Chapter 16

Analyzing DNA Replication Checkpoint in Budding Yeast

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Abstract

Checkpoints are conserved mechanisms that prevent progression into the next phase of the cell cycle when cells are unable to accomplish the previous event properly. Cells also possess a surveillance mechanism called the DNA replication checkpoint, which consists of a conserved kinase cascade that is provoked by insults that block or slow down replication fork progression. In the budding yeast *Saccharomyces cerevisiae*, the DNA replication checkpoint controls the timing of S-phase events such as origin firing and spindle elongation. This checkpoint also upregulates dNTP pools and maintains the replication fork structure in order to resume DNA replication after replication block. Many replication checkpoint factors have been found to be tumor suppressors, highlighting the importance of this checkpoint pathway in human health. Here we describe a series of protocols to analyze the DNA replication checkpoint in *S. cerevisiae*.

Key words DNA replication checkpoint, Mec1, Rad53, Kinase assay, Budding yeast

1 Introduction

Cells are constantly exposed to DNA damage. DNA lesions can arise from either exogenous (e.g., DNA-damaging drugs) or endogenous (e.g., replication forks encountering barriers) agents [1]. To preserve the genetic information, cells have evolved mechanisms called DNA damage checkpoint that senses the damage, stops the cell cycle, and induces DNA repair pathways [2].

Depending on the cell cycle stage in which the damage occurs, cells arrest either in G1 (before starting DNA replication) or G2 (before entering mitosis) stages of the cell cycle. During S phase, cells have a mechanism called the DNA replication checkpoint that is provoked by the insults that block or slow down replication forks.

The checkpoint signaling cascades include the human PI3K-like (PIKK) kinases ATM and ATR and their homologs Tel1 and Mec1 in budding yeast, respectively (Fig. 1) [3]. ATR and Mec1 genes are essential for cell viability, although a Mec1 deletion mutant can be generated by simultaneously upregulating ribonucleotide reductase (e.g., by deleting the gene encoding the
PIKKs are generally large proteins (about 2,500 amino acids in length) with a C-terminal kinase domain flanked by FRAPP ATM TRAPP (FAT) and FAT C-terminal (FATC) domains [3, 4]. Other members of this kinase family are mTOR kinases (budding yeast Tor1 and Tor2) and the catalytic subunit of DNA-PK [3].

While ATM becomes activated in response to DNA double-strand breaks (DSBs), ATR can sense a variety of lesions [3] that contain single-stranded DNA (ssDNA) coated by the single-strand DNA-binding protein replication protein A (RPA). ATR binds to these ssDNA-containing structures via its binding partner ATR-interacting protein (ATRIP, or Ddc2 in budding yeast) [5]. Experiments in *Xenopus* egg extracts indicated an additional

![Fig. 1 Current model of the replication checkpoint in budding yeast. Ddc1, Dpb11, and Dna2 activate Ddc2–Mec1 when it is recruited to stalled replication forks. Phosphorylation of the downstream kinase Rad53 requires the checkpoint mediator Mrc1. Sgs1 also aids to activate Rad53. Tel1 can partially substitute for Mec1 in the activation of Rad53, as does Rad9 for Mrc1. Active Rad53 leads to its autophosphorylation, thus amplifying the checkpoint signal. The replication checkpoint suppresses late origin firing, inhibits spindle elongation, upregulates dNTP pools, and promotes recovery from the fork arrest.](image-url)
requirement of double-stranded DNA adjacent to the ssDNA stretch for ATR-dependent checkpoint activation [6]. The Rad17–RFC2-5 clamp-loading complex (Rad24–Rfc2-5 in budding yeast) recognizes such DNA structures and loads the 9-1-1 clamp (the Rad9–Rad1–Hus1 complex in Schizosaccharomyces pombe and metazoans, Rad17–Mec3–Ddc1 in budding yeast) [7]. Phosphorylation of the Rad9 (Saccharomyces cerevisiae Ddc1) subunit of 9-1-1 recruits TopBP1 (S. cerevisiae Dpb11) [8–10]. TopBP1 can activate ATR–ATRIP (S. cerevisiae Mec1–Ddc2) in vitro, and mutations that abolish the activation were mapped in both ATR and ATRIP [11].

In S. cerevisiae, Ddc1 not only recruits the TopBP1 homolog, Dpb11, but also enhances Mec1 kinase activity in certain conditions in vitro [7, 12]. A C-terminal unstructured region in both Ddc1 and Dpb11 was shown to activate Mec1, and it has been suggested that the mode of Mec1 activation depends on the cell cycle stage [12–15]. Recently, another protein, Dna2, has been reported to activate Mec1 specifically in S phase [16] (Fig. 1).

