9

In Situ Assay for Analyzing the Chromatin Binding of Proteins in Fission Yeast

Stephen E. Kearsey, Lydia Brimage, Mandana Namdar, Emma Ralph, and Xiaowen Yang

Summary

An *in situ* technique for studying the chromatin binding of proteins in single fission yeast cells (*Schizosaccharomyces pombe*) is described. Cells are permeabilized by enzymatic digestion and extracted with a detergent-containing buffer. This procedure removes soluble proteins, but proteins that are bound to insoluble cell structures such as chromatin are retained, and overall cell morphology is maintained. Extraction of proteins is monitored by fluorescence microscopy, either using fluorescently tagged proteins or by indirect immunofluorescence. This method allows the chromatin association of proteins to be correlated with other cell cycle events without the need for cell synchronization.

Key Words

DNA replication; chromatin; cell cycle; *Schizosaccharomyces pombe*; fission yeast; GFP.

1. Introduction

The process of eukaryotic DNA replication involves stepwise assembly of replication proteins at origins in the process leading to initiation. An important protein complex involved in initiation is the origin recognition complex (ORC), which in budding and fission yeasts is bound to chromatin throughout the cell cycle and acts as a site where additional proteins needed for initiation bind. First, Cdt1 and Cdc6/Cdc18 bind to chromatin via ORC, and subsequently the minichromosome maintenance (Mcm)2–7 complex assembles onto origins, to form pre-replicative complexes (pre-RCs). The pre-RC is competent for initiation in S-phase upon activation by Cdc7 and Cdc2 protein kinases, and this process involves the assembly of additional proteins onto chromatin such as Cdc45 and the GINS (Sld5-PSF1-PSF2-PSF-3) complex. Thus, monitoring the chromatin binding of Mcm2–7 allows acquisition of replication com-

From: *Methods in Molecular Biology, vol. 296, Cell Cycle Protocols*
Edited by: T. Humphrey and G. Brooks © Humana Press Inc., Totowa, NJ

petence to be followed, and Cdc45 can be used to indicate the onset of DNA synthesis. Analyzing the chromatin binding of replication factors in mutants can be used to establish functional dependencies and to help establish when a replication factor functions. For instance, inactivation of Cdc23/Mcm10 has no effect on Mcm2–7 chromatin binding, but Cdc45 chromatin association is blocked, implying that Cdc23/Mcm10 functions after pre-RC formation at around the time of initiation ***(1)***.

A number of methods for detecting chromatin association of specific proteins have been developed ***(2)***. Chromatin immunoprecipitation uses formaldehyde to crosslink proteins to DNA, and, following shearing and immunoprecipitation of a specific protein, the association of specific DNA sequences with that protein can be established by polymerase chain reaction (PCR; e.g., *see* **ref. *3***). An alternative approach is to use Western blotting to examine whether proteins fractionate with crude chromatin preparations ***(2)***. Proteins that are in the chromatin fraction before but not after nuclease treatment can be considered to be chromatin-associated. Finally, cytological methods using fluorescence microscopy allow the chromatin association of proteins in single cells to be examined. Mammalian and other cultured cells can be permeabilized with nonionic detergents prior to fixation, which extracts soluble nuclear proteins. This allowed the demonstration that Mcm2–7 proteins, which remain in the nucleus throughout interphase in mammalian cells, are only bound to chromatin during the telophase/S-phase interval (e.g., *see* **ref. *4***). This approach is more difficult in yeasts, in which the cell wall prevents simple detergent extraction. In the chromosome-spreading technique, yeast cell walls are removed by enzymatic digestion, and spheroplasts are simultaneously lysed and fixed ***(5)***. This treatment destroys cell structure, but chromatin association of specific proteins can be detected by subsequent staining with antibodies.

