Chapter 22

Telomere Regulation During the Cell Cycle in Fission Yeast

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Abstract

The fission yeast *Schizosaccharomyces pombe* has emerged as a useful model organism to study telomere maintenance mechanisms. In this chapter, we provide detailed protocols for quantitative ChIP and BrdU incorporation analyses to investigate how fission yeast telomeres are regulated during the cell cycle by utilizing *cdc25-22* synchronized cell cultures.

Key words Cell cycle synchronization, *cdc25-22*, BrdU incorporation, Antibodies, Chromatin immunoprecipitation, Dot blot analysis, DNA hybridization, Real-time quantitative PCR

1 Introduction

Telomeres are the natural ends of eukaryotic chromosomes that must be protected from DNA repair enzymes and checkpoint response factors [1]. In most eukaryotic cells, telomeric DNA consists of G-rich repetitive sequences, and the telomere-specific reverse transcriptase known as telomerase is utilized to extend telomeric repeats to counteract loss of telomeric DNA during DNA replication by replicative DNA polymerases [2]. Telomeric repeat sequences provide binding sites for telomere-specific protective complexes, such as shelterin and CST (CTC1/Cdc13-Stn1-Ten1), which in turn regulate the accessibility of telomeres to DNA damage response factors and telomerase [3, 4]. While studies in the telomere field are often performed with asynchronous cell cultures, it is important to recognize that telomere maintenance mechanisms are carefully regulated during cell cycle, and in fact, recruitment of telomerase to telomeres has been found to be intimately linked to DNA replication [2, 5]. Therefore, in order to fully understand how cells ensure stable maintenance of telomeres, we must study them in the context of the cell cycle.

In recent years, the fission yeast *Schizosaccharomyces pombe* has emerged as an attractive model organism to study telomere biology, since it utilizes telomere factors that show high degree of

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evolutionary conservation with mammalian telomere factors [4]. In this chapter, we describe our detailed protocol for chromatin immunoprecipitation (ChIP) assays on samples taken from synchronized cell cultures to characterize how the protein composition of telomeres is dynamically regulated during the cell cycle. While various ways to synchronize fission yeast cell cultures have been described [6, 7], we prefer the use of the temperature-sensitive *cdc25-22* allele, since no other method easily allows for collection of sufficient quantity of cells from synchronized cell cultures to carry out ChIP experiments. Cdc25 is a key regulator of cell cycle progression in fission yeast [8], and its inactivation causes cells to arrest in G2 phase. In addition, we describe our protocol for monitoring the replication timing of telomeric DNA by quantitatively determining the timing of BrdU incorporation after release from the *cdc25-22* cell cycle arrest in G2 phase.

Finally, both real-time quantitative PCR (qPCR)- and dot blot hybridization-based methods to analyze ChIP and BrdU incorporation experiments are described. While wild-type fission yeast cell telomeres are relatively short (~300 bp) and can be effectively characterized by both methods, the dot blot hybridization-based method should be carried out for mutant cells with longer (≥1 kb) telomeres. Although we only describe ChIP and BrdU incorporation protocols in this chapter, it should be noted that samples obtained from synchronized *S. pombe* cultures can also be used for other types of experiments, such as western blot analysis to monitor protein levels and/or protein modifications, co-immunoprecipitation analysis to monitor telomere complex formation, and Southern blot analysis to monitor telomere length variations. Thus, we hope that the protocols described here prove useful in elucidating cell cycle regulation of fission yeast telomeres in future studies.

2 Materials

2.1 Cell Cycle Synchronization of cdc25-22 Cells

1. YES medium: 5 g Yeast extract, 30 g glucose (dextrose), 100 mg leucine, 100 mg uracil, 100 mg histidine-HCl, and 100 mg Adenine per 1 L. Autoclave for 20 min.
2. Hydroxyurea (HU) stock solution: 1 M HU in ddH$_2$O. Filter-sterilize with a 0.22 μm filter, and store at −20 °C until use.

2.2 Chromatin Immunoprecipitation Analysis

1. Formaldehyde solution: 11 % Formaldehyde (v/v), 100 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA, 50 mM Tris–HCl pH 8.0.
2. 2.5 M glycine: Dissolve 93.8 g glycine in 500 mL ddH$_2$O, filter-sterilize, and store at room temperature.
3. Tris-buffered saline (TBS): 20 mM Tris–HCl pH 7.6, 150 mM NaCl. Store at 4 °C.