In the checkpoint cascade, Mec1 activation leads to phosphorylation of the downstream effector kinases Rad53 (human CHK2) and Chk1 (human CHK1) in budding yeast (Fig. 1) [2]. While Rad53 is commonly referred to as the CHK2 homolog, human CHK1 acts as the Rad53 functional homolog in the replication-associated checkpoint. Activation of those effector kinases requires mediator proteins [17, 18]. In the case of Mec1 activation in budding yeast, in response to DSBs or DNA adducts such as those caused by methyl methanesulfonate (MMS), the checkpoint protein Rad9 (human 53BP1) recruits Rad53 and facilitates its phosphorylation by Mec1 at the site of damage [19–21]. Once Mec1 has primed Rad53 for activation, Rad9 also provides a scaffold that accommodates Rad53 autophosphorylation, which is necessary for full Rad53 activation [19]. In this process, Rad53 becomes heavily phosphorylated, and this can be monitored by a band shift on Western blots. Alternatively, Rad53 activation can be monitored by testing its autophosphorylation capability. Rad9 has little role in Rad53 activation in response to fork-stalling agents such as hydroxyurea (HU) [17, 22]. Instead, the replication fork components Mrc1 and Sgs1 facilitate Rad53 phosphorylation in response to HU-induced replication stress [23–25]. Both Mrc1 and Rad9 enhance enzyme–substrate interaction rather than increasing enzymatic activity of Mec1 [20, 26]. Sgs1 contributes to Rad53 activation, both by regulating the amount of ssDNA at the fork (by reversing fold-back structures and aberrant annealing) and by directly binding to Rad53 [27]. Once activated, the replication checkpoint inhibits the firing of late origins [28, 29] and maintains replication fork integrity by preventing the conversion of forks into DSBs and/or by reducing recombination events [30, 31]. This checkpoint also upregulates dNTP pools [32] and suppresses spindle elongation [33] (see also review 34).
Besides the downstream kinases Chk1 and Rad53, Mec1/ATR phosphorylates multiple targets, many of which are replication proteins (see review 34). Therefore, a Mec1 in vitro kinase assay can be used to identify direct targets of Mec1.

Here, we describe a series of protocols to monitor replication checkpoint activation in S. cerevisiae. First we describe means to monitor the activation of the effector kinase Rad53, and then we describe three tractable readouts of the replication checkpoint: cell growth on HU (drop assay), recovery from fork arrest (colony-forming assay), and spindle elongation in response to HU. Finally, we provide a protocol to measure Mec1 kinase activity in vitro.

2 Materials

2.1 α-Factor Synchronization of Budding Yeast Cells

1. YPAD medium: 20 g/L Bacto peptone, 10 g/L yeast extract, 20 g/L dextrose, 25 mg/L adenine-hemisulfate salt. Dissolve in ddH₂O, and autoclave at 120 °C for 15 min.
2. Spectrophotometer to measure optical density (OD₆₀₀) of yeast culture or other cell-counting device.
3. YPAD medium pH 5.0: Adjust YPAD medium to pH 5.0 with HCl then autoclave at 120 °C for 15 min.
4. Sterile tubes with air-permeable lids (50 mL) or sterile (autoclaved) Erlenmeyer flasks (50–100 mL).
5. α-Factor stock solution: 5 mg/mL α-Factor in 10 mM HCl, 1 mM β-mercaptoethanol, 0.2 mM EDTA. Make aliquots, and store at −20 °C.
6. Light microscope with a 20× objective.
7. 1.5-mL tubes.
8. 70 % ethanol.
9. RNase A solution: 200 μg/mL RNase A, 50 mM Tris–HCl pH 7.5.
10. PI solution: 10 μg/mL propidium iodide, 50 mM citrate–acetate pH 7.0.
12. Flow cytometer (e.g., FACSCalibur, Becton Dickinson).

2.2 Phospho-Shift Analysis of Rad53 Phosphorylation

1. Cell culture tools, medium, and equipment as listed in Subheading 2.1 (items 1–6).
2. 15- and 50-mL tubes.
3. Hydroxyurea (see Note 1).
4. 2-mL tubes.
5. Microfuge (4 °C).
6. Solution I: 1.85 M NaOH, 7.4 % (v/v) β-mercaptoethanol (see Note 2).
7. Solution II: 50 % trichloroacetic acid (TCA).
8. Solution III: 80 % (v/v) acetone, 10 % (v/v) methanol, 10 mM Tris–HCl pH 7.5.
9. 1× NuPAGE LDS sample buffer (Life Technologies) supplemented with 50 mM dithiothreitol (DTT) freshly added (see Note 3).
11. 6 % acrylamide SDS-PAGE gels or commercially available low-percent gradient gels (e.g., NuPAGE 3–8 % Tris–acetate protein gels, Life Technologies).
12. SDS-PAGE gel-running apparatus.
13. Protein transfer apparatus.
14. PVDF (polyvinyl difluoride) or nitrocellulose membrane.
15. TEN + Tween-20 buffer: 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.05 % Tween-20.
16. Blocking buffer: TEN + Tween-20 buffer supplemented with 5 % nonfat dry milk powder (skim milk).
17. Anti-Rad53 antibody (Santa Cruz, sc-6749).
19. ECL-developing reagent.
20. Films and film-developing system or CCD camera-based detection system (e.g., ChemiDoc XRS system, Bio-Rad).