We have developed an *in situ* chromatin binding assay for use with fission yeast that is much less destructive to cell structure ***(6)***. The method involves partial removal of the cell wall using a β-glucanase (zymolyase), which leaves α-glucan polymers intact. This enables the cells to withstand detergent extraction, which removes soluble nucleoplasmic proteins, but proteins retained on chromatin can be subsequently detected using a green fluorescent protein (GFP) tag, or by indirect immunofluorescence (**Fig. 1**). Since cell structure is maintained, the chromatin binding of a specific protein can be correlated with other cell cycle events such as anaphase spindle elongation or septation, and in many experiments the need for cell synchronization can be avoided. In addition to analysis of replication proteins ***(7,8)*** this method has also been used with proteins involved in checkpoint responses ***(9,10)*** and sister chromatid cohesion ***(11)***.

Fig. 1

This procedure is simplest when the protein of interest is tagged with a fluorescent protein, since no further processing of the samples is necessary after extraction and fixation (**Subheading 3.1.**). However, indirect immunofluorescence can be used to detect proteins after detergent extraction and fixation (**Subheading 3.2.**). Retention of a nuclear protein after detergent extraction could reflect chromatin binding; alternatively, the protein could be associated with the nuclear matrix or be insoluble under the conditions used. Therefore it is important to show that retention of a protein after detergent extraction is abolished upon nuclease digestion of chromatin (**Subheading**

Job: Humphrey 144-8
Chapter: 09 (Kearsey)
Template: MiMB/6x9/CondTempW/S/Rev.04.03
Compositor: Pius
Date: 7/13/4
Revision: 1st Pass

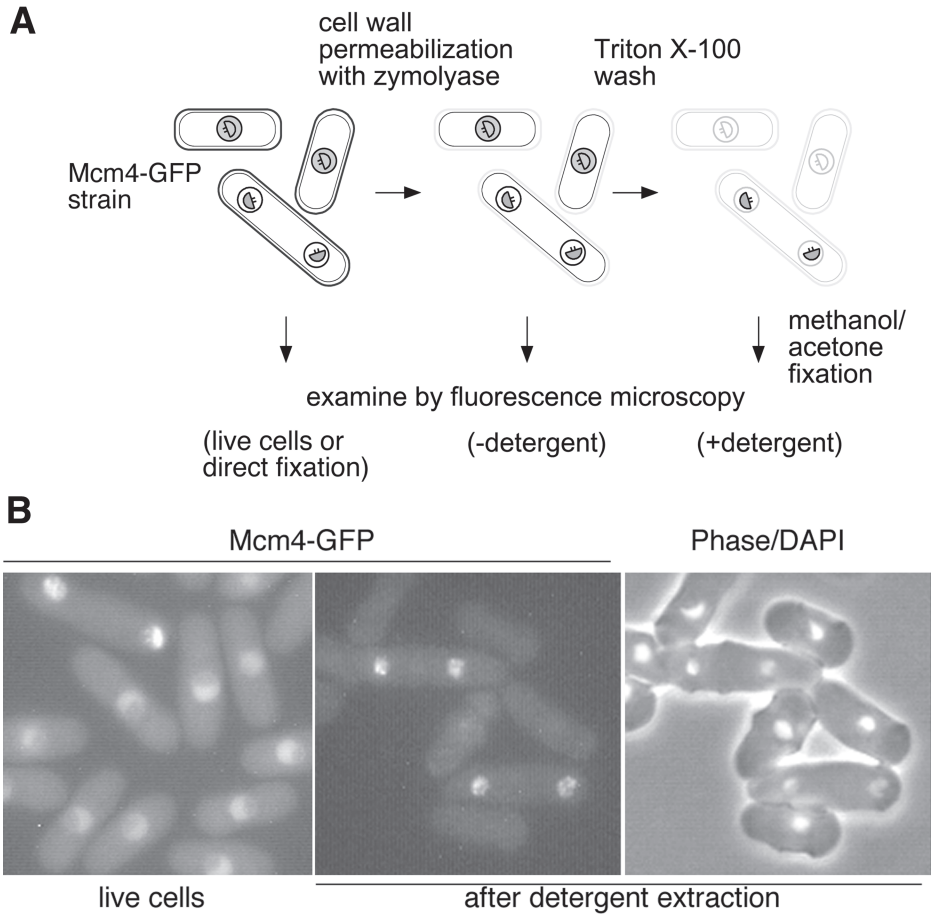

Fig. 1. **(A)** Chromatin binding assay. **(B)** Example of assay result using an Mcm4/GFP-tagged strain.

