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Assays Used to Study the DNA Replication Checkpoint in Fission Yeast

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Summary

The DNA replication checkpoint, also known as the intra-S or S-phase checkpoint, plays a central role in ensuring the accuracy of DNA replication. When replication is impeded by DNA damage or other conditions, this checkpoint delays cell cycle progression and coordinates resumption of replication with DNA repair pathways. One of its critical functions is to stabilize stalled replication forks in a replication-competent state, presumably by maintaining proper assembly of replisome components and preserving DNA structures. Here we describe a series of assays used to study the replication checkpoint. These assays allow us to investigate the specific functions of proteins involved in the replication checkpoint in fission yeast.

Keywords

The DNA replication checkpoint; S-phase stress response; Cds1; Chk1; Rad3; Cds1 kinase assays; Pulsed-field gel electrophoresis; Rad22-YFP DNA repair foci

1. Introduction

Environmental toxins or drugs can cause DNA damage and lead to an arrest of DNA replication forks (1–3). Arrested forks are among the most serious of threats to genomic integrity because they can collapse, break, or rearrange (4). To suppress these genome-destabilizing events, all eukaryotic cells are equipped with a DNA replication stress response pathway, termed the DNA replication checkpoint or the S-phase checkpoint (1–3). In humans, defects in this checkpoint cause genetic instability, leading to a strong predisposition to cancer (3, 5–11).

The replication checkpoint is activated when replication forks are arrested by DNA damage, protein complexes bound to chromatin, starvation of deoxyribonucleotides, or other conditions. This checkpoint arrests cell cycle progression, specifically preventing the onset of mitosis when DNA is not fully replicated, while at the same time regulating other less well-understood processes that are required for recovery from fork arrest (1–3). Central to this system are protein kinases such as human ATM and ATR (1–3, 5, 12). In humans, major ATM/ATR downstream targets include p53, Chk1, and Chk2. Both Chk1 and Chk2 arrest the cell cycle by phosphorylating Cdc25, which inhibits its phosphatase activity and in some cases promotes its degradation, thereby preventing it from activating Cdc2 (Cdk1), a kinase essential for mitotic onset. The checkpoint proteins are also thought to facilitate DNA repair and recombination pathways (3, 5, 6). In the model organism *Schizosaccharomyces pombe*, the ATM/ATR homolog Rad3 also controls downstream effector kinases Cds1 (Chk2 homolog) and Chk1 (Fig. 1). Cds1 and Chk1 define redundant pathways of checkpoint activation in response to fork arrest, although Cds1 acts as the main kinase for

activation of the replication checkpoint (Fig. 1) (1, 3, 13–15). Thus, the mechanisms of checkpoint responses appear to be highly conserved throughout evolution.

Recent studies have identified a group of proteins that are involved in the activation of the replication checkpoint kinase Cds1 and the stabilization of replication forks. In fission yeast, Mrc1, a mediator of the replication checkpoint, is essential for Cds1 activation in a manner dependent on Rad3 (16, 17). Swi1 forms a replication fork protection complex with Swi3 and is required for proper activation of Cds1 and replication fork stabilization (18, 19). Hsk1-Dfp1, the Cdc7-Dbf4-related kinase, functions in conjunction with the Swi1–Swi3 complex and is also important for activation of Cds1 and fork stabilization (20, 21). Furthermore, Ctf18, a component of an alternative replication factor C complex, has shown to be involved in these mechanisms. Ctf18 and Swi1–Swi3 function in separate and redundant pathways required for the replication checkpoint and sister chromatid cohesion (22). Taken together, these facts suggest that a complicated network of proteins is involved in checkpoint signaling and fork stabilization, ensuring accuracy in copying the genome. In this chapter, we will describe a collection of experiments that we have used to investigate the replication checkpoint in *S. pombe*. These experiments include assays to evaluate sensitivities of cells to S-phase stressing agents, epistasis analysis involving checkpoint defective mutants, Cds1 kinase assay, pulsed-field gel electrophoresis (PFGE) of chromosomes, and visualization of DNA damage during S-phase.

2. Materials

2.1. Serial Dilution Growth Assays

1. *YES (yeast extract and supplements medium)*. 5 g/L yeast extract, 30 g/L glucose, 187.5 mg/L leucine, 187.5 mg/L histidine, 187.5 mg/L adenine, and 100 mg/L uracil.
2. 1 M hydroxyurea (HU): dissolved in water and stored at −20°C (Sigma-Aldrich, St. Louis, MO).
3. Methylmethane sulfonate (MMS) (straight solution) (Sigma-Aldrich).
4. 10 mM camptothecin (CPT) (Sigma-Aldrich): dissolved in DMSO and stored at −20°C.
5. Hemacytometer.
6. 10-cm petri dishes.
7. 100% ethanol.
8. Replica plater for 96-well plate (8 × 6 Array, 48-pin) (R2383, Sigma-Aldrich).
9. 8-channel pipetter.
10. UV crosslinker (we used a Stratalinker from Stratagene, La Jolla, CA).

2.2. Cds1 Kinase Assay

1. 1 M HU.
2. *STOP buffer*. 150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3.
3. Anti-Cds1 antibody (kindly provided by Dr. Teresa Wang, Stanford University).
4. Protein A-Sepharose.
5. *Lysis Buffer*. 50 mM Tris–HCl, pH 7.5, 80 mM β-glycerol phosphate, 250 mM NaCl, 15 mM nitrophenylphosphate, 50 mM NaF, 5 mM EDTA, 1 mM DTT, and

0.1% NP-40 supplemented with protease inhibitor cocktail (complete EDTA-free protease inhibitor cocktail from Roche, Basel, Switzerland) and *p*-4-amidoinophenyl-methane sulfonyl fluoride hydrochloride monohydrate (*p* APMSF) (Sigma-Aldrich): prepare fresh before use.

