18*Δ *cds1*Δ, *ctf18*Δ *chk1*Δ, and *ctf18*Δ *rad3*Δ double mutant cells showed stronger CPT sensitivity than either single mutant (Figure 3B and Table 1), indicating that Ctf18's role in recovery from fork breakage during S phase cannot solely be accounted for by the defect in cell cycle checkpoint controls. Together, our results indicate that Ctf18 constitutes an S phase DNA damage response pathway that is independent of checkpoints.

### *Replication Abnormalities in ctf18Δ Cells*

Our data thus far demonstrate that Ctf18 plays an important role in cell survival after fork arrest or damage. Importantly, the genetic interaction involving *ctf18*Δ and *rad3*Δ (Figure 3) has suggested that Ctf18 has a checkpoint-independent

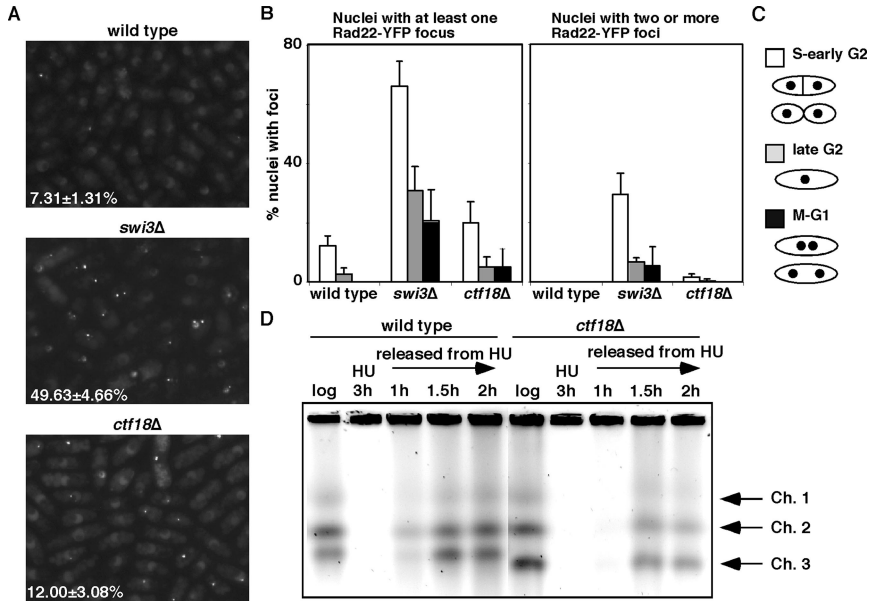


**Figure 4.** Ctf18 is involved in stabilization of replication forks. (A) Rad22-YFP foci formation was significantly elevated in *ctf18*Δ cells. Cells of the indicated genotype expressing genomic Rad22-YFP were grown in YES medium at 25°C until mid-log phase. The percentages of nuclei with at least one Rad22-YFP focus are shown. The standard deviations were obtained from four independent experiments (B) Quantification of Rad22-YFP foci according to cell cycle stages. S and early G2 cells had the most Rad22-YFP foci. The percentages of nuclei that have at lest one focus or harbor two or more foci are shown. At least 200 cells were counted for each strain. Error bar corresponds to the standard deviation obtained from four independent experiments. (C) Schematic drawing for nuclear and morphological changes during the *S. pombe* cell cycle. (D) Ctf18 is required for the efficient resumption of replication after fork arrest. Chromosome samples from either wild-type or *ctf18*Δ cells were examined by PFGE. Cells were grown until mid-log phase and then incubated in the presence of 12 mM HU for 3 h at 30°C. Cells were then washed and released into fresh medium. Chromosomal DNA samples were prepared at the indicated times. Representative results from repeat experiments are shown.