3.3.). Cells processed for this assay can also be analyzed by flow cytometry to determine their DNA content (**Subheading 3.4.**).

2. Materials

1. EMMSorb buffer: 15 m*M* KH phallate, 15 m*M* Na_2HPO_4, 90 m*M* NH_4Cl, 1.2 *M* sorbitol, pH 7.0.
2. Extraction buffer 1 (no Mg^{2+}): 20 m*M* PIPES-KOH, pH 6.8, 0.4 *M* sorbitol, 1 m*M* EDTA, 150 m*M* KAc, 0.5 m*M* spermidine HCl, 0.15 m*M* spermine HCl. Store at –20°C.
3. Extraction buffer 2 (containing Mg^{2+}): 20 m*M* PIPES-KOH, pH 6.8, 0.4 *M* sorbitol, 150 m*M* KAc, 2 m*M* MgAc. Store at –20°C.
4. Nuclease extraction buffer: 20 m*M* PIPES-KOH, pH 6.8, 0.4 *M* sorbitol, 150 m*M* KAc, 2 m*M* MgAc, 2 m*M* $CaCl_2$, 250 m*M* NaCl. Store at –20°C.

5. 20,000 U/g Zymolyase 20-T (ICN, cat. no. 320921) at 20 mg/mL in EMMSorb buffer. Store 50-µL aliquots at –70°C.
6. 10% Triton X-100 (in extraction buffer).
7. 2% Sodium dodecyl sulfate (SDS).
8. 1 *M* Dithiothreitol (DTT).
9. 1 *M* Sodium azide.
10. 1 *M* deoxy-glucose.
11. Protease inhibitor tablets (Complete mini, cat. no. 1 836 153, Roche).
12. Mounting solution: 50% glycerol in phosphate-buffered saline (PBS), 5–100 ng/mL DAPI (4'6'-diamidino-2-phenylindole).
13. Microscope slides, poly-L-lysine–coated (Sigma, cat. no. P 8920), or alternatively, SuperFrost Plus (Menzel-Glaser, Germany, cat. no. 041300; wash in acetone before use.
14. Poly-L-lysine–coated cover slips. Soak 13-mm cover slips in 0.1% poly-L-lysine (Sigma, cat. no. P 8920) for 10 min. Drain, dry, rinse in water, and dry.
15. PBSBAL buffer: PBS (10 m*M* Na_2HPO4, 2 m*M* KH_2PO4, 3 m*M* KCl, 0.14 *M* NaCl) containing 100 m*M* lysine hydrochloride, 10 m*M* NaN_3, 1 % essentially fatty acid-free bovine serum albumin (BSA; Sigma, cat. no. A0281), pH 6.9.
16. 5 m*M* Sytox green in DMSO (Molecular Probes, S-7020; store at –20°C). Dilute to 2 µ*M* in 10 m*M* EDTA, pH 8.0, before use (keep solution in the dark).
17. 10 m*M* EDTA, pH 8.0.
18. 10 mg/mL RNase A (Roche, cat. no. 109169); boil for 10 min, cool to room temperature, filter, and store at –20°C. Dilute to 0.1 mg/mL in 10 m*M* EDTA, pH 8.0, before use.
19. Micrococcal nuclease (Sigma, cat. no. N3755). Dissolve 50 U in 0.2 mL 20 m*M* HEPES-KOH, pH 7.4, 50 m*M* KCl, 50% glycerol. Store 10-µL aliquots at –70°C.
20. 0.2 *M* EGTA, pH 7.5.
21. Yeast EMM or YES media ***(12)***.