4. 0.5 mm Glass beads.
5. Fast Prep® FP120 (Qbiogene).
6. Misonix Sonicator 3000 with cup horn device.
7. Lysis buffer: 50 mM Hepes–KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton X-100, 0.1 % (w/v) sodium deoxycholate, "Complete" protease inhibitor (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF). Prepare fresh on ice.
8. Lysis buffer 500: Replace 140 mM NaCl in lysis buffer (**item 7** above) with 500 mM NaCl and no protease inhibitors. Prepare fresh on ice.
9. Wash buffer: 10 mM Tris–HCl pH 8.0, 0.25 M LiCl, 0.5 % NP-40, 0.5 % sodium deoxycholate, 1 mM EDTA. Prepare fresh on ice.
10. TE buffer: 10 mM Tris–HCl, 1 mM EDTA pH 7.5. Store at 4 °C.
11. Antibodies: We commonly use monoclonal anti-myc (9B11, Cell Signaling 2276S) or anti-FLAG (M2, Sigma F1804) antibodies for myc- or FLAG-tagged proteins, respectively. Other antibodies that efficiently purify the protein of interest can also be used.
12. Dynabeads® Protein G (Life Technologies, 30 mg/mL).
13. Magnetic stand.
14. Chelex 100 Resin 10 %: 0.1 mg/mL in ddH_2O (BioRad). Prepare fresh.
15. Proteinase K: Dissolve Proteinase K (Invitrogen) at 10 mg/mL in 10 mM Tris–HCl pH 7.5, 20 mM calcium chloride, 50 % glycerol. Store at −20 °C.

2.3 Analysis of 5-Bromo-2-Deoxyuridine-Incorporated DNA

1. 5-Bromo-2-deoxyuridine (BrdU): 10 mM BrdU stock solution (3.07 mg/mL) in ddH_2O. Prepare fresh.
2. 1 M Sodium azide: Store at room temperature.
3. SP1 buffer: 1.2 M sorbitol, 50 mM sodium citrate, 50 mM sodium phosphate (Na_2HPO_4), 40 mM EDTA. Adjust pH to 5.6 using citric acid.
4. Zymolyase 100T (Amsbio).
5. 5× TE: 50 mM Tris pH 8.0, 5 mM EDTA.
6. 10 % Sodium dodecyl sulfate (SDS): 10 g SDS in 100 mL ddH_2O. Store at room temperature.
7. 5 M Potassium acetate: Dissolve 49.1 g KCH_3CO_2 in 100 mL ddH_2O. Autoclave, and store at room temperature.
8. Isopropanol.
9. DNase free RNase A (Fermentas).

10. Phenol: Equilibrated with 10 mM Tris pH 8.0, 1 mM EDTA.
11. Phenol, chloroform, and isoamyl alcohol mix (25:24:1), saturated with 10 mM Tris pH 8.0, 1 mM EDTA.
12. Chloroform and isoamyl alcohol mix (24:1).
13. Ethanol: Store at −20 °C.
14. 10 mM Tris–HCl pH 8.0: Store at room temperature.
15. Anti-BrdU antibody: 25 μg/mL (Becton Dickinson, 347580).
16. Dynabeads® Protein G: 30 mg/mL (Life Technologies).
17. Magnetic stand.
18. TBS: 20 mM Tris–HCl pH 7.6, 150 mM NaCl. Store at 4 °C.
19. 1× Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per 1 L ddH_2O. Dissolve components in 800 mL ddH_2O, adjust to pH 7.4 with HCl, and bring to 1 L with ddH_2O. Sterilize by autoclaving, and store at 4 °C.
20. IP buffer: Add 0.05 % Triton X-100 to 1× PBS.
21. TBSE: 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA.
22. TBSE + Triton: TBSE + 1 % Triton X-100.
23. TE buffer: 10 mM Tris–HCl, 1 mM EDTA pH 7.5.
24. Chelex 100 Resin 10 %: 0.1 mg/mL in ddH_2O (BioRad). Prepare fresh.
25. Proteinase K: Dissolve Proteinase K at 10 mg/mL in 10 mM Tris–HCl pH 7.5, 20 mM calcium chloride, 50 % glycerol. Store at −20 °C.

2.4 Quantitative PCR Analysis

1. iQ SYBR Green Supermix (Bio-Rad).
2. PCR primers: jk380 (5′-TAT TTC TTT ATT CAA CTT ACC GCA CTT C-3′) and jk381 (5′-CAG TAG TGC AGT GTA TTA TGA TAA TTA AAA TGG-3′) [9].
3. iQ5 Real-Time PCR Detection System with iQ5 Optical Software (Bio-Rad).