2.3 Rad53 Autophosphorylation by In Situ Kinase Assay

1. Cell culture tools, medium, and equipment as listed in Subheading 2.1 (items 1–6).
2. 10 % Acrylamide SDS-PAGE gels.
3. SDS-PAGE gel-running apparatus.
4. PVDF membrane.
5. Protein transfer apparatus.
6. Denaturing buffer: 7 M Guanidine–HCl, 50 mM Tris–HCl pH 8.0, 2 mM EDTA, 50 mM DTT (see Note 4).
7. Tris-buffered saline (TBS): 50 mM Tris–HCl pH 7.5, 150 mM NaCl.
8. Renaturing buffer: 10 mM Tris–HCl pH 7.5, 140 mM NaCl, 2 mM EDTA, 0.04 % w/v Tween-20, 1 % w/v bovine serum albumin (BSA), 2 mM DTT (see Note 5).
9. 30 mM Tris–HCl pH 7.5.
10. Kinase buffer: 40 mM HEPES–NaOH pH 8.0, 20 mM MgCl₂, 20 mM MnCl₂, 0.1 mM EGTA, 100 μM sodium orthovanadate, 1 mM DTT (freshly added from powder or 1 M –20 °C frozen stock).
11. γ-³²P-ATP (370 MBq (10 mCi)/mL).
12. 30 mM Tris–HCl pH 7.5, 0.01 % NP-40.
13. 1 M KOH.
14. 10 % TCA.
15. Plastic wrap (e.g., Saran wrap).
16. Phosphor-imaging system (e.g., Typhoon, GE Healthcare).

### 2.4 Testing Cell Sensitivity to DNA-Damaging Agents by Drop Assay

1. YPAD plates: 20 g/L Bacto peptone, 10 g/L yeast extract, 20 g/L dextrose, 25 mg/L adenine-hemisulfate salt, 20 g/L Bacto Agar. Suspend in ddH₂O, and autoclave at 120 °C for 15 min (see Note 6). Let it cool down about 50 °C, and pour about 20 mL in each petri dish.

2. Cell culture tools, medium, and equipment as listed in Subheading 2.1 (items 1, 2 and 4).

3. 96-well plates.

4. 8-channel pipette.

5. YPAD plates with DNA-damaging reagents: Autoclave YPAD-agar, cool down to about 50 °C, and then add DNA-damaging agents (HU, MMS, bleomycin, etc.) to the desired concentration. Pour about 20 mL in each petri dish in the ventilation hood.

### 2.5 Cell Recovery Assay

1. YPAD plates (see Subheading 2.4, item 1).

2. Cell culture tools, medium, and equipment as listed in Subheading 2.1 (items 1–6).

3. Hydroxyurea (see Note 1).

### 2.6 Monitoring Spindle Elongation

1. YPAD plates (see Subheading 2.4, item 1).

2. Cell culture tools, medium, and equipment as listed in Subheading 2.1 (items 1–6).

3. Budding yeast strain expressing the GFP-TUB1 fusion protein. If not available, wild-type cells can be used (see Note 7).

4. Hydroxyurea (see Note 1).

5. 20 % Paraformaldehyde solution: Dissolve 5 g of paraformaldehyde powder in 15 mL of H₂O, then add 25 μL of 10 N NaOH, and heat at 60 °C until the solution becomes clear. Add H₂O filling up to 25 mL, and keep at 4 °C.

6. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.1 mM KH₂PO₄, pH 7.4.

7. 10 % Triton X-100.

8. (Optional) DAPI: 4′,6-Diamidino-2-phenylindole, dihydrochloride.

9. Fluorescent microscope and image analysis software (e.g., Image J).
1. Cell culture tools, medium, and equipment as listed in Subheading 2.4 (items 1, 2 and 4).
2. Budding yeast strain expressing the Ddc2–GFP fusion protein (see Note 8).
3. 500-mL Erlenmeyer flask.
4. PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.1 mM KH₂PO₄, pH 7.4.
5. 2-mL screw-cap tubes.
7. Dynabeads Protein G (Life Technologies).
8. Lysis buffer: 50 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5 % (w/v) NP-40, protease inhibitor cocktail (complete, Roche).
9. Magnet tube holder (e.g., Dynamag, Life Technologies).
10. Anti-GFP antibody (Roche, 1181446001).
11. 0.5-mm Zirconia/silica beads.
13. 25-gauge needles.
14. 4× NuPAGE LDS sample buffer (Life Technologies) supplemented with 200 mM DTT (freshly added) or equivalent 4× SDS-PAGE sample buffer (see Note 3).
15. Heating block.
16. 10× Kinase buffer: 200 mM Tris–HCl pH 7.4, 100 mM magnesium acetate, 0.5 % (w/v) Tween-20, 40 mM MnCl₂, 1 mM DTT (freshly added from powder or 1 M −20 °C frozen stock).
17. 10 mM adenosine triphosphate (ATP).
18. γ⁻³²P-ATP (370 MBq (10 mCi)/mL).
19. Recombinant substrate (e.g., human EIF4EBP1 recombinant protein, Abnova, H00001978-P01) (see Note 9).
20. 300 mM caffeine.
21. 10 % SDS gels or commercially available gradient gels (e.g., 4–12 % Bis/Tris gels, Life Technologies).
22. SDS-PAGE gel-running apparatus.
23. Protein gel dye (e.g., Instantblue, Expedeon).
25. Phosphor-imaging system (e.g., Typhoon, GE Healthcare).
26. Protein transfer apparatus.
3 Methods

The activity of the effector kinase Rad53 is commonly used as readout of the activation of the replication checkpoint in budding yeast. As mentioned in Subheading 1, once activated, Rad53 undergoes autophosphorylation and becomes hyperphosphorylated (Fig. 1). Hyperphosphorylated Rad53 migrates more slowly than its less phosphorylated form in SDS-PAGE. In this section, we first describe how to synchronize budding yeast cells. We then describe two methods to monitor Rad53 activation and two different survival assays: drop assay and recovery assay, both of which reflect the activity of the replication checkpoint. In budding yeast, unlike other eukaryotes, microtubules (spindles) start to elongate in S phase, and the replication checkpoint inhibits, once activated, premature spindle elongation. We also describe a method to monitor spindle elongation in response to HU. Finally, we provide a protocol to test Mec1 kinase activity on a recombinant substrate in vitro.