function during the S phase stress response. Because Ctf18 is essential in cells defective for the replication fork protection complex that consists of Swi1 and Swi3 (Figure 1), we speculated that Ctf18 has a critical role in stabilization of replication forks in a manner independent of Swi1-Swi3. In previous studies, we have shown that *swi1*Δ or *swi3*Δ cells accumulate spontaneous DNA damage near replication forks (Noguchi *et al*., 2004). Therefore, we investigated whether *ctf18*Δ cells also accumulate DNA damage in the absence of genotoxic agents. *ctf18*Δ cells were engineered to express Rad22-YFP from its genomic locus. Rad22 is a homologue of budding yeast Rad52 and shown to bind single-stranded DNA (ssDNA) during homologous recombination and at double-strand breaks and other sites that have an exposed ssDNA segment, leading the formation of Rad22-YFP DNA repair foci at the site of DNA damage (Ostermann *et al*., 1993; Kim *et al*., 2000; Noguchi *et al*., 2003). As reported previously (Noguchi *et al*., 2004), a dramatic increase in spontaneous Rad22-YFP foci was detected in *swi3*Δ cells (49.63 ± 4.66% of total *swi3*Δ nuclei compared with 7.31 ± 1.31% of wild-type nuclei) (Figure 4A). A significant increase in Rad22-YFP foci was also observed in *ctf18*Δ cells (12.00 ± 3.08% of *ctf18*Δ nuclei) (Figure 4A). Particularly, we reproducibly found that *ctf18*Δ cells but not wild-type cells contained nuclei with multiple Rad22 foci (Figure 4B). These data indicate that both *swi3*Δ and *ctf18*Δ cells accumulate spontaneous DNA damage although a smaller number of *ctf18*Δ cells displayed Rad22-YFP foci. To address whether Rad22 foci arose from replication abnormalities, the cell cycle position of cells containing Rad22-YFP foci was evaluated (Figure 4B). Cell cycle position in fission yeast can be estimated by noting cell length, nuclear morphology, and the appearance of septum as shown in Figure 4C. This analysis demonstrated that Rad22-YFP foci formed predominantly in S or early G2 phase (Figure 4B). Importantly, we found that accumulation of Rad22-YFP foci in *ctf18*Δ cells was specifically noticeable in S phase, suggesting that replication fork abnormality causes spontaneous DNA damage in *ctf18*Δ cells. After S phase, the number of foci then decreased throughout the cell cycle. Similar results have been obtained with *swi1*Δ and *swi3*Δ cells (Figure 4B) (Noguchi *et al*., 2003, 2004).

To further address the role of Ctf18 in the stabilization of replication forks, we examined the recovery of DNA replication after fork arrest provoked by HU exposure. Chromosome samples of wild-type and *ctf18*Δ cells were prepared before (log) and at 3 h after HU treatment, and also at different time points during recovery after the removal of HU (Figure 4D). These chromosomes were then resolved by PFGE, which allows only a fully replicated chromosome to enter the gel. Chromosomes from exponentially growing cells (log) in both wild-type and *ctf18*Δ migrated into the gel, indicating that *ctf18*Δ cells have no significant defect in replicating DNA (Figure 4D, log). HU treatment caused an arrest of DNA synthesis, leading to the reduction in the amount of chromosomes migrated into the gel in both wild-type and *ctf18*Δ cells (Figure 4D, HU 3 h). When cells are released into fresh medium without HU, chromosomes from wild-type cells entered into the gel at 1 h after the HU removal due to the completion of DNA synthesis. However, chromosomes from *ctf18*Δ cells did not migrate at this time point and displayed a reduced capacity to enter the gel at either 1.5 or 2 h during recovery (Figure 4D). We also performed PFGE of chromosomes prepared from *swi1*Δ cells and found that *swi1*Δ cells show even more severe delay in recovery of DNA replication after fork arrest (Supplemental Figure S1). These data are consistent with our aforementioned results that *swi1*Δ cells had a greater number of Rad22-YFP foci and stronger HU sensitivity compared with *ctf18*Δ cells (Figures 1B and 4, A and B). We have also observed that *ctf18*Δ cells harbor a shorter chromosome III that contains ribosomal DNA repeats in *S. pombe*. We and other groups have reported similar findings in a number of mutants defective for replication and replication fork stabilization including *swi1*Δ, *swi3*Δ, and *sap1-48* (Supplemental Figure S1) (Sommariva *et al*., 2005; Noguchi and Noguchi, 2007). Taken together, the fact that *ctf18*Δ cells accumulate DNA damage during S phase, are delayed in recovering from replication arrest, and are sensitive to fork damaging