6. 0.5-mm glass beads.
7. FastPrep cell disruptor (Qbiogene, Irvine, CA).
8. Protein Assay Dye Reagent Concentrate (BioRad, Hercules, CA).
9. *2× Kinase Buffer*. 20 mM HEPES, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT.
10. *1× Kinase Buffer*. 10 mM HEPES, pH 7.5, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT.
11. γ-^{32}P-ATP (3,000 Ci/mmol).
12. 10 mM ATP.
13. 10 mg/mL MBP (Myelin Basic Protein, a Cds1 substrate): dissolved in water and stored at −20°C (Sigma-Aldrich).
14. *2× SDS-PAGE sample buffer*. 100 mM Tris–HCl, pH 8.0, 4% SDS, 4 mM EDTA, 20% glycerol, 0.005% bromophenol blue, and 10% mercaptoethanol.
15. PhosphorImager system (e.g., Storm 840 from GE Healthcare).
16. Liquid scintillation counter.

2.3. Pulsed-Field Gel Electrophoresis

1. CSE buffer: 20 mM citric acid, 20 mM Na_2 HPO_4, adjusted to pH 5.6, 1.2 M sorbitol, 40 mM EDTA.
2. 1 M HU.
3. Zymolyase 100T (Seikagaku, Tokyo, Japan).
4. *TSE buffer*. 10 mM Tris–HCl, pH 7.5, 0.9 M sorbitol, 45 mM EDTA, pH 8.0.
5. Low Melt Agarose (BioRad).
6. Plug molds for CHEF gel system (BioRad).
7. *Tris–EDTA–SDS buffer*. 0.25 M EDTA, pH 8.0, 50 mM Tris–HCl, pH 7.5, 1% SDS.
8. *NDS buffer*. 10 mM Tris base, 0.5 M EDTA, adjusted to pH 9.5, 1% lauryl sarcosine.
9. 20 mg/mL Proteinase K (Invitrogen, Carlsbad, CA): dissolved in water and stored at −20°C.
10. 0.5 M EDTA, pH 8.0.
11. *TE buffer*. 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0.
12. Megabase agarose (BioRad).
13. *1× TAE buffer*. 40 mM Tris–Acetate, pH 8.3, 1 mM EDTA
14. 50 mg/mL Ethidium bromide.
15. Hemacytometer.

16. Pulsed-field gel electrophoresis system (we used a CHEFDR II system from BioRad).

2.4. Rad22-YFP Foci Detection

1. Glass slides.
2. Glass coverslips.
3. Hemacytometer.
4. Fluorescence microscope.
5. *S. pombe rad22-YFP* strain (will be available from National BioResource Project, Japan)

3. Methods

3.1. Serial Dilution Growth Assays Used to Determine the Effects of Fork Stalling Agents

Stalled replication forks activate the replication checkpoint. Therefore, many checkpoint mutants show sensitivity to hydroxyurea (HU), which inhibits ribonucleotide reductase, thereby depleting the dNTP pool available for DNA synthesis and leading to stalled replication forks (1–3). Some mutants also show sensitivity to methylmethane sulfonate (MMS, which promotes alkylation of DNA templates, causing stalled replication forks), ultraviolet (UV, which causes the formation of cyclobutane dimers and other lesions leading to an arrest of replisome progression), or camptothecin (CPT, which induces replication fork breakage by trapping topoisomerase I-DNA complexes) (23–26). In *S. pombe*, mutations in Cds1, a master kinase for the replication checkpoint, render cells highly sensitive to HU (24). There is a group of proteins that are required for activation of Cds1 in fission yeast. These include Mrc1, Swi1–Swi3, and Ctf18, whose mutations also render cells sensitive to HU (16, 18, 19, 22). In addition, cells with mutations in proteins involved in fork stabilization, including Swi1–Swi3 and Ctf18, display sensitivity to MMS and CPT (22, 27). In this section, we describe the use of serial dilution growth assays, colloquially known as "spot assays," to evaluate sensitivity of *S. pombe* mutant cells to S-phase stressing agents. This approach serves as a first step toward understanding the possible role of a gene of interest in the activation of Cds1 and/or fork stabilization.

Cds1 and Chk1 define redundant pathways of checkpoint activation in response to fork arrest (1, 3, 13–15). Therefore, *cds1*sΔ *chk1*Δ double mutant cells display extreme sensitivity to HU and UV, both of which cause stalled replication forks. Because both Cds1 and Chk1 pathways are controlled by the Rad3 kinase (Fig. 1), cds1Δ chk1Δ and *rad3*Δ show similar sensitivities to HU or UV (1, 3, 13–15). Therefore, epistasis analysis with checkpoint mutants in HU and UV survival assays determines whether a gene of interest functions in the Cds1 or Chk1 pathway. For example, we have previously shown that the Ctf18 protein is involved in the Cds1-dependent checkpoint pathway (22). As shown in Fig. 2, ctf18Δ chk1Δ cells are more sensitive to HU and UV than either single mutant, while *ctf18*Δ*cds1*Δ and *ctf18*Δ *rad3* double mutant cells show HU and UV sensitivity similar to either single mutant. These results suggest that Ctf18 is involved in the Cds1-dependent replication checkpoint.

1. Inoculate *S. pombe* cells into 5 mL of YES, and grow cells overnight until the OD_{600} reaches ~1.0. If cells are overgrown (OD_{600} of more than 2.0), dilute cells into YES at OD_{600} of 0.4, and grow cells for 4 h.