2.5 Dot Blot Analysis

1. 1 M NaOH.
2. 500 mM EDTA.
3. Amersham Hybond-XL membrane (GE Healthcare).
4. Bio-Dot Microfiltration Apparatus (Bio-Rad).
5. 2× Saline sodium citrate (SSC): 300 mM NaCl, 30 mM Na citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$).
6. UV Stratalinker 1800 (Stratagene).
7. Church buffer: 0.25 M Sodium phosphate buffer pH 7.2, 1 mM EDTA, 1 % BSA, 7 % SDS. Aliquot, and store at −20 °C.
8. Stratagene Prime It II Labeling Kit.

9. [α-^{32}P]-dCTP (3,000 Ci/mmol).
10. ProbeQuant G-50 Micro Columns (GE).
11. Telomeric probe template DNA: Gel purify a ~300 bp SacI-ApaI DNA fragment from pTELO plasmid and resuspend in 10 mM Tris, pH 8.5 (*see* **Note 1**).
12. 0.1× SSC, 0.1 % SDS.
13. PhosphorImager (Molecular Dynamics) and phosphorImager cassettes.

3 Methods

3.1 Cell Cycle Synchronization of cdc25-22 Cells

1. Grow a 500 mL culture of *cdc25-22* cells in YES medium overnight at 25 °C to OD_{600} = ~0.25 (early- to mid-exponential phase). Shift the cell culture to 36 °C for 3 h to arrest cells in G2 (*see* **Note 2**). Shift the culture back to 25 °C rapidly (*see* **Note 3**), incubate at 25 °C for the ongoing experiment, and take 30 mL aliquots every 20 min for 4 h (*see* **Note 4**).
2. In order to prevent telomere replication, HU can be added to the culture (15 mM final concentration) immediately after it has been shifted back to 25 °C. HU causes stalling of ongoing replication forks due to dNTP depletion and inhibits replication of late-replicating regions, including telomeres [5, 10].
3. Cell cycle progression should be monitored by measuring the septation index (% of cells with septum). *S. pombe* cells form a septum during G1/S phase, which can be easily monitored microscopically. The septation index should reach 60–80 % during S phase (Fig. 1). At every time point take ~5 μL culture to determine the septation index.

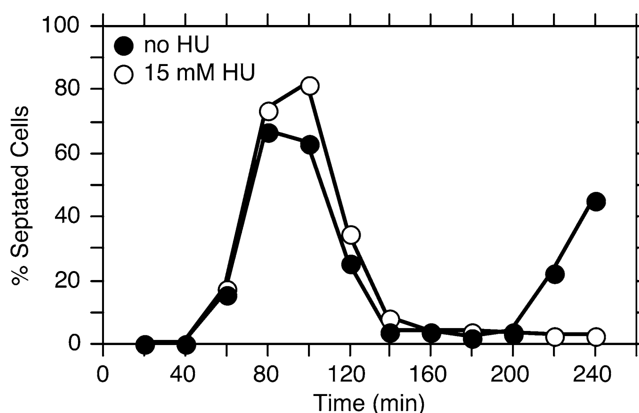

Fig. 1 Typical changes in the percentage of septated cells after release from a *cdc25-22* cell cycle arrest

3.2 Chromatin Immunoprecipitation Analysis

Cell cycle-regulated association of telomere proteins can be monitored by performing ChIP analysis of samples obtained from synchronized cell cultures (*see* Subheading 3.1). In ChIP analysis, proteins are first cross-linked to DNA with formaldehyde and then purified with protein-specific antibodies. DNA is recovered and analyzed for the presence of telomeric DNA sequences. While many ChIP protocols exist, we prefer a protocol that is short, is less toxic, and produces highly reproducible results by using Chelex 100 resin [11], rather than phenol–chloroform extraction or various commercially available spin columns designed to purify dsDNA to recover DNA (*see* **Note 5**).