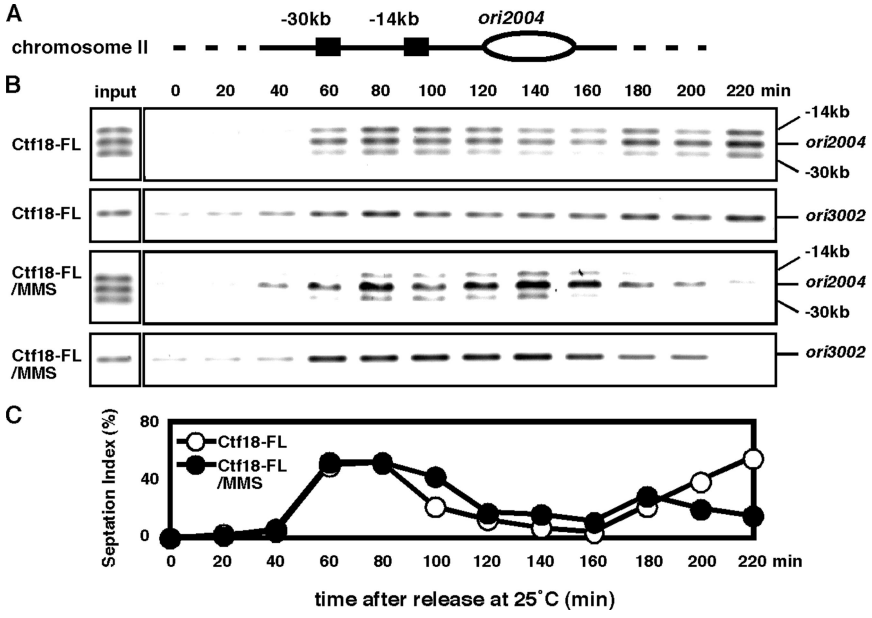


**Figure 5.** Ctf18 associates with chromatin during S phase. (A) Diagram of the *ori2004* region on *S. pombe* chromosome II used in ChIP is shown. (B) ChIP assay of Ctf18-5FLAG were performed at *ori2004*, at sites located 14 or 30 kb away from this origin and at *ori3002* as indicated. *cdc25-22* cells were synchronized at the G2-M boundary by incubation at 36°C for 4 h and then released into fresh YES medium containing 0 or 0.03% MMS at 25°C as indicated. ChIP assays were performed at the indicated times. ChIP of input (whole-cell extract) samples shows that 3 PCR products at *ori2004* amplify equally with the primers. Representative results from repeat experiments are shown. (C) An increase in the septation index indicates the onset of S phase.

agents, we conclude that Ctf18 is involved in the stabilization of replication forks.

We have previously shown that Swi1-Swi3 is required for the stabilization of replication forks and that *swi1*Δ or *swi3*Δ shows strong synthetic genetic interaction with *rqh1*Δ (Table 1) (Noguchi *et al*., 2003, 2004). Rqh1, RecQ-like DNA helicase, and its *S. cerevisiae* orthologue Sgs1, are thought to be required for the stabilization of replication forks (Doe *et al*., 2002; Cobb *et al*., 2003; Bjergbaek *et al*., 2005). Therefore, to understand pathways involved in fork stabilization or S phase stress response, we examined genetic interaction between *rqh1*Δ and *ctf18*Δ. *rqh1*Δ cells showed significant sensitivity to MMS and CPT (Supplemental Figure S2A). *rqh1*Δ *ctf18*Δ double mutant cells were much more sensitive to MMS and CPT than either single mutant cells (Table 1 and Supplemental Figure S2A). Together with the fact that *swi1*Δ or *swi3*Δ has strong genetic interaction with *rqh1*Δ (Table 1), we conclude that Swi1-Swi3, Ctf18, and Rqh1 play separate but redundant roles in the stabilization of replication forks.

### *Ctf18 Is Recruited to Chromatin during S Phase*

In budding yeast, Ctf18 has been shown to be recruited to replication forks in response to HU, although whether Ctf18 is localized at replication forks in the absence of genotoxic agents is unknown (Lengronne *et al*., 2006; Ogiwara *et al*., 2007). Therefore, we examined whether Ctf18 is associated with chromatin in unperturbed fission yeast cells. The *cdc25-22* strain was engineered to express Ctf18-5FLAG via its endogenous promoter. *ctf18-5FLAG* strains showed normal growth rate and no detectable sensitivity to UV, HU, MMS, or CPT, indicating that Ctf18-5FLAG is functional (data not shown). The *cdc25-22* allele was used to synchronize cells at the G2-M boundary. The localization of Ctf18-5FLAG was monitored by ChIP analysis at the well-characterized replication *origin 2004* (*ori2004*) and at two positions 14 and 30 kb away on *S. pombe* chromosome II (Figure 5A). On release from the *cdc25-22* arrest, Ctf18-5FLAG was observed to strongly associate with the *ori2004* region at 60 min, which subsequently declined between 120 and 160 min and increased again at 180 min (Figure 5B). We also examined septation, which in fission yeast occurs in S phase, to monitor cell cycle progression (Figure 5C). The level of Ctf18 association with the *ori2004* region was found to correlate with an increase in the septation index, which also coincided with the onset of S phase, and this association was found to decline as the septation index decreased (Figure 5, B and C), indicating that Ctf18 tightly associates with the *ori2004* region during unperturbed S phase. Similar association of Ctf18 with chromatin was observed at the *ori3002* region (Figure 5B), another active replication origin (Dubey *et al*., 1996), indicating that Ctf18 interacts with chromatin during DNA replication. A weaker association of Ctf18 with chromatin was also observed at 14- and 30-kb positions near *ori2004* (Figure 5B). It should be noted that similar chromatin association has been reported with Mcm6, a component of putative replicative DNA helicase in *S. pombe*. Therefore, our results suggest that Ctf18 is involved in DNA replication in unperturbed cells. To further understand the role of Ctf18 in S phase response pathways, we performed ChIP assays of Ctf18-5FLAG in the presence of MMS. As shown in Figure 5B, Ctf18-5FLAG was observed to associate with *ori2004* and *ori3002* as the septation index increase. This association seemed to be slightly stronger than that in unperturbed cells and persisted throughout extended S phase due to the MMS treatment, further strengthening our conclusion that Ctf18 tightly associate with chromatin during S phase. This result also suggests that the association of Ctf18 with chromatin plays an important role in the S phase stress response.