2. Prepare 1/10 dilution of culture, and use 10 μL of diluted cell suspension to measure cell density in a hemacytometer (*see* Fig. 2 in Chapter "Chromatin Immunoprecipitation of Replication Factors Moving with the Replication Fork").
3. Adjust cell density to 2.0×10^7 cells/mL in a 1.5-mL microcentrifuge tube. If the cell density of a culture is less than 2.0×10^7, then centrifuge the culture and suspend cells in an appropriate amount of YES to obtain the cell density of 2.0×10^7 cells/mL.
4. Prepare two 10-cm petri dishes, one containing sterilized water and the other one containing 100% ethanol.
5. Sterilize the pins of a 48-pin-replica plater with 100% ethanol and the flame of a Bunsen burner. Allow the plater to cool to room temperature.
6. Using an 8-channel pipetter, add 200 μL of sterilized water from the 10-cm petri dish to columns #2 to #6 of a sterilized 96-well plate (*see* Fig. 3).
7. Add 250 μL of cell suspension (2.0×10^7 cell/mL) to column #1.
8. Mix the cell suspension by pipetting up and down using an 8-channel pipetter set to 50 μL, and transfer 50 μL of cell suspension to column #2 to make fivefold dilution. Repeat fivefold serial dilutions until column #6 (*see* Note 1 and Fig. 3).
9. Place the replica plater in cell suspensions on the 96-well plate to transfer cell suspension to pins.
10. Touch the replica plater to a YES agar plate or a YES agar plate containing a drug. Repeat the transfer of cells from the 96-well plate to another YES plate containing a different drug. It is important to wash pins of the replica plater with water and then with ethanol at each replication to avoid crosscontamination of different drugs. For UV sensitivity assays, replica plate cells to YES agar medium, and expose the YES plates to short-wavelength (254-nm) UV in a Stratalinker (*see* Note 2).
11. Allow agar plates to absorb cell suspension. Incubate agar plates at appropriate temperatures to allow cell growth. Compare cell growth of different cell lines after several days of incubation. Document using a digital camera or a scanner linked to a computer. Growth of eight strains can be simultaneously compared on a single plate (rows A through H, *see* Fig. 3).

3.2. Cds1 Kinase Assay

If a gene of interest is implicated in the Cds1-dependent replication checkpoint pathway, it is obviously important to test whether the mutant cells show a decreased level of Cds1 activity. In this section we describe a Cds1 kinase assay using myelin basic protein (MBP) as a substrate. We will treat cells with HU to activate Cds1. Figure 4 shows an example of Cds1 kinase assays using *swi1*Δ and *cds1*Δ mutants.

3.2. 1.Preparation of Cells

1. Inoculate *S. pombe* cells into 5 mL of YES, and grow cells overnight. Next day, dilute cells into 100 mL of YES and grow overnight until the OD_{600} reaches ~1.0. If cells are overgrown (OD of more than 2.0), dilute cells into YES at OD_{600} of 0.4, and grow cells for 4 h.

[1]We use water to dilute cells on a 96-well plate. Water has more surface tension compared to YES medium, allowing a better transfer of cells from a 96-well plate to a 48-pin replica plater.
[2]The cell suspension should be absorbed into the agar medium before UV irradiation. After UV irradiation, the plates should be incubated in dark in an incubator to avoid possible photoreactivation repair although this pathway may not exist in *S. pombe*.

2. Dilute cells at OD_{600} of 0.4 in 200 mL of YES and grow cells.
3. Add 2.4 mL of HU (final concentration 12 mM) and continue to grow cells.
4. Transfer 50 mL of cell culture to a centrifuge tube at indicated times (0, 1, 2, and 4 h after the addition of HU). Centrifuge at 1,000 × *g* for 3 min at 4°C to collect cells.
5. Resuspend cells in 10 mL of STOP buffer and centrifuge again.
6. Resuspend cells in 1 mL of STOP buffer, transfer to a 1.5-mL screw top tube (*see* Note 3), aspirate buffer, and immediately freeze cell pellet at −80°C.

3.2.2. Immunoprecipitation of Cds1

1. Prepare anti-Cds1 antibody-bound protein A beads a day before immunoprecipitation (*see* Note 4): Mix 20 μL bed volume of protein A sepharose (prewashed with Lysis buffer), 20 μL of Lysis buffer, and 1 μL of anti-Cds1 antibody for one sample. Rotate overnight at 4°C.
2. Suspend cell pellet with 200 μL of Lysis buffer.
3. Add glass beads until they reach the surface of the buffer.
4. Break cells using a FastPrep cell disruptor at 4°C (output 6.0, 20 s, two cycles, 2 min interval between cycles) (*see* Note 5).
5. To recover cell lysate, pierce the bottom of the tube with a heated needle, and place tube in a new 1.5-mL microcentrifuge tube. Centrifuge at 800 × *g* using a microcentrifuge for 30 s to collect cell lysate in the new tube.
6. Discard the tube containing glass beads, add 400 μL of Lysis Buffer to the cell lysate, and mix well.
7. Centrifuge at 16,000 × *g* for 5 min at 4°C, transfer supernatant to a new 1.5-mL microcentrifuge tube.
8. Centrifuge again at 16,000 × *g* for 10 min at 4°C, transfer supernatant to a new 1.5-mL microcentrifuge tube.
9. Measure protein concentration using BioRad Protein Assay Dye Reagent Concentrate and adjust concentrations to the lowest concentrated sample.
10. Add 40 μL of Cds1-antibody-bound protein A beads (50% slurry) prepared at **step 1** to each sample.
11. Rotate samples for 1–2 h at 4°C.
12. Wash beads three times with 500 μL of ice-cold Lysis Buffer
13. Wash beads three times with 500 μL of ice-cold 1× Kinase Buffer

3.2.3. Kinase Reaction

1. *Prepare Kinase Reaction Cocktail*. Mix 10 μL of 2× Kinase Buffer, 2 μL of γ-^{32}P-ATP (5 μCi), 0.2 μL of 10 mM ATP, 0.5 μL of 10 mg/mL MBP, and 7.3 μL of H_2O for one kinase reaction.

[3]It is important to use tubes that fit properly into the FastPrep cell disruptor.
[4]If the anti-Cds1 antibody is not available, use strains that are engineered to express tagged Cds1. In this case, an antibody against or an affinity column for the tag should be used. The *cds1–2HA6His* strain is available from National BioResource Project, Japan.
[5]It is important to monitor cell disruption under a microscope. More than 90% of cells should be disrupted.

2. Add 20 μL of Kinase Reaction Cocktail to antibody-bound protein A beads prepared earlier.
3. Incubate for 15 min at 30°C. Mix every 1–3 min to avoid precipitation of beads.
4. Stop the kinase reaction by adding 25 μL of 2× SDS Sample Buffer
5. Boil samples for 5 min, and store samples at −20°C.

3.2.4. Detection of Cds1 Kinase Activity

1. To visualize MBP, run a 15% SDS-PAGE gel using 10 μL of samples prepared earlier.
2. After SDS-PAGE, stain the gel with Coomassie Brilliant Blue, and dry the gel.
3. Wrap the dried gel with plastic wrap and detect radioactivity incorporated in MBP with a phosphorImager.
4. After imaging, cut out MBP bands. The radioactivity levels (cpm) of MBP bands should be determined in a liquid scintillation counter.