3. Methods

3.1. In Situ Chromatin Binding Assay

1. Inoculate 25 mL YES or EMM (*see* **Note 1**) with yeast strain, and shake overnight (*see* **Note 2**). Culture should be in early log phase when cells are taken for assay. Typically 25 mL of culture at OD_{595} = 0.2–0.5 is enough for one to five assays.
2. It is useful to fix an aliquot of cells directly as a control for examining the protein distribution in nonextracted cells and to process the remaining cells for the chromatin binding assay. Fix an aliquot of cells (5 mL) directly by spinning down (3000*g*, 5 min) and resuspending in methanol (0°C). After 10 min, spin down and resuspend in acetone (0°C). Examine cells as in **step 11**.
3. Spin down remaining cells (3000*g*, 5 min), and wash in 2 mL ice-cold EMMSorb containing 10 m*M* DTT (*see* **Note 3**). Transfer to a 2-mL Eppendorf tube.
4. Resuspend in 0.45 mL EMMSorb containing 10 m*M* DTT, and add 50 µL of 20 mg/mL zymolyase 20-T. Digest at 32°C for 10–20 min. Test for adequate digestion by mixing a few microliters of cells with an equal volume of 2% SDS on a slide and examining under phase contrast microscopy. Cells should go phase dark. (Aim for >95% cells going phase dark.) If digestion is going slowly, add more zymolyase.
5. When adequate digestion has been achieved, add 1.5 mL EMMsorb buffer (at 4°C), and spin down. (All subsequent spins are 1000*g* for 1 min in an Eppendorf centrifuge at 4°C). Resuspend cells in EMMsorb (4°C), and spin down (*see* **Note 4**).

6. Resuspend in 2 mL extraction buffer, and spin down (*see* **Note 5**).
7. Resuspend in 0.9 mL extraction buffer containing protease inhibitors. Transfer 0.45 mL of this cell suspension to a 2-mL eppendorf tube containing 50 µL 10% Triton X-100. Mix and transfer to 20°C water bath for 5 min. Remaining cells can be used as minus detergent control (*see* **Note 6**).
8. Spin cells down (both plus and minus detergent samples), and resuspend in 2 mL methanol (0°C). Keep on ice for 10 min (*see* **Note 7**).
9. Spin down cells and resuspend in 1 mL acetone (0°C). Cells can be stored at this stage at –20°C for a few days.
10. To examine the cells by fluorescence microscopy, vortex cells in acetone to resuspend, and spin down 0.1–0.3 mL. Take off acetone leaving 10–20 µL. Cells can be gently sonicated at this stage to break up cell clumps.
11. Spread a thin film of cells on poly-L-lysine–coated or Superfrost microscope slide. Apply 10 µL mounting solution and a 22-mm cover slip. The cover slip can be sealed with nail varnish, but this is not necessary if the slides are to be viewed immediately. Obtain images of cells, taking first a phase image, then a green fluorescent protein (GFP) image, and finally a DAPI image for each field of cells (*see* **Note 8**).

3.2. Immunostaining Cells After Detergent Extraction and Fixation

1. Process cells to **step 9** in **Subheading 3.1.** Resuspend cells and transfer approx 0.2 mL to 1.5 mL Eppendorf tube. Spin down (1000*g*, 1 min), and take off most acetone; leave about 20–50 µL.
2. Resuspend cells by vortexing, and mildly sonicate to break up cell clumps. Spread about 10 µL of the cell suspension onto a poly-L-lysine–coated 13-mm cover slip.
3. Rinse cover slip in PBS, and then incubate in PBSBAL for 30 min. (It is convenient to keep the cover slips in small Petri dishes or multiwell plates for **steps 3–7**.)
4. Remove PBSBAL, add 20 µL primary antibody to the cover slip, and incubate in a humid container for at least 1 h.
5. Wash twice in PBS and once in PBSBAL (three times, 5 min).
6. Add 20 µL secondary antibody to the cover slip (conjugated with fluorescent dye). Incubate in dark humid container for at least 1 h.
7. Wash three times in PBS (three times, 5 min).
8. Drain the cover slip well. Mount in DAPI/PBS. Seal the edges of cover slip with nail varnish. Obtain images of cells taking first a phase image, then a GFP image, and finally a DAPI image for each field of cells.