### *Chl1 Is Involved in an S Phase Stress Response Pathway*

The dosage suppression of *swi1*Δ cells by *chl1*$^+$ in HU and MMS sensitivity assays (Figure 1B) suggested that Chl1 is also involved in the tolerance of S phase stresses. To confirm this idea, we have performed a series of drug sensitivity assays of *chl1*Δ cells. *chl1*Δ cells seemed to have no significant sensitivity to MMS or CPT (Figure 6A). However, when combined with *chk1*Δ, *chl1*Δ *chk1*Δ double deletion cells showed a significant increase in MMS and CPT sensitivity, suggesting that, in response to S phase DNA-damaging agents, *chl1*Δ cells accumulate unusual DNA structures that must be repaired by the Chk1-dependent G2-M checkpoint. Moreover, *chl1*Δ *rad3*Δ cells are much more sensitive to

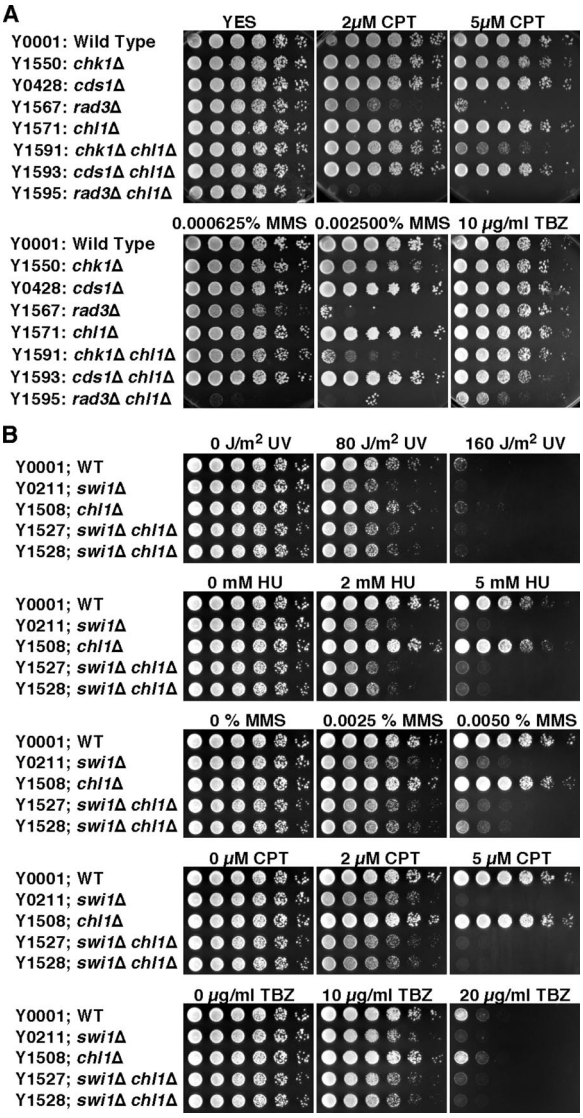


**Figure 6.** Chl1 is involved in an S phase response pathway. (A and B) For drug sensitivity assays, fivefold serial dilution of cells were incubated on YES agar medium supplemented with the indicated amounts of MMS, CPT, HU, or TBZ for 2–4 d at 32°C. For UV survival assays, fivefold serial dilution of cells of the indicated genotypes were spotted onto YES agar medium and exposed to the indicated doses of UV irradiation. The plates were then incubated for 2–3 d at 32°C. Representative images of repeat experiments are shown.

MMS and CPT than either single mutant (Figure 6A and Table 1), suggesting that the involvement of Chl1 in an S phase DNA damage response is independent of cell cycle checkpoints. To further investigate the role of Chl1 in S phase stress response pathway, we examined whether *swi1*Δ *chl1*Δ double mutant cells showed increased levels of sensitivity to S phase-stressing agents and found that there was no significant genetic interaction between *swi1*Δ and *chl1*Δ in drug sensitivity assays including UV, HU, MMS, CPT, and TBZ (Figure 6B and Table 1). Considering the fact that an increased dosage of *chl1*$^{+}$ suppresses the HU and MMS sensitivity of *swi1*Δ cells (Figure 1B), our results are consistent with the notion that Chl1 and Swi1 are in the same pathway required for tolerance of S phase stresses. It should also be noted that *swi1*Δ, *swi3*Δ, and *chl1*Δ all showed synthetic lethal interaction with *ctf18*Δ (Figure 1A), again strengthening the idea that the Chl1-dependent S phase stress response pathway is independent of, but partially redundant with, the Ctf18-regulated mechanism.