3.3. Pulsed-Field Gel Electrophoresis

One of most important functions of the replication checkpoint is to stabilize replication forks by maintaining proper assembly of replisome components and preserving DNA structures when problems are encountered (28–32). Therefore, it is also important to determine whether a protein involved in Cds1 activation is also required for replication fork stabilization. The replication fork stalls in the presence of HU; however, wild-type cells can recover from fork arrest because forks are maintained in a state competent for resumption of DNA synthesis. In contrast, if replication forks are not stably maintained in the absence of the proper replication checkpoint, forks may collapse or rearrange, resulting in a defect in replication recovery after fork abnormality. To evaluate fork stability in *S. pombe* mutants, we utilize pulsed-field gel electrophoresis (PFGE). Figure 5 shows an example of PFGE analysis of *ctf18*Δ cells treated with HU.

1. Prepare a midlog phase *S. pombe* cell culture (OD_{600} = 0.4) in 300 mL YES.
2. Monitor cell density using a hemacytometer, and collect a sample of 2.5×10^8 cells by centrifugation (1,000 × *g*, 3 min, 4°C) for a log-phase sample. Wash cells with CSE buffer once, and store cell pellet at −80°C, and proceed to **step 7**.
3. Add HU to the culture to a final concentration of 12 mM, and grow cells for additional 3 h in a 30°C shaker.
4. Monitor cell density using a hemacytometer, and collect a sample of 2.5×10^8 cells by centrifugation (1,000 × *g*, 3 min, 4°C) for an HU-treated sample. Wash cells with CSE buffer once, and store cell pellet at −80°C, and proceed to **step 7**.
5. Wash remaining cells twice with fresh YES medium, and return the culture to the 30°C shaker.
6. In 1, 2, and 4 h, monitor cell density using a hemacytometer, and collect a sample of 2.5×10^8 cells by centrifugation (1,000 × *g*, 3 min, 4°C). Wash cells with CSE buffer once, store cell pellet at −80°C, and proceed to **step 7**.
7. Suspend cells from the collected samples in 1 mL of CSE containing 1 mg/mL of Zymolyase 100T, and incubate at 37°C for 2 h.
8. Pellet cells by centrifugation (1,000 × *g*, 3 min, 4°C).

9. Resuspend cells at a concentration of 8×10^8 cells/mL in 300 μL TSE.
10. Warm the cell suspension to 42°C.
11. Add 300 μl of 1.1% low melting temperature agarose in TSE.
12. Dispense aliquots into plug molds (five aliquots per sample), and allow plugs to solidify for 30 min at 4°C.
13. Transfer plugs into a centrifuge tube containing 3 mL of Tris–EDTA–SDS, and incubate at 55°C for 90 min.
14. Replace the buffer with 3 mL of NDS supplemented with 1 mg/mL Proteinase K (Invitrogen, Carlsbad, CA), and incubate plugs at 55°C for 24 h (*see* Note 6).
15. Replace the buffer with 3 mL of fresh NDS supplemented with 1 mg/mL Proteinase K, and incubate plugs at 55°C for 24 h (*see* Note 6).
16. To analyze chromosome DNA embedded in plugs, equilibrate plugs in 5 mL TE three times for 30 min each (*see* Note 7).
17. Run on 0.8% Megabase agarose gel (BioRad, Hercules, CA) in 1× TAE using a CHEF-DR II system (BioRad, Hercules, CA) at the following settings: block 1, 2 v/cm, initial, and final switch time of 1,800 s, 14°C, pump speed 70, for 72 h.
18. Stain gels with 0.5 μg/mL ethidium bromide in H_2O for 30 min, then destain with water for 1–2 h.
19. Visualize chromosomes using a UV transilluminator.

3.4. Detection of Rad22-YFP DNA Repair Foci

Some factors involved in the replication checkpoint are also involved in replication fork stabilization even in the absence of genotoxic stress (18–20, 22). When cells experience collapsed replication forks, cells show an increase in DNA damage because of an accumulation of abnormal DNA structures at the replication forks (18, 19). To visualize DNA damage in live cells, we utilize cells expressing Rad22-YFP fusion protein from its endogenous promoter at its genomic locus. Rad22 is a homolog of budding yeast Rad52 and is shown to bind single-stranded DNA (ssDNA) during homologous recombination at double-strand breaks and other sites that have exposed ssDNA segments, leading to the formation of Rad22-YFP DNA repair foci at the site of DNA damage during S-phase (18, 33, 34). Figure 6 shows an example of spontaneous Rad22-YFP foci accumulated in *swi1* cells.

1. Inoculate cells expressing Rad22-YFP in 5 mL YES and grow cells at 25°C until midlog phase (*see* Note 8).
2. Collect cells by centrifugation (*see* Note 9) and keep cell pellet (with small amount of YES) on ice.
3. Place 2 μL of cell suspension (from the bottom of tube) on a glass slide. Cover the cell suspension with a 22 mm × 22 mm coverslip.
4. Observe cells with a fluorescence microscope, and capture Rad22-YFP fluorescence images. In total, more than 200 cells should be monitored.

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[6]To activate Proteinase K, samples containing Proteinase K should be preincubated for 30 min at 37°C.
[7]If plugs are not used for electrophoresis immediately, store the plugs in 5 mL 0.5 M EDTA at 4°C. Equilibrate plugs again as described in **Subheading 3.3.16** before electrophoresis.
[8]We grow cells at 25°C to obtain stronger yellow fluorescent protein (YFP) signals.
[9]We increase cell density by centrifugation. This allows us to monitor many cells in one image.

5. Estimate the cell cycle position of cells containing Rad22- YFP foci by analyzing the cell length, number, and position of nuclei, and the presence of a division plate (see Fig. 2 in Chapter “Chromatin Immunoprecipitation of Replication Factors Moving with the Replication Fork”).