1. Fix 30 mL aliquot samples (*see* Subheading 3.1) immediately with 1 % formaldehyde by adding 1/10 sample volume of formaldehyde solution and incubating for 20 min at room temperature by gently shaking.
2. Add 2.5 M glycine to a final concentration of 125 mM and incubate for 5 min at room temperature while shaking.
3. Collect cells by centrifugation (2,100 × *g* for 5 min) and transfer into a 1.5 mL screw top microcentrifuge tube.
4. Wash three times with 1 mL of TBS on ice (*see* **Note 6**). Snap-freeze the cell pellets in liquid nitrogen and store at −80 °C until further use (*see* **Note 7**).
5. Thaw cell pellets on ice. For all subsequent steps, work should be performed on ice or at 4 °C using ice-cold buffers.
6. Resuspend each cell pellet in 250 μL of lysis buffer, add glass beads, and lyse cells in Fast Prep® (*see* **Note 8**).
7. Recover lysate in a new microcentrifuge tube by punching a small hole into the bottom of the tube with a hot needle, putting the tube into a receiver tube (snap cap), and spinning at 376 × *g* for a few seconds in a microcentrifuge.
8. Sonicate lysate in an ice bath (Misonix Sonicator 3000 with cup horn device) to obtain DNA fragments ~500 bp (*see* **Note 9**). Centrifuge at 16,200 × *g* for 10 min at 4 °C. Recover supernatant.
9. Prepare 1–2 mg of lysate in 200 μL of lysis buffer for each ChIP (*see* **Note 10**). For input control, save 5 μL and process as described below. Until it is ready to be processed, it should be stored at −20 °C.
10. For ChIP samples, add 1 μg of antibody to the lysate and incubate with gentle rocking for 2 h at 4 °C.
11. Add 25 μL of Dynabeads® Protein G (*see* **Note 11**), and incubate for 1 h at 4 °C. Alternatively, preincubate the antibody with Dynabeads® Protein G for 1 h at 4 °C, wash the beads using the magnetic stand with 1 mL of lysis buffer to remove

excess antibody, add the beads to lysates, and incubate for 2–3 h at 4 °C. The amount of antibody and beads can be adjusted depending on the protein of interest and its epitope.

12. Using the magnetic stand, wash beads successively 2× with 1 mL of lysis buffer (*see* **Note 12**), 2× with 1 mL of lysis buffer 500, 2× with 1 mL of wash buffer, and 2× with 1 mL of TE buffer. For washing with lysis buffer 500 and wash buffer, samples should be incubated for 10 min at 4 °C on a rotating device. Remove all liquid after the final wash (*see* **Note 13**).
13. Add 100 μL of 10 % Chelex 100 Resin to input (*see* **step 9** above) and bead samples, mix, and boil for 15 min to reverse the formaldehyde cross-link in order to recover DNA (*see* **Note 14**).
14. Cool samples to room temperature, add 2 μL of Proteinase K, and incubate at 55 °C for 30 min while shaking.
15. Add an additional 100 μL of ddH_2O. Boil for 10 min, and recover 130 μL of supernatant (*see* **Note 14**). Store samples at −20 °C.
16. Samples are now ready for qPCR analysis (*see* Subheading 3.4) or dot blot analysis (*see* Subheading 3.5).

3.3 Analysis of BrdU-Incor-porated DNA

BrdU is a thymidine analog, and its incorporation into newly synthesized DNA during cell cycle progression allows for monitoring the progression of the DNA replication fork. Anti-BrdU antibodies can be utilized in immunoprecipitation to purify BrdU-labeled DNA from bulk DNA samples. The protocol described here has been used to determine the timing of replication at telomeres in our lab [5], but it can also be adapted to monitor replication timing of other genomic loci.

For this experiment, modified fission yeast cells carrying the human equilibrative nucleoside transporter 1 (*hENT1*) gene and the *Herpes simplex* virus thymidine kinase (*hsv-tk*) gene are used. Expression of hENT is required for efficient entry of BrdU into cells, and expression of TK is necessary to incorporate BrdU into DNA [12, 13].

1. Synchronize *cdc25-22 hENT hsv-tk* cells as described above (*see* Subheading 3.1).
2. After the arrested cell culture has been shifted back to 25 °C, add BrdU to the culture to a final concentration of 50 μM. Incubate cells at 25 °C, and take 30 mL of aliquots every 20 min.
3. Add sodium azide to each aliquot to a final concentration of 10 mM.
4. Spin at 2,100 × *g* for 5 min.
5. Wash pellets in 1 mL of SP1 buffer, transfer to a 1.5 mL microcentrifuge tube, and centrifuge to collect cells as pellets.