### *Swi1-Swi3, Ctf18, and Chl1 Are Involved in Sister Chromatid Cohesion*

In budding yeast, Ctf18 has been shown to be required for proper chromosome cohesion (Hanna *et al*., 2001; Mayer *et al*., 2001). It has also been reported that some S phase checkpoint proteins are essential in mutants defective in the sister chromatid cohesion pathway (Mayer *et al*., 2004; Warren *et al*., 2004), suggesting a tight link between DNA replication and sister chromatid cohesion. Consistent with this notion, we found that *swi1*Δ or *swi3*Δ are synthetically lethal with a mutation in Rad21/Scc1 (*rad21-K1*) (Table 1), a subunit of the cohesin complex. In addition, we have also found that *ctf18*Δ or *chl1*Δ shows synergistic genetic interaction with *rad21-K1* (Table 1 and Supplemental Figure S2B), suggesting the importance of Swi1-Swi3, Ctf18, and Chl1 in sister chromatid cohesion in *S. pombe*. To monitor cohesion defects in *swi1*, *swi3*, *ctf18*, and *chl1* mutants, we used a strain that has the bacterial LacO tandem repeat sequences inserted at the *lys1* locus located in the vicinity of the centromere on chromosome I. This strain is engineered to express the LacI repressor fused to GFP-NLS, which is recruited to LacO repeat sequences, allowing us to visualize the centromere I (Ding *et al*., 2004). If sister chromatids are properly adhered to one another, the GFP signal should occur as a single focus in the nuclei of metaphase cells. However, if sister chromatids are prematurely separated, two distinct GFP foci will occur in the nuclei of metaphase cells (Figure 7A). Using this system, we determined the effect of *swi1*, *swi3*, *ctf18*, or *chl1* mutations on cohesion at the centromere region. For synchronization, cells were first arrested at the beginning of S phase by a 2-h incubation in the presence of HU. To obtain metaphase-arrested cells, cells were then released into medium containing TBZ, a compound that depolymerizes tubulin. Because sister chromatids are still attached to one another at metaphase, most of wild-type cells showed a single centromere focus in nuclei (Figure 7, A and B). In contrast, the experiments revealed a significant increase in the number of *swi1*Δ nuclei with two foci (Figure 7, A and B). We also obtained similar results with *swi3*Δ, *ctf18*Δ, and *chl1*Δ cells (Figure 7, A and B), indicating a higher frequency of precocious sister chromatid separation in these mutants. To further confirm the importance of these factors in cohesion pathways, we repeated cohesion assays using two additional methods. In the first method (Figure 7C), *cdc25-22* cells were synchronized at G2-M and released into medium containing TBZ. As reported previously (Takeda *et al*., 2001), *rad21-K1*, which contains a temperature-sensitive mutation in a cohesin subunit, showed a strong defect in sister chromatid cohesion. There is an increase in the percentage of cells with two centromere I foci in *swi1*Δ, *swi3*Δ, *ctf18*Δ, and *chl1*Δ cells (Figure 7C), further confirming our conclusion that these factors play important roles in sister chromatid cohesion. In the second method (Figure 7D), *nda3-KM311* cold-sensitive cells (Hiraoka *et al*., 1984) were arrested at prophase by culturing them at 20°C. Again, we observed reproducible precocious sister chromatid separation in all the mutants we tested.

Interestingly, *swi1*Δ, *swi3*Δ, and *ctf18*Δ cells, and *rad21-K1* cells, were found to be sensitive to TBZ (Figure 7E). Although *chl1*Δ cells were not sensitive to TBZ, *chl1*Δ showed synergistic genetic interaction with *rad3*Δ in TBZ sensitivity assays (Figure 6A and Table 1). TBZ sensitivity is found