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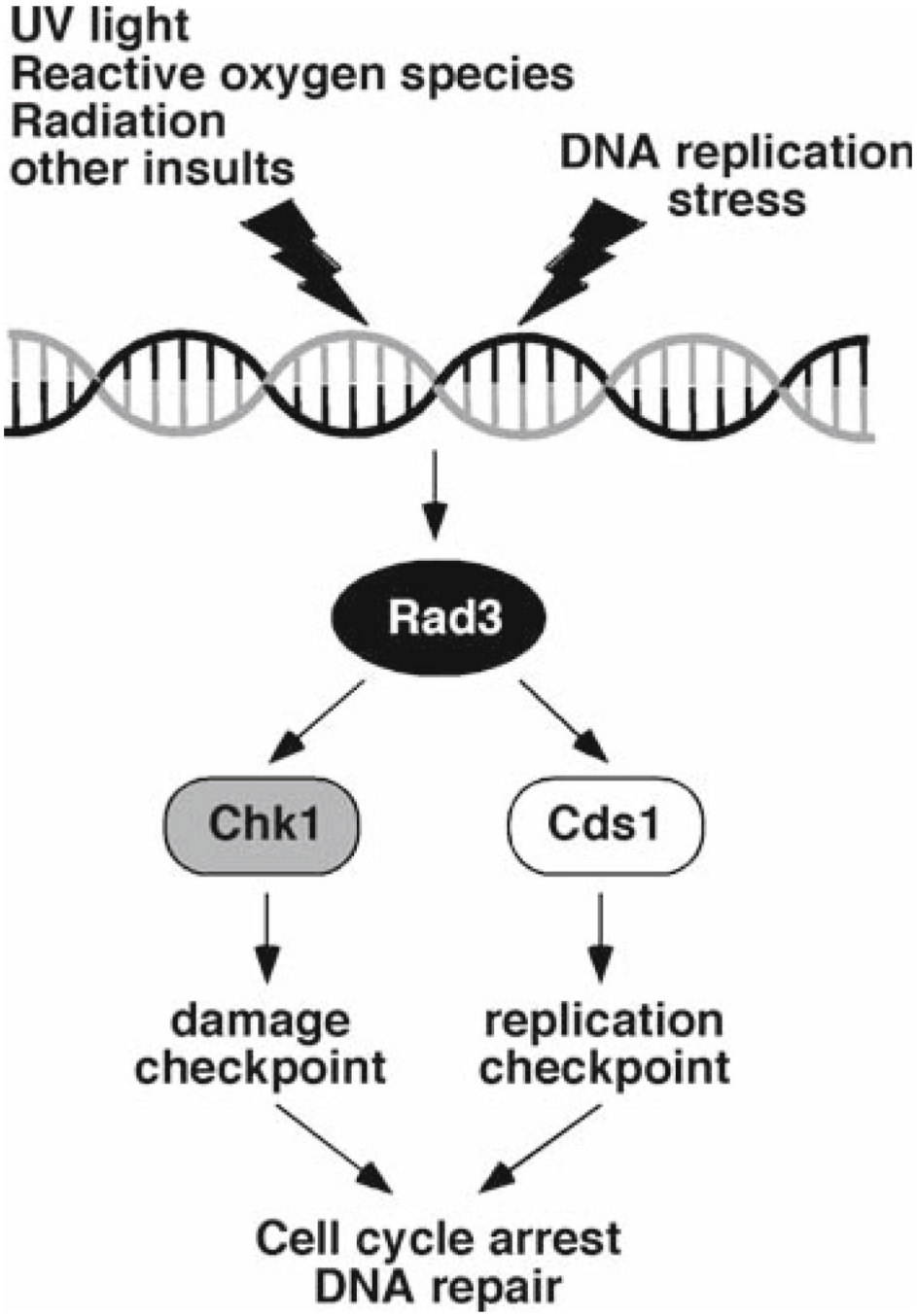

Fig. 1.

S. pombe checkpoint pathway. DNA damage or replication stress activates the Rad3-dependent checkpoint pathway. Rad3 sends a checkpoint signal to downstream checkpoint effectors, Chk1 and Cds1 to arrest the cell cycle and facilitate DNA repair pathways.