3.1 α-Factor Synchronization of Budding Yeast Cells

We use α-factor to synchronize budding yeast cells in G1 phase. Efficacy of α-factor varies from one batch to the other. To ensure efficacy, every batch of α-factor should be tested to confirm its effect on cell synchronization. In this section, we provide a protocol to determine an optimal α-factor concentration.

3.1.1 α-Factor Treatment of Budding Yeast Cells

1. Culture MATα yeast cells in 5 mL of YPAD medium overnight.
2. Dilute cells in 30 mL of YPAD (OD$_{600}$~0.2) in a 250-mL flask, and then culture until an OD$_{600}$ of 0.5–0.6 is achieved (see Note 10).
3. Centrifuge at 1,500 × $g$ for 3 min at room temperature to collect cells, and resuspend cells in 30 mL of YPAD medium pH 5.0 (see Note 11).
4. Take 1 mL of cell culture for FACS analysis (random culture sample). Follow Subheading 3.1.2 for FACS analysis.
5. Distribute 5 mL of cell culture to 50-mL sterile tubes with air-permeable lids, and add different amounts of α-factor stock solution to each tube (e.g., 5, 2.5, 1, 0.5 μL to achieve final concentrations of 5, 2.5, 1, 0.5 μg/mL) (see Note 12).
6. Culture cells for 90 min at 30 °C.
7. Take 1 mL of each cell culture for FACS analysis (see Subheading 3.1.2). Also, monitor cell cycle arrest under the microscope (see Note 13).
8. Centrifuge the cultures at 1,500 × $g$ for 2 min at room temperature, and remove the culture supernatant.
9. Suspends cells with 5 mL of YPAD and then spin at 1,500 × $g$ for 2 min at room temperature to wash and collect cells.
10. Resuspend cells in 5 mL of YPAD and culture at 30 °C.

11. Take 1 mL of each cell culture at 15, 30, 45, and 60 min after release for FACS analysis (see Subheading 3.1.2).

3.1.2 FACS Analysis to Determine an Optimal α-Factor Concentration

1. Centrifuge cells at 1,500 × g for 2 min at room temperature to collect cells in a 1.5-mL tube.

2. Remove the medium from each sample, suspend cells in 500 μL of 70 % ethanol, and keep the samples at 4 °C for 30 min. The samples can be kept at 4 °C for several days.

3. Spin samples at 8,000 × g for 1 min at room temperature, and remove the ethanol.

4. Add 250 μL of RNaseA solution, and incubate at 37 °C for 2 h.

5. Spin samples at 8,000 × g for 1 min, remove supernatant, and add 250 μL of PI solution.

6. Pulse sonicate each sample for 1 s (see Note 14).

7. Dilute the samples by five- to tenfold, 50–100 μL of samples, in 500 μL of PI solution, and then analyze the cell-cycle profile by flow cytometry (see Note 15).

8. Determine the optimal α-factor concentration based on cell-cycle profiles of synchronized cells and cells released from the α-factor arrest (see Note 16).

3.2 Phospho-Shift Analysis of Rad53 Phosphorylation After Replication Stress

3.2.1 Synchronization and Hydroxyurea Treatment of Budding Yeast Cells

Rad53 activation can be monitored by its hyperphosphorylation. Hyperphosphorylated Rad53 migrates more slowly in an SDS-PAGE gel, resulting in a band shift (phospho-shift). This section describes a method to detect hyperphosphorylated Rad53 by Western blotting.

1. Streak yeast cells freshly on YPAD plates. Incubate for 1–2 days at 30 °C (see Note 17).

2. Inoculate a colony of yeast into 5 mL YPAD medium in a sterile (autoclaved) Erlenmeyer flask (50–100 mL) or into a sterile tube with air-permeable lids (50 mL). Shake the culture at 30 °C overnight.

3. Measure OD_{600} after overnight culture, and dilute the culture to an OD_{600} of 0.3 in 15 mL of YPAD in a sterile (autoclaved) Erlenmeyer flask (50–100 mL) or a sterile tube with air-permeable lid (50 mL); shake culture at 30 °C for about 3 h (see Note 18).

4. Measure OD_{600}, and transfer a culture volume equivalent to 15 mL of an OD_{600} 0.5 culture into a fresh 50-mL tube (see Note 18).

5. Spin at 1,500 × g for 2 min at room temperature.

6. Wash cells once with 15 mL of YPAD.

7. Resuspend in 15 mL of YPAD pH 5.0, and add the appropriate amount of α-factor.
8. Shake the culture at 30 °C for about 75 min, and check cells under the microscope for absence of small buds (see Note 13).

9. Spin at 1,500 × g for 2 min.

10. Wash cells once with 15 mL of YPAD.

11. Resuspend cells in 15 mL of YPAD supplemented with 0.2 M HU or intra-S damage checkpoint can be induced with other DNA-damaging drugs such as 0.03 % MMS. Culture cells at 30 °C.