6. Snap-freeze the cell pellets in liquid nitrogen and store at −80 °C.
7. Thaw cell pellets on ice, resuspend each in 1 mL of SP1 buffer supplemented with 0.2 mg/mL Zymolyase 100T, and incubate at 37 °C for 30–60 min while shaking in order to obtain protoplasts by digesting the cell wall. To monitor completion of the cell wall digestion, mix 2 μL of the protoplasts and 2 μL of 2 % SDS, and then check under a phase-contrast microscope (*see* **Note 15**).
8. Gently spin the protoplasted cells in a microcentrifuge at 1,500 × *g* for 2 min, and resuspend them in 0.9 mL of 5× TE. Bring to 1 % SDS by adding 100 μL of 10 % SDS, and incubate at 65 °C for 15 min.
9. Add 306 μL of 5 M potassium acetate (1.17 M final), and incubate on ice for 10 min. Spin sample at 16,200 × *g* for 10 min at 4 °C, and recover 800 μL of supernatant.
10. Add 1 volume (800 μL) of isopropanol to each sample, and incubate on ice for 30 min.
11. Spin sample at 16,200 × *g* for 15 min at 4 °C. Remove all liquid, and air-dry pellet for 10–15 min. When the color of the pellet turns from white to clear, move on to the next step.
12. Resuspend each sample in 500 μL of 5× TE + RNaseA (20 μg/mL) and incubate at 37 °C for 2 h. Wrap samples in aluminum foil to minimize exposure of BrdU-labeled DNA to light.
13. Extract samples sequentially with phenol, phenol/chloroform/isoamyl alcohol, and chloroform/isoamyl alcohol (500 μL each), and recover aqueous (top) phase, which contains DNA (*see* **Note 16**).
14. Precipitate DNA by adding 2.5× volume of −20 °C ethanol and incubating at −20 °C for 30 min.
15. Spin at 16,200 × *g* for 15 min at 4 °C, and wash pellet with 500 μL of 70 % ethanol. Remove any residual liquid, and air-dry pellet for 10–15 min.
16. Resuspend pellet in 50 μL of 10 mM Tris pH 8.0, and measure DNA concentration (*see* **Note 17**). Samples can be stored at −20 °C.
17. Prepare 2 μg of each DNA sample in 50 μL 10 mM Tris pH 8.0.
18. Sonicate samples to obtain DNA fragments ~500 bp (*see* **Note 9**). Fragmentation of DNA should be verified by running 4 μL of sample on a 1.5 % agarose gel. Samples can be stored at −20 °C.
19. For every sample, preincubate 1 μg (40 μL) of the anti-BrdU antibody with 30 μL of pre-washed Dynabeads® Protein G at 4 °C overnight. This step should be scaled up for the number of samples (= *n*) for each experiment (*see* **Note 18**).

20. Next day, using the magnetic stand wash beads with 1 mL of TBS, resuspend in 1 ml of TBS, and split into *n* times aliquots. Remove all liquid from the beads and resuspend in 100 µL of IP buffer each.
21. Use 6 µL of sonicated sample (*see* **step 18**), and add 54 µL of IP buffer. Heat denature at 95 °C for 5 min (*see* **Note 14**), and put immediately on ice.
22. Add 50 µL of the sonicated and denatured DNA (*see* **step 21**) to the 100 µL of the anti-BrdU/beads/IP buffer (*see* **step 20**). Incubate at room temperature for 2 h with gentle rocking. Reserve the remaining sonicated and denatured DNA sample as input and store at −20 °C.
23. Using the magnetic stand, wash beads successively 2× with 1 mL of TBSE, 4× with 1 mL of TBSE + Triton, and 1× with 1 mL of TE buffer. For each wash, incubate the beads for 10 min at 4 °C on a rotating device. After the final TE buffer wash, remove all liquid and keep beads only.
24. To recover DNA, add 100 µL of 10 % Chelex 100 Resin to input (*see* **step 22**) and bead samples (*see* **step 23**), mix, and boil for 15 min (*see* **Note 14**). Cool samples to room temperature.
25. Add 2 µL of Proteinase K, and incubate at 55 °C for 30 min while shaking.
26. Add an additional 100 µL of ddH_2O, boil for 10 min (*see* **Note 14**), and using the magnetic stand, recover 130 µL supernatant. Store samples at −20 °C.
27. Samples are now ready for qPCR analysis (Subheading 3.4) or dot blot analysis (Subheading 3.5).

3.4 Quantitative PCR Analysis

In this analysis, we quantify the amount of telomeric DNA that has been coprecipitated with a given protein in ChIP or telomeric DNA that has been pulled down with the anti-BrdU antibody. Primers have been designed against a region within the subtelomere immediately adjacent to the G-rich telomeric repeat sequence, ~400 bp from the end of wild-type length telomeres. It would be difficult to provide a universal protocol that covers various real-time PCR setups available in other labs. Thus, we will only describe our protocol, which utilizes SYBR Green to quantify PCR products. However, we hope that the following protocol may serve as a useful guideline for researchers who wish to utilize other types of real-time PCR reagents.