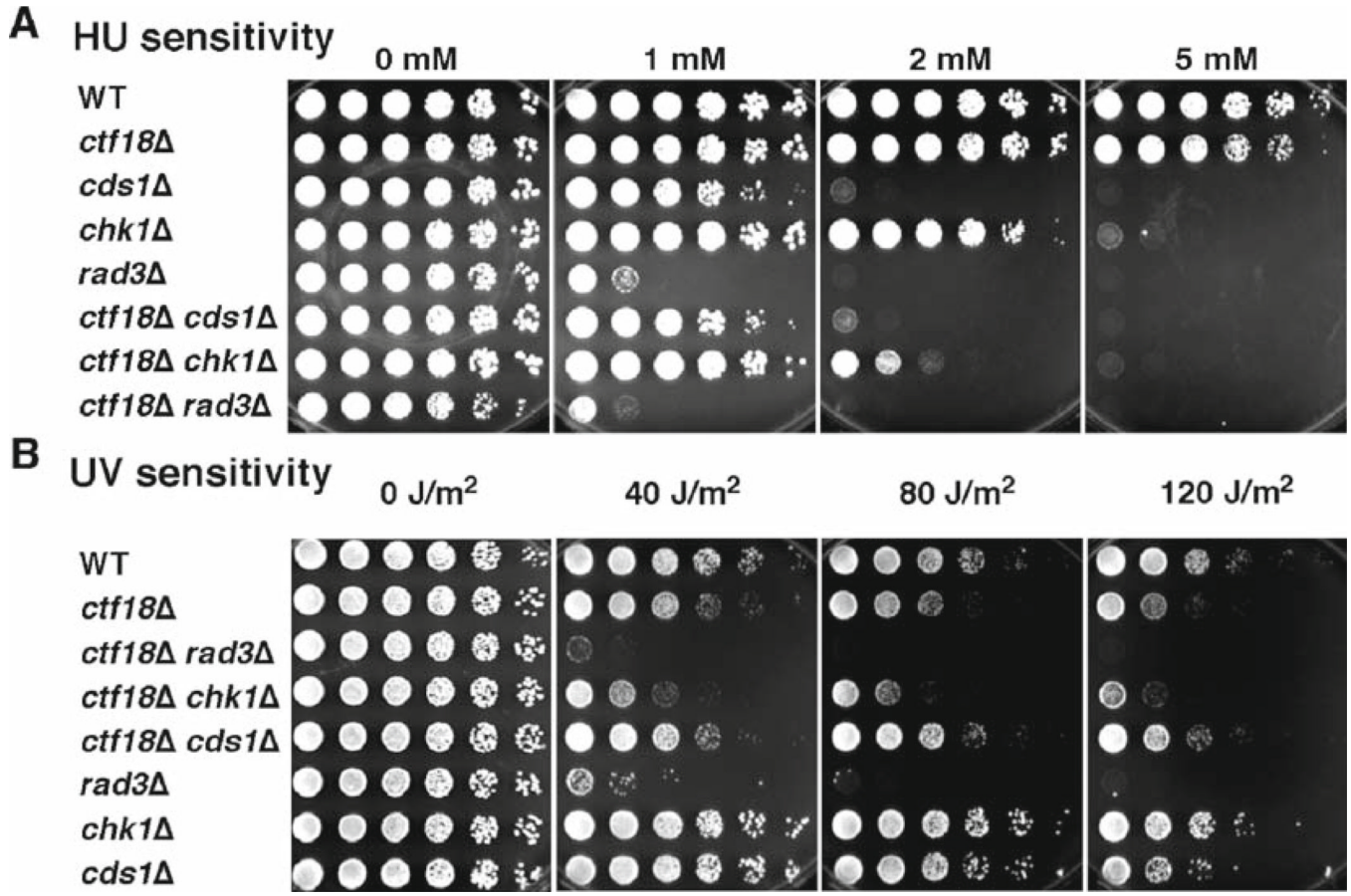

Fig. 2.

Ctf18 is involved in the replication checkpoint enforced by the Cds1 kinase. Synergistic interaction of *ctf18*Δ and *chk1*Δ in HU (**A**) and UV (**B**) survival assays shows that Ctf18 is required for survival of replication fork arrest. For HU sensitivity assays, fivefold serial dilution of cells was incubated on YES agar medium supplemented with the indicated amounts of HU for 2–4 days at 32°C. For UV survival assays, fivefold serial dilution of cells was plated on YES agar medium and exposed to the indicated doses of UV. Agar plates were then incubated for 2–3 days at 32°C. *ctf18*Δ showed a strong synergistic interaction with *chk1*Δ, but not with *cds1*Δ and *rad3*Δ, suggesting that Ctf18 is involved in the Cds1 pathway.

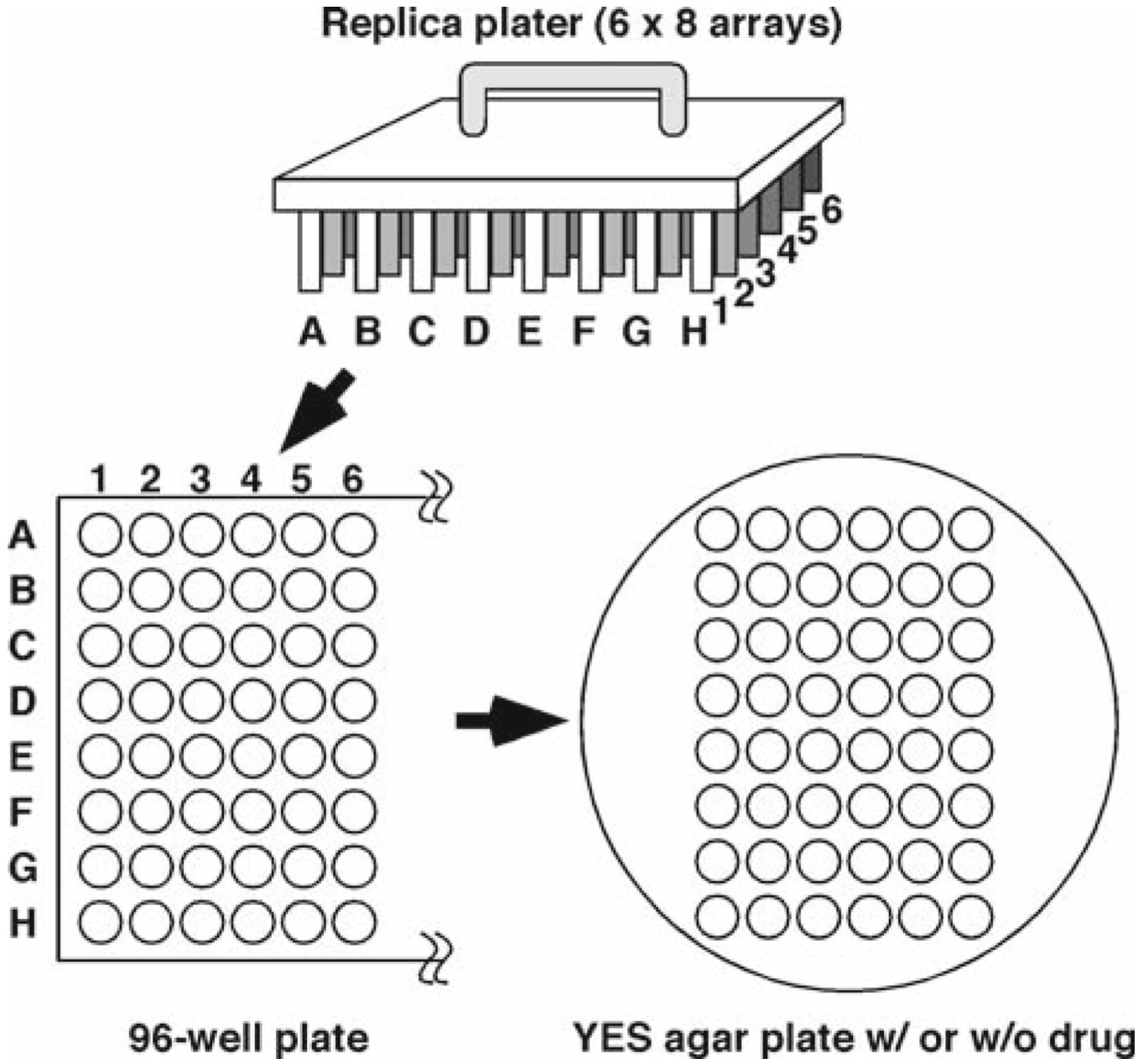

Fig. 3.