3.2.2  Protein Sample Preparation by NaOH/Trichloroacetic Acid Precipitation

1. Take 2 mL of the culture (see Subheading 3.2.1, step 11) after 0, 15, 30, 60, and 90 min of HU or MMS treatment into 2-mL tubes.

2. Spin at max speed in a microfuge for 1 min at 4 °C, remove the medium, and add 100 μL of ice-cold solution I.

3. Suspend cells, and keep the samples on ice for 10 min.

4. Add 100 μL of solution II, vortex to mix, and then keep samples on ice for 10 min. After adding solution II, samples can be kept on ice until the end of the time-course experiment (see step 1) and processed all together.

5. Spin down samples at max speed for 1 min at 4 °C in a microfuge, and remove supernatant.

6. Wash with 500 μL of solution III (do not disturb the pellet).

7. Spin at max speed for 1 min at 4 °C in a microfuge, and remove supernatant completely (see Note 19).

8. Let samples dry at room temperature with the tube lids open.

9. Resuspend samples in 50 μL of 1× NuPAGE sample buffer supplemented with 50 mM DTT (see Note 3).

10. Heat samples at 70 °C for 10 min (see Note 3).

11. Spin shortly, and store whole samples at −20 °C until SDS-PAGE and Western blot analysis. Repeat step 10 and spin shortly just before loading sample on SDS-PAGE.

3.2.3  SDS-PAGE and Western Blotting

1. If samples were stored at −20 °C, heat samples at 70 °C for 10 min again (see Note 3).

2. Spin down briefly, load 2.5–5 μL of sample on a 6 % acrylamide SDS-PAGE gels, and perform electrophoresis according to standard procedures.

3. Transfer proteins to PVDF or nitrocellulose membrane according to the instructions for the protein transfer apparatus.

4. Block the membrane by incubating with blocking buffer for 30 min at room temperature (see Note 20).

5. Incubate the membrane with blocking buffer supplemented with the anti-Rad53 antibody (see Note 21) at room temperature for 1 h or at 4 °C overnight.
6. Wash the membrane with TEN-Tween 20 buffer for 10–20 min at room temperature at least three times with buffer exchange.

7. Incubate the membrane with blocking buffer containing the secondary antibody for 30 min to 1 h at room temperature.

8. Wash the membrane with TEN-Tween 20 buffer for 5–10 min at room temperature at least five times with buffer exchange.

9. Detect Western blot signal using ECL-developing reagent on either films or a CCD camera-based system (Fig. 2).

Rad53 activation can also be monitored by its autophosphorylation, which can be detected by an in situ kinase assay (ISA) described in this section. This procedure has been adapted from a previously described method [22].

1. Follow all the steps in Subheadings 3.2.1 and 3.2.2.

2. Load 5–10 μL of sample on 10 % SDS-PAGE gel, and perform electrophoresis according to standard procedures.

3. Transfer proteins to PVDF membrane according to the instructions for the protein transfer apparatus (see Note 22).

4. Incubate membrane in denaturing buffer for 1 h at room temperature (see Note 20).

5. Wash membrane twice for 10 min in TBS.

6. Incubate membrane in renaturing buffer at 4 °C overnight. Change this buffer more than four times during the incubation.

7. Wash membrane in 30 mM Tris–HCl pH 7.5 for 30–60 min at room temperature.

8. Incubate membrane with kinase buffer for 30 min at room temperature.

9. Incubate membrane with kinase buffer supplemented with 0.37 MBq/mL (10 μCi/mL) of [γ-32P]ATP for 1 h at room temperature (10 mL of kinase buffer is typically used for a mini-gel size of PVDF membrane, 10 × 8 cm).

Fig. 2 Rad53 phospho-shift and Rad53-ISA. Wild-type and mec1-100 cells were arrested in G1 phase with α-factor and released into YPAD medium supplemented with 0.2 M HU. Samples were taken at indicated time points, and proteins were extracted using TCA. Protein samples were subjected to SDS-PAGE and Western blot with anti-Rad53 antibody (left). An example of Rad53-ISA results is also shown (right).
10. Wash membrane with 30 mM Tris–HCl pH 7.5 twice for 10 min.
11. Wash membrane with 30 mM Tris–HCl pH 7.5 supplemented with 0.01 % NP-40 once for 10 min.
12. Wash membrane with 30 mM Tris–HCl pH 7.5 once for 10 min.
13. Wash membrane with 1 M KOH once for 10 min.
14. Rinse membrane with ddH$_2$O.
15. Wash membrane with 10 % TCA once for 10 min.
16. Rinse membrane with ddH$_2$O, and wrap it in plastic wrap.
17. Expose the membrane to a Phosphorimager screen overnight, and detect radioactivity by a phosphor-imaging system (see Note 23 and Fig. 2).