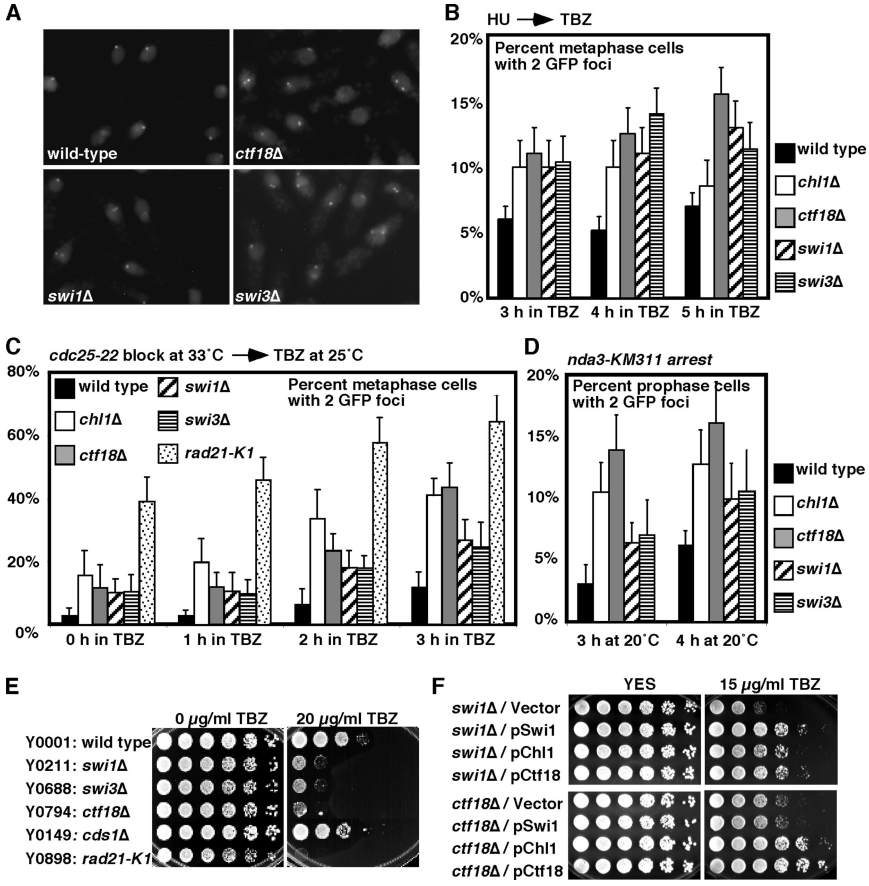


**Figure 7.** Ctf18, Chl1, and FPC play an important role for proper sister chromatid cohesion. (A) Cells of the indicated genotypes that had LacO repeats near centromere 1 and expressed LacI-GFP-NLS were grown to mid-log phase and supplemented with 10 mM HU for 2 h to synchronize cells in early S phase. Cells were then released into fresh YES medium containing 100 $\mu$g/ml TBZ for 3, 4, and 5 h to obtain metaphase cells. Representative images at 4 h in TBZ are shown for cells of the indicated genotypes. (B) Quantification of metaphase cells that had two GFP foci shown in A. At least 200 cells were counted for each strain. Error bar corresponds to the standard deviations obtained from three independent experiments. (C) *cdc25-22* cells with the indicated genotypes were arrested at G2-M and released into the cell cycle in the presence of 100 $\mu$g/ml TBZ. Quantification of metaphase cells that had two GFP foci was performed at the indicated times as described above. (D) *nda3-KM311* cells with the indicated genotypes were arrested at prophase, and cells with two GFP foci was quantified at the indicated times as described above. (E) Swi1-Swi3 and RFC$^{Ctf18}$ are required for cellular tolerance to a microtubule drug, TBZ. Fivefold serial dilution of cells of the indicated genotypes were incubated on YES agar medium supplemented with 0 or 20 $\mu$g/ml TBZ for 3 d at 30°C. (F) Genetic interaction between *swi1*, *chl1* and *ctf18*. *swi1*Δ or *ctf18*Δ cells were transformed with the indicated plasmid and plated on YES medium containing 0 or 15 $\mu$g/ml TBZ. Fivefold serial dilution of cells were incubated for 2–3 d at 32°C. Representative images of repeat experiments are shown.

among mutants that affect general sister chromatid cohesion and segregation (Tatebayashi *et al*., 1998; Wang *et al*., 2002; Williams and McIntosh, 2002; Silverstein *et al*., 2003). Therefore, we used TBZ sensitivity to further understand the pathways involving Swi1-Swi3, Ctf18, and Chl1 (Figure 7F and Table 2). TBZ sensitivity of *swi1*Δ was partially rescued by a dosage increase in *chl1*$^+$ or *ctf18*$^+$, whereas TBZ sensitivity of *ctf18*Δ is strongly rescued by *chl1*$^+$, but not by *swi1*$^+$. This is a striking contrast with our earlier observation that a dosage increase in *chl1*$^+$ failed to rescue HU or MMS sensitivity of *ctf18*Δ (Figure 1B and Table 2). Therefore, our results suggest that Ctf18 and Chl1 have partially redundant roles in cellular tolerance to tubulin poison TBZ.