Using a 48-pin replica plater, serial dilution of cells can be transferred from a 96-well plate to a YES agar medium. Fivefold serial dilutions of cells are added from column #1 through #6. Eight strains (rows A through H) can be tested simultaneously.

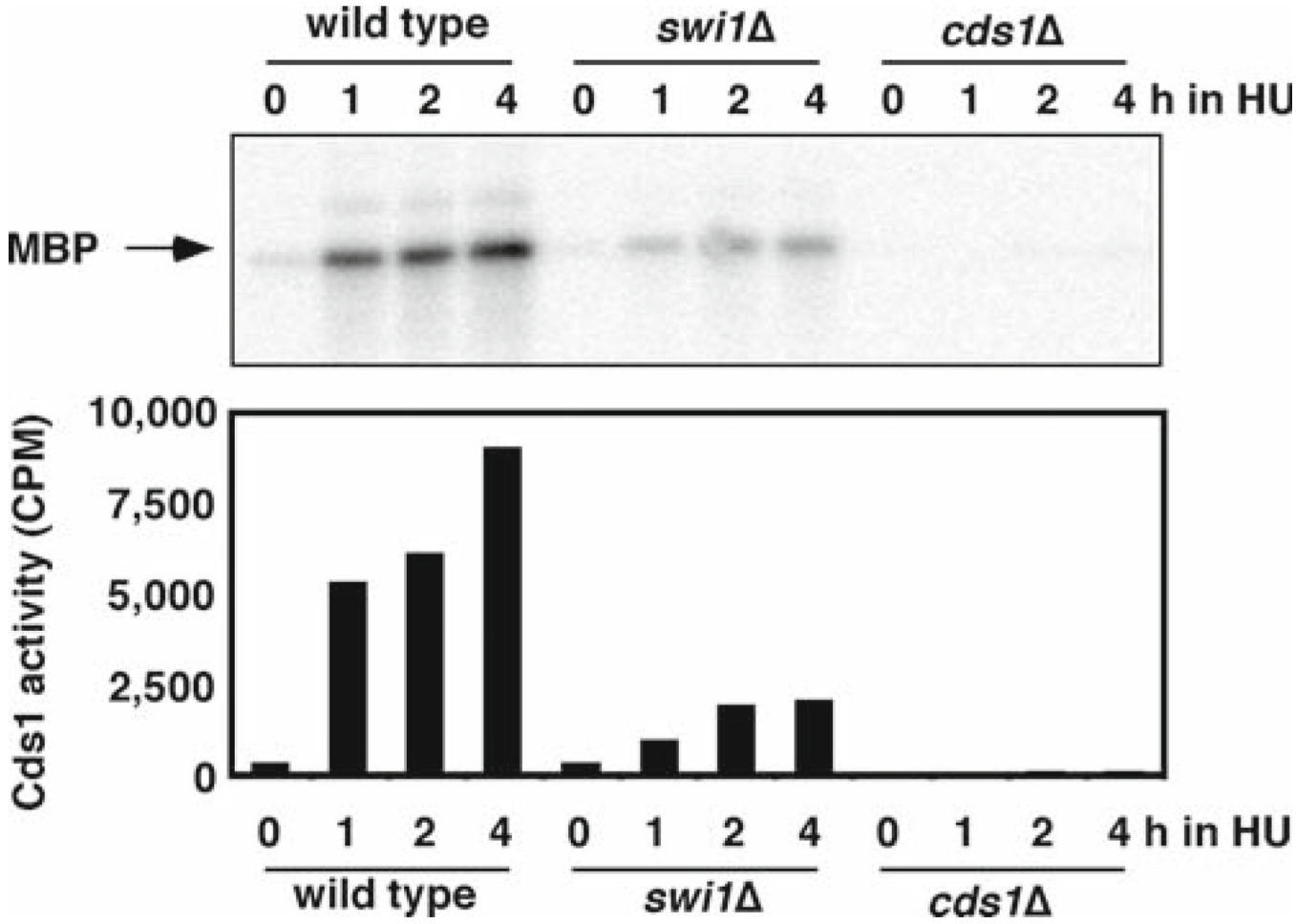

Fig. 4.

Cds1 activation is strongly reduced in *swi1*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 myelin basic protein (MBP) as a substrate. The radiolabeled MBP was detected after gel electrophoresis (*upper panel*). The radioactivity levels (counts per minute, CPM) of MBP were then determined in a liquid scintillation counter (*lower panel*).