3.4 Drop Assay to Test Cellular Sensitivity to DNA-Damaging Agents

1. Streak yeast cells fresh on YPAD plates. Incubate for 1–2 days at 30 °C (see Note 17).
2. Inoculate a colony of yeast into 5 mL of YPAD in a sterile (autoclaved) Erlenmeyer flask (50–100 mL) or a sterile tube with air-permeable lids (50 mL); shake the culture at 30 °C overnight.
3. Measure OD$_{600}$ after overnight culture, and dilute all cultures to the same OD$_{600}$ or cell number (e.g., OD$_{600}$ 1.0) (see Note 10).
4. Prepare a tenfold serial dilution in a 96-well plate: Add 100 μL of cell density-adjusted cultures (e.g., OD$_{600}$ 1.0) in the wells of the first column. Then, using an 8-channel pipette, add 90 μL of water in the wells of the second through the sixth columns (each column has 8 wells).
5. Using an 8-channel pipette, add 10 μL of cultures from the first column into the wells of the second column, and mix cells by pipetting up and down several times.
6. Repeat step 5 until the sixth column (10 μL from the second to third, 10 μL from the third to fourth, and so on).
7. YPAD control plates and YPAD plates with DNA-damaging agent should be prepared the day before, and the surface should be free of water droplets. If you have water droplets, let plates dry in a fume hood or a laminar flow hood.
8. Drop 2–3 μL of the serially diluted cultures column by column on the YPAD plates using an 8-channel pipette (see Note 24). Finally, every row on the plate will show a tenfold serial dilution of cells of one strain.
9. Incubate plates at 30 °C for 2–3 days, and take pictures of the plates after incubation (Fig. 3a) (see Note 25).
1. Follow Subheading 3.2.1 for synchronization and hydroxyurea treatment of budding yeast cells.

2. Determine cell density by a cell-counting device (can be done while cells are still in α-factor arrest).

3. Make dilutions of cells at 1 × 10^3 cells/mL, and plate 100–200 cells (100–200 μL) on three YPAD plates for each strain after 0, 2, 4, and 6 h of hydroxyurea treatment (see Note 26).

4. Incubate plates at 30 °C for 2–3 days before counting colonies.

5. Calculate average colony number of the three plates of each strain and each time point.

6. Calculate percentages of cell survival based on 0-h time point for each strain (Fig. 3b) (see Note 27).

### 3.5 Cell Recovery Assay from HU Treatment

1. Grow GFP-TUB1-expressing cells (see Note 7) to exponential phase, and treat cells with α-factor to induce G1 arrest in ~40 mL of YPAD (see Subheading 3.2.1 for details).

2. Take 1 mL of exponentially growing cells and α-factor-arrested cells for FACS analysis (see Subheading 3.1.2).

3. Release cells in ~40 mL of YPAD containing 0.2 M HU (see Subheading 3.2.1 for details).

### 3.6 Monitoring Spindle Elongation

**Fig. 3** HU sensitivity assays of budding yeast cells. (a) For drop assays, tenfold serial dilutions of wild-type and mec1-100 cells were spotted and grown on YPAD-agar medium containing 0 (control) or 50 mM HU (see text for details). Pictures were taken after 2 days of incubation at 30 °C. (b) For cell recovery assays, wild-type and mec1-100 cells were arrested in G1 phase with α-factor and released into YPAD medium containing 0.2 M HU. Samples were taken at 0, 2, 4, and 6 h after release from the α-factor arrest and subjected to colony-forming assay. Plating efficiency of each strain was normalized to samples at time 0 h. Error bar represents standard deviation of three independent experiments. Drop assay represents tolerance/outgrowth of cells on replication stress, whereas recovery assay reflects the replication fork stability/restart. The DNA replication checkpoint is important for both aspects.

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<th>wild-type</th>
<th>YPAD (control)</th>
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<td>mec1-100</td>
<td>+50 mM HU</td>
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<table>
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<tr>
<th>time in 0.2M HU</th>
<th>%</th>
<th>wild-type</th>
<th>mec1-100</th>
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<td>0</td>
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Drop assay represents tolerance/outgrowth of cells on replication stress, whereas recovery assay reflects the replication fork stability/restart. The DNA replication checkpoint is important for both aspects.
4. Take 5 mL of culture at 0, 1, 2, 3, 4, and 5 h after release from α-factor, and fix cells with 1% paraformaldehyde (0.25 mL of 20% paraformaldehyde solution) for 5 min at room temperature. Take also 1 mL of culture for FACS analysis at each time point to determine whether DNA synthesis is inhibited (see Subheading 3.1.2).

5. Spin fixed samples at 1,500 × g for 5 min at room temperature, and then remove the culture medium.

6. Add 5 mL of ice-cold PBS to wash the samples, and spin at 1,500 × g for 5 min at room temperature. Repeat this step once more.

7. (Optional) DAPI staining: Resuspend cells in 100 μL of PBS supplemented with 0.1% Triton and 1 μg/mL DAPI and transfer to a 1.5-mL tube. Incubate for 5 min at room temperature, and then spin at 6,000 × g for 1 min at room temperature.

8. Resuspend cells in 100 μL of PBS, and keep the samples at 4 °C until analysis with a fluorescent microscope.

9. Take pictures with a fluorescent microscope, and measure the spindle length (Fig. 4) (see Note 28).

### 3.7 In Vitro Mec1 Kinase Assay

Immunoprecipitated Mec1–Ddc2 complex can be used to monitor its kinase activity on recombinant substrates. This protocol was adapted from a previously described assay [27].