1. Input samples should be diluted 1:100 in ddH_2O, while IP samples can be taken straight for qPCR analysis (*see* **Note 19**).
2. PCR reaction: 2 µL sample, 1× iQ SYBR Green Supermix, 0.3 µM primers (*see* **Note 20**). PCR condition: 3 min 95 °C, followed by 40 cycles [20 s 95 °C, 40 s 60 °C].

3. Calculate % of precipitated DNA values based on ΔCt between input and IP samples with the following formula: [% of Precipitated DNA] = $[100 \times E\Delta^{Ct}]/[D \times R]$, where E = amplification efficiency of primer pairs, ΔCt = [Ct Input] − [Ct ChIP], D = [dilution factor of ChIP]/[dilution factor of input], and R = [Sample volume used in IP]/[Sample volume set aside for input control] (*see* **Note 21**).

3.5 Dot Blot Analysis

Dot blot analysis is preferred over qPCR for fission yeast strains that carry significantly longer telomeres (≥1 kb) than wild-type cells. This is because telomeric ends may be too distant from the subtelomeric region that is amplified in our qPCR analysis.

1. Use 120 μL of DNA sample, and bring to 0.4 M NaOH and 10 mM EDTA in a total volume of 300 μL. Heat sample at 100 °C for 10 min (*see* **Note 14**), and rapidly cool on ice.
2. Pre-wet Hybond-XL membrane in ddH_2O for 15 min.
3. Set up the Bio-Dot Microfiltration Apparatus according to the manufacturer's instructions. Lay the membrane on the gasket in the apparatus so that it covers all the holes, and remove all air bubbles underneath. Place the sample template on top and screw into place. All sample wells that are not needed should be tightly taped off. Attach your vacuum source (house vacuum or pump).
4. Apply 500 μL of ddH_2O to all sample wells to rehydrate the membrane. Remove all ddH_2O gently by applying vacuum. Release the vacuum.
5. Apply 300 μL of denatured DNA (*see* **step 1** and **Note 22**) with the vacuum, wash wells with 500 μL of 0.4 M NaOH, and remove membrane from the blotting apparatus.
6. Rinse the membrane one time in 2× SSC, and allow to air-dry.
7. UV-cross link (Stratalinker, autocrosslink, 120 $\mu J/cm^2$) samples to the membrane.
8. Hybridize membrane in Church buffer for 30 min at 65 °C.
9. To prepare the telomeric probe, we use the Stratagene Prime It II Labeling Kit according to the manufacturer's instructions. For removal of excess unincorporated [α-^{32}P]-dCTP, we use illustra ProbeQuant G-50 Micro Columns. Denature the double-stranded probe by heating at 100 °C for 5 min, and then cool rapidly on ice.
10. Add the telomeric probe to the Church buffer at 10^6 cpm/mL, and incubate for 24 h at 65 °C.
11. Wash membrane in 0.1× SSC and 0.1 % SDS for 30 min at 65 °C.
12. Expose membrane on a PhosphorImager cassette for 24–48 h.

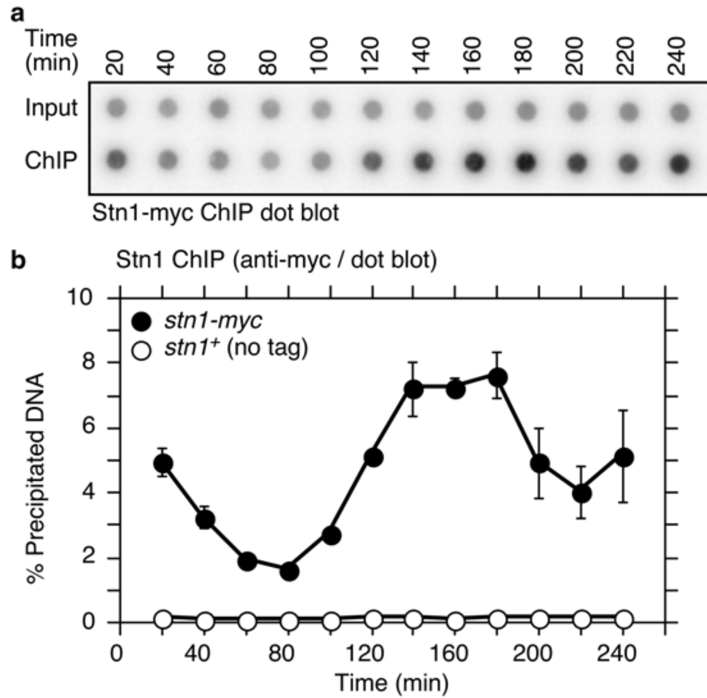

Fig. 2 Cell cycle ChIP analysis of the fission yeast shelterin subunit Stn1 by dot blot hybridization to a telomeric DNA probe. (**a**) Stn1-myc ChIP dot blot hybridized to telomere probe. (**b**) Cell cycle ChIP data (Stn1-myc and no tag control) obtained from *cdc25-22* synchronized samples. Error bars represent standard deviation

13. Quantify using ImageQuant software (GE Healthcare Life Sciences) or similar software. *See* Fig. 2 for an example of a dot blot-based cell cycle ChIP analysis.