## DISCUSSION

### *Roles of RFC$^{Ctf18}$ and Swi1-Swi3 in Activation of Replication Checkpoint and Fork Stabilization*

Ctf18 is an RFC1-like subunit of the alternative replication factor C complex and thought to be involved in loading or unloading of PCNA, a trimeric sliding clamp required for DNA synthesis in the budding yeast *S. cerevisiae* and humans (Bermudez *et al*., 2003; Shiomi *et al*., 2004; Bylund and Burgers, 2005). Ctf18 is also implicated in the replication checkpoint and functions redundantly with Rad24 to activate Rad53, a budding yeast homolog of Cds1 (Naiki *et al*., 2001), although it is not clear whether Ctf18 is involved in replication fork stabilization in this organism. The results of the present study suggest that fission yeast Ctf18 also forms an alternative RFC complex, RFC$^{Ctf18}$ (Figure 1D) and that it is involved in the Cds1-dependent replication checkpoint pathway and stabilization of stalled replication forks. As with *swi1*Δ, *ctf18*Δ shows a significant decrease in HU-induced activation of Cds1 (Figure 2C) and a significant delay in resumption of DNA replication after fork arrest (Figure 4D). The decrease of Cds1 activity in *ctf18*Δ cells may be explained by one of the following mechanisms, or a combination of the three: 1) RFC$^{Ctf18}$ directly interacts with Cds1 to modulate its activity. 2) RFC$^{Ctf18}$ interacts with Mrc1, which is the mediator of Cds1 activation, to promote the replication checkpoint. 3) Inactivation of RFC$^{Ctf18}$ causes fork structures to become unstable, which in turn attenuates replication checkpoint activation. In this third model, we envision that RFC$^{Ctf18}$ preserves replication fork structures in a configuration that is recognized by the replication checkpoint sensors (Figure 8). *ctf18*Δ cells showed a significant increase in S phase-dependent accumulation of Rad22 DNA repair foci (Figure 4B), indicating that these cells generate ssDNA stretches during DNA synthesis in the absence of genotoxic agents. This suggests that Ctf18 has a checkpoint independent function, which is required for stabilization of replication forks during normal S phase. Therefore, we prefer the third model in which RFC$^{Ctf18}$ acts at replication forks during normal DNA synthesis to modulate replication fork or replisome structures in a replication competent state to facilitate proper Cds1 activation (Figure 8). Consistently, our result suggests that Ctf18 may associate with replication origins during both unperturbed and arrested S phase (Figure 5). Although we are not able to detect relocation of Ctf18 along the chromosomes, it is still possible that Ctf18 moves with replication forks to stabilize them as in the case of Swi1-Swi3. Similarly, Mcm6 has been observed

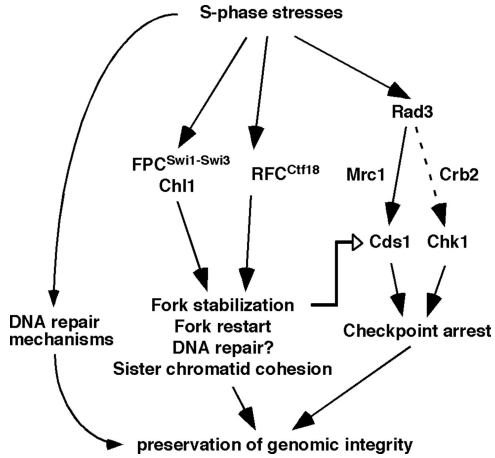


**Figure 8.** Models for S phase stress response mechanisms in *S. pombe*. RFC$^{Ctf18}$ is involved in Cds1-dependent replication checkpoint. FPC$^{Swi1-Swi3}$, RFC$^{Ctf18}$, and Chl1 also have checkpoint independent functions that are important for fork protection and DNA repair. In this model, FPC$^{Swi1-Swi3}$, RFC$^{Ctf18}$, and Chl1 stabilize replication forks in a configuration that is recognized by replication checkpoint sensors. Chl1 may work together with FPC$^{Swi1-Swi3}$to facilitate fork protection. In addition, FPC$^{Swi1-Swi3}$and RFC$^{Ctf18}$ act in parallel to facilitate proper chromosome cohesion.

to display no significant association with sites at 14 or 30 kb away from *ori2004* in *S. pombe* (Ogawa *et al*., 1999), although budding yeast Mcm proteins have been shown to travel with replication forks (Aparicio *et al*., 1997). It is possible that not all cells initiate DNA synthesis at *ori2004* in fission yeast but all *ori2004* sites recruits Ctf18, resulting in dilution of fork-bound Ctf18 proteins in our cell extract preparation. Therefore, we speculate that Ctf18 recognizes replication origins and relocates along the chromosome. In contrast, we have previously shown that Swi1, Swi3, and RPA relocate along the chromosome (Noguchi *et al*., 2004), suggesting that these proteins may recognize replication fork structures after origin unwinding.