![Fig. 4 Spindle elongation in checkpoint-deficient cells in response to HU. Wild-type and rad53-11 cells were synchronized in G1 and released into S phase in YPAD medium supplemented with 0.2 M HU. Samples were taken after 4 h for immunofluorescence using TAT-1 that reacts with α-tubulin. DNA was counterstained with DAPI. Scale bar, 5 μm. Checkpoint-deficient rad53-11 cells exhibit an elongated spindle, while wild-type cells retain a short spindle.](image-url)
### 3.7.1 Harvesting Cells

1. Streak yeast cells fresh on YPAD plates. Incubate overnight at 30 °C.
2. Inoculate a colony of Ddc2–GFP-expressing cells (see Note 8) into 5 mL YPAD in a sterile (autoclaved) Erlenmeyer flask (50–100 mL) or a sterile tube with air-permeable lid (50 mL); shake the culture at 30 °C overnight.
3. Measure OD$_{600}$ of overnight culture, and dilute to an OD$_{600}$ of 0.3 in 100 mL of YPAD in sterile (autoclaved) Erlenmeyer flasks (500 mL); shake the culture at 30 °C for about 2–3 h (see Note 10).
4. Measure OD$_{600}$, and harvest cells equivalent to 100 mL of an OD$_{600}$ 0.5/mL culture (50 ODs, about $7 \times 10^8$ cells) by centrifugation at $1,500 \times g$ for 2 min at room temperature.
5. Resuspend cell pellets in 1 mL cold PBS, and transfer cells to 2-mL screw-cap tubes.
6. Spin at max speed in a microfuge for 1 min at 4 °C.
7. Remove supernatant. At this stage the cell pellet can be frozen in liquid nitrogen and stored at −80 °C.

### 3.7.2 Anti-GFP Bead Preparation

1. Transfer 50 μL of Dynabeads Protein G slurry per sample into a fresh 1.5-mL tube.
2. Wash beads twice with lysis buffer using a magnet tube holder. If necessary, centrifuge tubes only briefly.
3. Resuspend beads in 50 μL (original bead slurry volume) of lysis buffer, and add 5 μg of the anti-GFP antibody.
4. Incubate for 1 h at room temperature with rotation. In the meantime, extracts can be prepared (see Subheading 3.7.3).
5. Using a magnet tube holder, collect beads, remove supernatant, and wash beads twice with lysis buffer.
6. Wash beads once with lysis buffer supplemented with protease inhibitor cocktail.

### 3.7.3 Cell Extract Preparation

1. Resuspend the cell pellets (prepared in Subheading 3.7.1) in 200 μL ice-cold lysis buffer supplemented with protease inhibitor cocktail. Perform all the subsequent steps on ice.
2. Add ~200 μL of zirconia/silica beads.
3. Break cells using a FastPrep-24 system (see Note 29).
4. To separate zirconia/silica beads from crude cell extracts, make a hole at the tube bottom using a 25-gauge needle and assemble the tube onto a second tube. Spin at $1,500 \times g$ for 2 min at 4 °C, and collect the extracts in the second tube (see Note 30).
5. Transfer the crude extracts to a fresh 1.5-mL tube, and clarify the cell extract by spinning at max speed in a microfuge for 10 min at 4 °C.
6. Transfer the cleared supernatant into a fresh 1.5-mL tube.
7. Take 5 μL of cleared lysate (input sample), dilute into 40 μL lysis buffer, and add 15 μL 4× NuPAGE sample buffer supplemented with 50 mM DTT or equivalent. Heat sample for 10 min at 70 °C (see Note 3).

3.7.4 Immuno-precipitation

1. Using a magnet tube holder, remove the supernatant from the anti-GFP antibody-coupled magnetic beads (see Subheading 3.7.2), and directly add 200 μL of cleared lysates (see Subheading 3.7.3, step 6).

2. Incubate for 1 h at 4 °C with rotation.

3. Using a magnet tube holder, wash the immunoprecipitated beads three times for 10 min with lysis buffer supplemented with protease inhibitor cocktail.

4. Rinse beads two times with 1× kinase buffer.

5. Resuspend beads in 60 μL of 1× kinase buffer, and keep them on ice.

3.7.5 Kinase Assay

1. Prepare 5× master reaction mix (4 μL per reaction) containing 5× ATP (250 μM), 5× recombinant substrate (200 ng/μL), and 5× [γ-32P]ATP (9.25 kBq/μL = 0.25 μCi/μL) in 1× kinase buffer.

2. Prepare a dilution series of kinase: Add 3.5, 7, and 14 μL of the immunoprecipitated kinase sample (prepared in Subheading 3.7.4, step 5) to new tubes. Prepare the last one in duplicate: in one of these two tubes add 1.8 μL of caffeine stock solution for “plus caffeine” as a Mec1 inhibited control.

3. Add 1× kinase buffer to all samples filling up to 16 μL.

4. Add 4 μL of 5× master reaction mix, and then incubate for 30 min at 30 °C with gentle agitation.

5. Stop the reaction by adding 6 μL of 4× NuPAGE sample buffer supplemented with 200 mM DTT. Heat the sample for 10 min at 70 °C (see Note 3).

6. Load 10 μL of each sample on an SDS-PAGE gel, and follow standard SDS-PAGE procedures.

7. Stain the gel with protein gel stain according to the manufacturer’s instructions, and check for equal amounts of substrate.

8. Dry the gel with a gel drying apparatus and expose to a phosphorimager screen to monitor the 32P incorporation into the substrate (Fig. 5).