4 Notes

1. The pTELO plasmid was generated by cloning ~300 bp SacI-ApaI telomere repeat DNA fragment from pNSU70 plasmid [14]. The pTELO plasmid is available upon request from our laboratory. Alternatively, pNSU70 can also be used to make a telomere repeat probe. While the pNSU70 plasmid was originally constructed by Dr. Neal Sugawara, it was only described in his Ph.D. thesis. However, the pNSU70 plasmid should be widely available from most laboratories that study fission yeast telomeres.
2. All cells should be elongated, and no septa should be visible after 3-h incubation at 36 °C. This can easily be determined by microscopic observation. If for any reason this is not the case, the culture can be incubated at 36 °C for an additional 30–60 min.

3. It is important that cell cultures are quickly equilibrated to their permissive temperature. To achieve this, we typically place our flasks in large 5 L autoclave pans filled with ice and water, and shake them gently by hand. Changes in temperature are carefully monitored by directly inserting a thermometer into the cell culture.
4. Although the cell cycle experiment can be performed for longer time periods, the synchrony of the culture will eventually diminish. Therefore, carrying out experiments beyond a second full round of cell cycle (~300 min after release from *cdc25-22* arrest) is not recommended. We have settled on 20-min time point intervals as it allows us to process samples for ChIP and determine the septation index. If shorter time intervals are required, it may be necessary to have two people to collect and process samples.
5. We found DNA purification columns that are often used in asynchronous ChIP protocols to be unsuitable for cell cycle ChIP for telomeres, as we are especially interested in localization of proteins during replication, and replication intermediates may purify differently from canonical dsDNA.
6. Between washes, perform low-speed spins at 1,500 × *g* for 30 s in a microcentrifuge to allow easy resuspension of pellets. After the final wash, perform a high-speed spin at 16,100 × *g* for 15 s and completely remove all excess liquid.
7. Since synchronization and sample collection require several hours, we usually collect and freeze samples at −80 °C in 1 day and perform ChIP and BrdU analysis on a later day. We found samples stored at −80 °C to be stable for at least several months.
8. When adding glass beads, we leave a little space at the top, so that the beads have room to move. Our FastPrep is set up in a 4 °C room. We usually perform lysis at setting 5.5 for 4× 15 s, with 2-min cooling intervals with an open lid. Other glass bead-based homogenizers may also work to lyse cells, but one should check carefully under a microscope to make sure that the majority of cells have been lysed.
9. Sonication conditions must be established depending on the sonicator used to ensure proper fragmentation of DNA. For ChIP analysis (*see* Subheading 3.2) using the Misonix Sonicator 3000 with cup horn device, we perform ten rounds of sonication for 15 s with 1-min cooling intervals at setting 5.0. For BrdU labeling (*see* Subheading 3.3) we perform six rounds of sonication for 15 s with 1-min cooling intervals at setting 6.0.
10. The amount of lysate used for ChIP might vary depending on the protein of interest, but 1–2 mg of lysate has worked well for us for most proteins we investigated.