### *Role of RFC$^{Ctf18}$ and Swi1-Swi3 in Sister Chromatid Cohesion*

Emerging evidence suggests that DNA replication is coupled with sister chromatid cohesion (Skibbens, 2005). Cohesin proteins are loaded onto chromatin during G1 phase, and chromosomal cohesion is established during DNA replication when replication forks pass through cohesin rings (Skibbens, 2005; Lengronne *et al*., 2006). This is probably when cohesin and fork components interact together to establish chromosomal cohesion.

In this study, we showed that both RFC$^{Ctf18}$ and Swi1-Swi3 play an important role in the activation of the replication checkpoint, fork stabilization, and sister chromatid cohesion. However, it is still unclear how Ctf18 is acting to facilitate chromosomal cohesion. Our data suggest that Ctf18 may recognize replication origins. This is interesting in light of the fact that the origin recognition complex functions in sister chromatid cohesion in budding yeast (Shimada and Gasser, 2007). Therefore, Ctf18-origin interaction might be important for proper establishment of cohesion. Another possibility is that Ctf18 is required to either recruit cohesin or related factors onto DNA, or to help maintain their association with chromosomes. Several different mechanisms can be proposed for the importance of replication fork maintenance in sister chromatid cohesion: 1) The cohesin ring may be an obstacle for replication fork progression. In this model, cohesin may cause a pausing of replication forks. Because paused replication forks are prone to collapse, there may be an increased requirement for fork stabilizing proteins, such as Swi1-Swi3 and RFC$^{Ctf18}$, at cohesin sites. 2) Components of the replication fork might be required to aid in stabilizing cohesin complexes during DNA synthesis. Lengronne *et al*. (2006) have proposed a model in which cohesin rings may transiently dissociate when forks pass through them. This is possible if the replisome complex is too large to fit through the cohesin ring. This suggests that fork components might preserve cohesin structures or tether cohesin-related proteins to DNA when forks pass through the ring. We speculate Ctf18 and/or the Swi1–Swi3 complex may be required for these functions, thereby facilitating proper establishment of sister chromatid cohesion. In support of this idea, a human Ctf18 homologue has been shown to interact with various cohesin proteins (Bermudez *et al*., 2003). 3) Ctf18 might be needed to act as a clamp loader or unloader through cohesin-rich regions. Bylund *et al*. has suggested that Ctf18-dependent unloading of PCNA might loosen the replication fork structure so that replication forks are able to pass through the cohesin ring without its temporal dissociation (Bylund and Burgers, 2005). Alternatively, Ctf18-dependent unloading and reloading of PCNA may facilitate a polymerase switch at cohesin sites. Consistent with this notion, it has been reported that human RFC$^{Ctf18}$ physically interact with DNA polymerase $\eta$ and stimulate its activity (Shiomi *et al*., 2007). In the future, it would therefore be interesting to examine whether RFC$^{Ctf18}$ and Swi1-Swi3 are required for this mechanism.

### *Role of Chl1 in Preservation of Genomic Integrity*

We have also identified a putative DNA helicase, Chl1, as a dosage suppressor of *lws1-1 swi1*Δ synthetic lethality. In budding yeast, Chl1 has been thought to be involved in DNA damage response, preservation of genomic integrity during S phase, and efficient sister chromatid cohesion (Mayer *et al*., 2004; Petronczki *et al*., 2004; Skibbens, 2004; Warren *et al*., 2004). In humans, Chl1 has been shown to exhibit DNA helicase activity and to be involved in sister chromatid cohesion (Hirota and Lahti, 2000; Parish *et al*., 2006). These Chl1 functions seemed to be evolutionarily conserved, because, in our present study, we have shown that fission yeast Chl1 is involved in the S phase stress response and efficient sister chromatid cohesion. Our data also suggest that *chl1*Δ cells accumulate abnormal DNA structures that activate the checkpoint response and that Chl1 is involved in the maintenance of replication forks. Interestingly, our genetic studies involving *chl1*$^+$, *ctf18*$^+$, and *swi1*$^+$ suggested the possibility that Chl1 and Swi1 are in the same pathway to preserve genomic integrity and that this pathway is working in parallel with the pathway involving Ctf18 (Figure 8). In support of this idea, budding yeast Chl1 and Tof1-Csm3 have been shown to be in the same genetic pathway required for sister chromatid cohesion (Xu *et al*., 2007). It has also been reported that Tof1-Csm3 and Ctf18 function in different pathways (Xu *et al*., 2007), indicating evolutional conservation in pathways involving Swi1-Swi3, Ctf18, and Chl1. Therefore, we speculate that Chl1 and Swi1 cooperate to stabilize replication forks as ancillary components of the replisome and promote proper establishment of sister chromatid cohesion, thereby preserving genomic integrity (Figure 8).

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