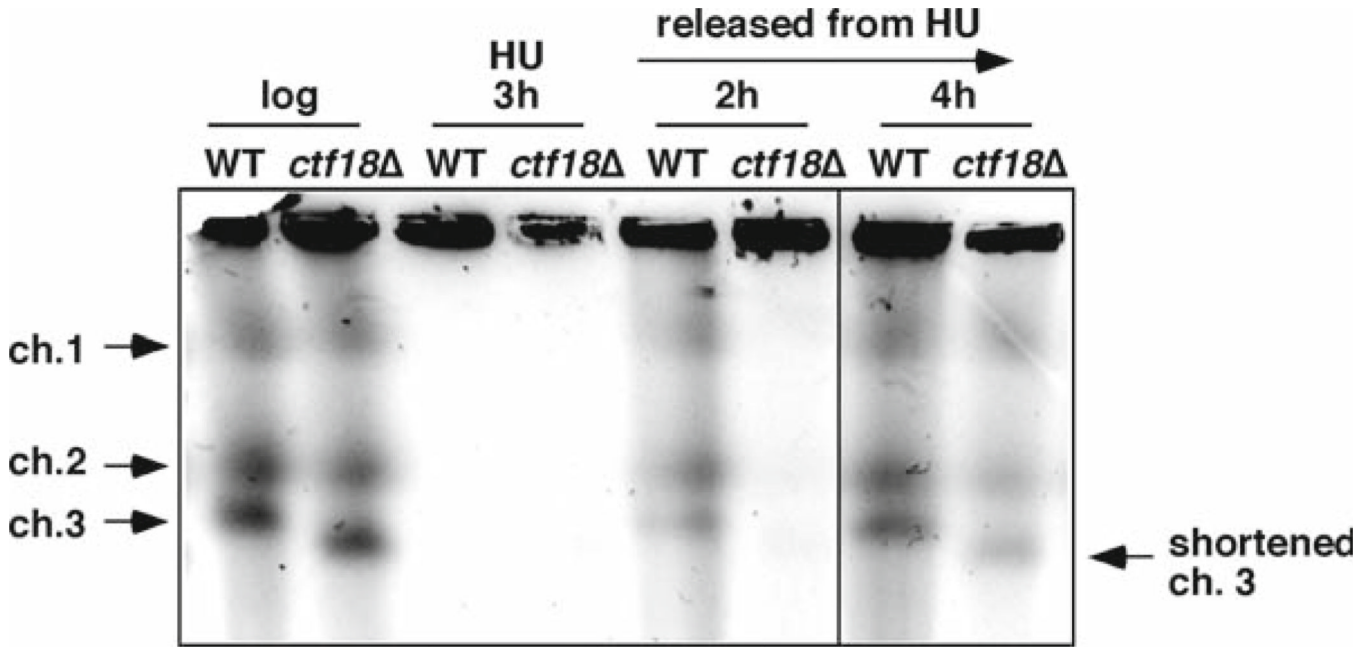

Fig. 5.

Ctf18 is required for the efficient resumption of replication following fork damage. Chromosome samples from either wild-type or *ctf18*Δ cells were examined by PFGE. Cells were grown until midlog 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. *ctf18*Δ cells showed a delay in recovery of DNA replication after fork arrest. *ctf18*Δ cells also displayed a short chromosome III probably due to replication and/or recombination defects.

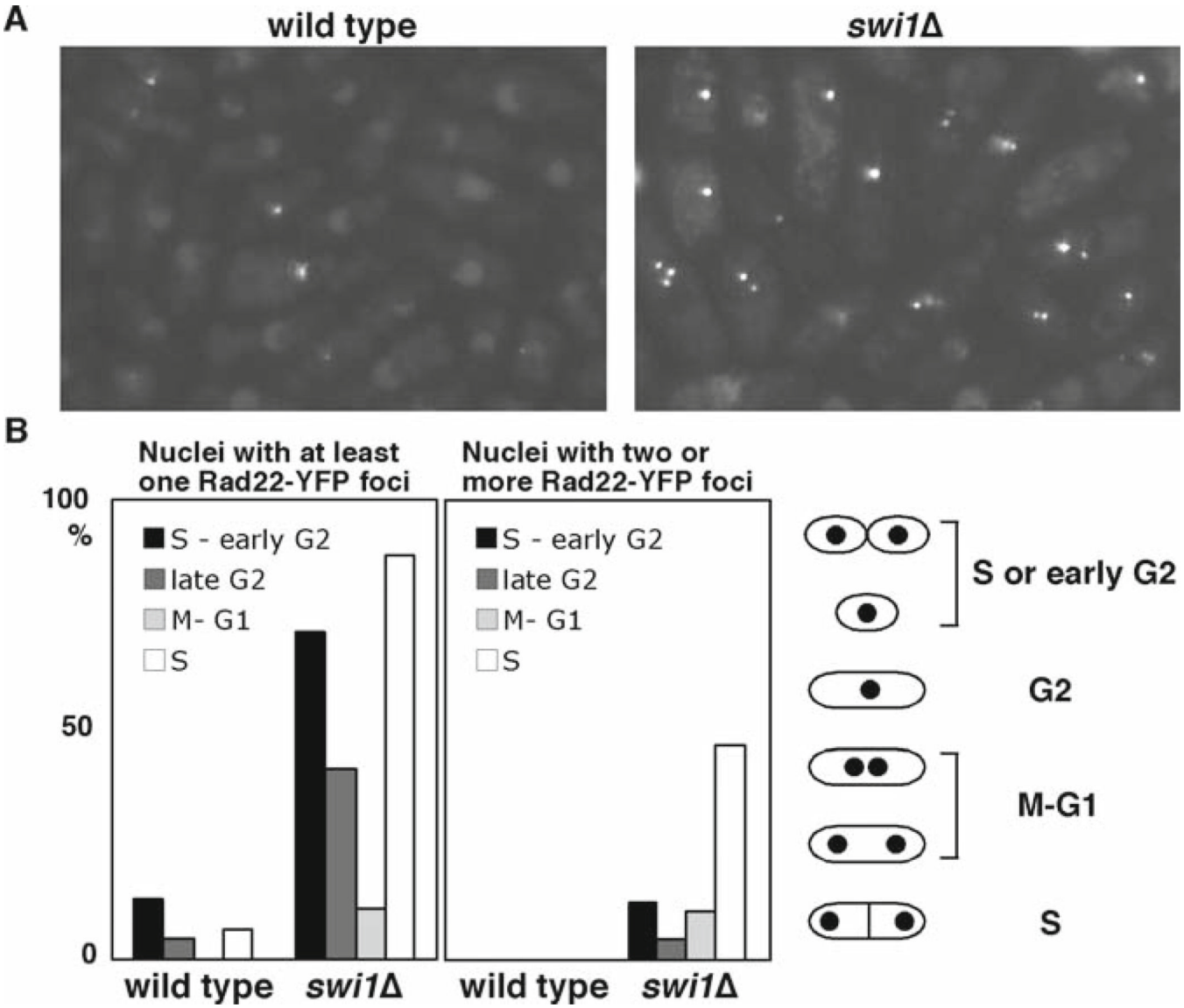

Fig. 6.

*swi1*Δ cells experience replication abnormality. (**a**) Rad22-YFP foci formation was significantly elevated in *swi1*Δ cells. Cells of the indicated genotype expressing genomic Rad22-YFP were grown in YES medium at 25°C until midlog phase. (**b**) Quantification of Rad22-YFP foci according to cell cycle stages. S and early G_2 cells had the most Rad22-YFP foci. The percentages of nuclei that have at least one focus or harbor two or more foci are shown.