11. We generally use 25 μL of Dynabeads® Protein G per ChIP sample. To prepare the beads, wash them with TBS and resuspend in lysis buffer. The use of magnetic beads is preferred over the use of Sepharose- or agarose-based beads, as magnetic beads result in significantly less background and therefore higher reproducibility. To wash and handle magnetic beads, we used a magnetic stand throughout our protocols. Magnetic stands are available from various vendors.
12. While the lysis buffer used to prepare the lysate should be supplemented with protease inhibitors, buffers used for sample washes do not require protease inhibitors.
13. As ChIP is a highly quantitative analysis, variation among samples has to be avoided. Therefore, it is important to avoid any loss of beads during the washes and to remove all liquid after the final wash.
14. It is important to avoid popping of tube lids during boiling, which would result in loss of sample. Use cap closures or a heavy weight to prevent the lids from popping.
15. Addition of SDS should cause protoplasted cells to lyse. Lysed cells will appear flat and less shiny (or dark) under a phase-contrast microscope.
16. The interface between the aqueous phase and the organic phase should become clear. If it is not, extra extraction steps must be carried out before moving on to the next step.
17. To measure DNA concentrations, we are currently using the Qubit® 2.0 Fluorometer from Life Technologies with dsDNA BR Assay Kit, which utilizes a fluorescent dye that only emits a signal when bound to DNA, even at low concentrations. This method is more reliable than measuring DNA concentration by UV absorbance at 260 nm and thus improved the reproducibility of our BrdU experiments by allowing us to more consistently utilize equal amounts of DNA across different time points.
18. To pre-wash, use n times 30 μL Dynabeads® Protein G in a 1.5 mL microcentrifuge tube, wash 2× with 1 mL TBS, and remove all liquid. Add n times 40 μL anti-BrdU antibody and n times 40 μL TBS. Incubate at 4 °C o/n.
19. When undiluted ChIP input samples are used for qPCR analysis, PCR reactions can be inhibited, possibly due to residual inhibitory factors in the sample. Therefore, we recommend performing an initial titration analysis of your samples to make sure that you are testing within the linear range.
20. While we are using SYBR Green for our qPCR analysis, conditions can be established based on your method of choice. Although most manufacturers recommend a reaction volume of 50 μL, we routinely perform 10 μL reactions, as we did not

find any noticeable difference in PCR results performed at either volume. For each time point, we set up triplicate PCR reactions to obtain average Ct values for input and IP samples.

21. For the ChIP protocol described in Subheading 3.2, $E=2$ (based on titration analysis, primer pairs jk380 and jk381 had ~100 % amplification efficiency), $D=1/0.01=100$ (ChIP samples are used undiluted, while input samples are diluted 1:100), and $R=195/5$ (195 μL extract was used in IP, while 5 μL extract was set aside as input control). These values should be adjusted if other primer pairs are used or protocols are modified. For example, if a new pair of primers is found to have only 80 % amplification efficiency, E should be 1.8.
22. During sample application, samples occasionally do not transfer well through the membrane although the vacuum is applied. In this case, carefully pipette the liquid in the well up and down a few times.

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References

1. Lydall D (2009) Taming the tiger by the tail: modulation of DNA damage responses by telomeres. EMBO J 28(15):2174–2187
2. Verdun RE, Karlseder J (2007) Replication and protection of telomeres. Nature 447(7147): 924–931
3. Price CM, Boltz KA, Chaiken MF, Stewart JA, Beilstein MA, Shippen DE (2010) Evolution of CST function in telomere maintenance. Cell Cycle 9(16):3157–3165
4. Moser BA, Nakamura TM (2009) Protection and replication of telomeres in fission yeast. Biochem Cell Biol 87(5):747–758
5. Moser BA, Subramanian L, Chang YT, Noguchi C, Noguchi E, Nakamura TM (2009) Differential arrival of leading and lagging strand DNA polymerases at fission yeast telomeres. EMBO J 28(7): 810–820
6. Forsburg SL (2003) Growth and manipulation of *S. pombe*. Curr Protoc Mol Biol Chapter 13:Unit 13.16
7. Alfa C, Fantes P, Hyams J, McLoed M, Warbrick E (1993) Experiments with Fission Yeast. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
8. Russell P, Nurse P (1986) *cdc25*+ functions as an inducer in the mitotic control of fission yeast. Cell 45(1):145–153
9. Kanoh J, Sadaie M, Urano T, Ishikawa F (2005) Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. Curr Biol 15(20):1808–1819
10. Hayashi M, Katou Y, Itoh T, Tazumi A, Yamada Y, Takahashi T, Nakagawa T, Shirahige K, Masukata H (2007) Genome-wide localization of pre-RC sites and identification of replication origins in fission yeast. EMBO J 26(5):1327–1339
11. Nelson JD, Denisenko O, Bomsztyk K (2006) Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat Protoc 1(1):179–185
12. Sivakumar S, Porter-Goff M, Patel PK, Benoit K, Rhind N (2004) *In vivo* labeling of fission yeast DNA with thymidine and thymidine analogs. Methods 33(3):213–219
13. Hodson JA, Bailis JM, Forsburg SL (2003) Efficient labeling of fission yeast *Schizosaccharomyces pombe* with thymidine and BUdR. Nucleic Acids Res 31(21):e134
14. Sugawara NF (1988) DNA sequences at the telomeres of the fission yeast *S. pombe*. Ph.D. thesis. Harvard University, Cambridge, Massachusetts