9. Denature by heating the residual immunoprecipitated kinase-bead sample (IP, see Subheading 3.7.4, step 5) with NuPAGE sample buffer for 10 min at 70 °C (see Note 3). Check the successful immunoprecipitation by Western blot of input (see Subheading 3.7.3, step 7) and IP sample.
1. Hydroxyurea is hygroscopic and decomposes in the presence of moisture. Let vessel equilibrate to room temperature before opening. Weigh the amount needed, and dissolve directly in the culture medium.

2. Prepare solution I fresh before each experiment and keep it on ice.

3. We normally use 1× (or 4×) NuPAGE LDS sample buffer. However, regular 1× (or 4×) SDS-PAGE sample buffer (for 1×: 50 mM Tris–HCl pH 6.8, 10 % glycerol, 2 % SDS, 1 % β-mercaptoethanol, 0.01 % bromophenol blue) can also be used. Heat samples at 70 °C for 10 min with 1× NuPAGE LDS sample buffer or at 95 °C for 5 min with 1× SDS-PAGE sample buffer.

4. Weigh guanidine-HCl and dissolve directly in the buffer. Add DTT freshly from powder or from 1 M stock solution stored at −20 °C.

5. Weigh BSA and dissolve directly in the buffer. Add DTT freshly from powder or from 1 M stock solution stored at −20 °C.

6. Agar completely dissolves only during autoclaving.

7. The localization of the GFP-TUB1 fusion protein can be monitored by fluorescent microscopy described in this protocol. GFP-TUB1 yeast strain can be obtained from Life Technologies or upon request [35]. When using wild-type...
cells, immunofluorescence with anti-TAT-1 antibody (anti-α Tubulin, Sigma 00020911) needs to be performed.

8. The budding yeast DDC2–GFP strain is available from Life Technologies or upon request.

9. We also use an Sgs1 peptide (amino-acid 404–604) as a Mec1 substrate [27].

10. Photometric measurements of OD\text{600} are usually not linear at higher values. Overnight cultures should be diluted in such a way that the measured OD\text{600} is below 1. The actual OD\text{600} can be calculated as indicated below:

\[
\text{Actual OD} = \frac{\text{Measured OD} \times \text{Final volume of diluted culture}}{\text{Volume of undiluted culture used for dilution}}
\]

11. α-Factor arrest is more efficient in YPAD pH 5.0 than in standard YPAD because the secreted protease Bar1, which cleaves α-factor, is less active in low pH conditions.

12. To increase the efficiency of α-factor arrest, Bar1-deficient cells can be used. In this case, α-factor can be used at a 50–100 times lower concentration.

13. In budding yeast bud growth is coupled to cell cycle progression. G1-arrested cells should not contain small buds. Cells should be unbudded or arrested as two equal-sized cells that are attached together.


16. After 90 min of α-factor treatment, cells should be arrested in G1. G1-arrested cells should not show small buds as can be seen by microscopic analysis. S or G2 populations should not be detected by flow cytometry. At 30–45 min after release from the α-factor arrest, the culture should be highly enriched with S-phase cells. At 60 min after the release, cells should reach G2 phase.

17. When using a temperature-sensitive strain, grow cells at 23 °C here and during the rest of cell culture steps in Subheading 3.2.1 before shifting up the temperature and inactivating a gene product.

18. An OD\text{600} of 0.7 corresponds to roughly 1 \times 10^7 cells/mL. Therefore, a 15 mL culture of OD\text{600} 0.3 (4.5 ODs) and 0.5 (7.5 ODs) roughly corresponds to 6 \times 10^8 and 1 \times 10^8 cells, respectively.

19. If necessary, spin down once more in order to remove supernatant completely.
20. Submerge membrane in 10–20 mL buffer for each step, and gently agitate at room temperature unless otherwise indicated.

21. If the anti-Rad53 antibody is not available, use a strain that is engineered to express tagged Rad53 (e.g., Rad53-GFP is available from Life Technologies and Rad53-13myc is available upon request) [24]. Antibodies against those tags are available from various commercial sources.

22. A wet Transfer system is preferred in order to obtain consistent protein-transfer efficiency. It should also be considered to use slightly elevated amperage than usual to ensure most efficient transfer. We use 400–500 mA for this assay instead of the usual 200 mA.

23. Alternatively an X-ray film can be used to detect radioactive signals with a standard film developer.

24. Alternatively, a stamping device, such as a 48-pin replicator, can be used.

25. The drop assay scores the tolerance of cells typically on low dose of replication stress or DNA-damaging agents. In contrast, the cell recovery assay described in Subheading 3.5 reflects cell ability to restart and stabilize stalled replication forks.

26. Cell numbers refer to wild-type strains. Mutant strains might be more sensitive to DNA-damaging agents. In this case a higher number of cells can be plated.

27. Percentage of colony formation (plating efficiency) can be normalized to wild-type colony formation unit.

28. Checkpoint-deficient cells exhibit elongated mitotic spindles, which are typically more than 5 μm in length, while checkpoint-proficient cells keep the short spindle (<2 μm) (Fig. 4).

29. For Fast Prep-24, use the following settings: level 6.5, 60 s, three times with 5-min cooling on ice in between beating steps.

30. At this step the silica beads should stay in the 2-mL screw-cap tube. For collection of cells, FACS sample tubes (e.g., Semadeni, 3190; 10 mm × 54 mm length) or normal 1.5-mL tubes can be used depending on the size of the centrifuge used for collection.

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