3.3. Micrococcal Nuclease Control to Determine Whether Retention of Proteins After Detergent Extraction is Chromatin-Dependent

1. Process cells to **step 6** in **Subheading 3.1.** Resuspend in 0.9 mL nuclease extraction buffer. (This has a higher salt concentration, which is necessary to solubilize digested chromatin ***[13]***, as well as Ca^{2+} for micrococcal nuclease activity.) Add Triton X-100 to 1%, and divide sample into three tubes. To tube 1 (plus nuclease), add micrococcal nuclease to 2.5 U/mL, and to tube 2 add first EGTA to 10 m*M* and then micrococcal nuclease to 2.5 U/mL (plus nuclease, inactive); tube 3 is minus nuclease control. Incubate at 20°C for 5 min, and then proceed as in **Subheading 3.1., step 8**.

Job: Humphrey 144-8
Chapter: 09 (Kearsey)
Template: MiMB/6x9/CondTempW/S/Rev.04.03
Compositor: Pius
Date: 7/13/4
Revision: 1st Pass

Table 1
pSM Vectors

	Selectable marker	Fluorescent protein	Ref. or accession no.
pSMUG2+	*ura4+*	eGFP	AJ306911
pSMRG2+	*kanMX6*	eGFP	AJ306910
pSMUC2+	*ura4+*	CFP	***1***[a]
pSMUY2+	*ura4+*	YFP	***1***[a]
pSMRC2+	*kanMX6*	CFP	
pSMRY2+	*kanMX6*	YFP	

[a]Vector sequences are available at URL: users.ox.ac.uk/~kearsey/plasmids/.

3.4. Flow Cytometric Analysis of Cells After Detergent Extraction and Fixation

1. Process cells to **step 9** in **Subheading 3.1.** Vortex cells and transfer 0.2 mL to 5 mL 10 m*M* EDTA, pH 8.0 (*see* **Note 9**). Spin down (3000*g*, 5 min) and resuspend in 0.5 mL 0.1 mg/mL RNase A in 10 m*M* EDTA, pH 8.0.
2. Incubate at 37°C for 2–24 h.
3. Add 0.5 mL 2 µ*M* Sytox green in 10 m*M* EDTA, pH 8.0 (*see* **Note 10**).
4. Sonicate and analyze in flow cytometer.

4. Notes

1. We have found that GFP-tagged strains grown in minimal medium (EMM) give lower cytoplasmic fluorescence in some experiments, such as those involving temperature shifts, than when rich (YES) medium is used.
2. Strain used should have a GFP (or other fluorescent tag) on the protein to be examined; alternatively, use protocol in **Subheading 3.2.** from **step 9** to detect protein via indirect immunofluorescence. A number of vectors have been developed for tagging fission yeast proteins with GFP (e.g., *see* **ref. *14***). **Table 1** and **Fig. 2** give details of vectors that we have developed for GFP, CFP, or YFP tagging. Genes can be tagged by long oligo PCR, using the PCR product for transformation ***(14)***. Alternatively, clone the 3' region of the gene to be tagged into the vector, linearize the construct in this 3' gene region, and transform fission yeast with this DNA.
3. For time-courses, e.g., after a temperature shift, it may be advisable to arrest cellular metabolism by adding 10 m*M* NaN_3 and 10 m*M* deoxy-glucose to cells just before the initial centrifugation.
4. An extra EMMSorb wash can be included at this stage.
5. The Mg^{2+}-containing extraction buffer is suitable for analysis of Mcm2-7 and Cdc45 proteins in log phase cells, whereas the minus Mg^{2+} buffer is recommended for analysis of these proteins in cells that have been synchronized by nitrogen starvation. Buffer modification may be necessary for analysis of other proteins.
6. Cells fixed directly, prior to zymolyase digestion, or viewed live, give the best indication of the in vivo distribution of the tagged protein. Cells fixed after zymolyase digestion and washing in extraction buffer without detergent may show some loss of soluble nucleoplasmic protein (possibly after cell lysis in the hypotonic extraction buffer).

Job: Humphrey 144-8
Chapter: 09 (Kearsey)
Template: MiMB/6x9/CondTempW/S/Rev.04.03
Compositor: Pius
Date: 7/13/4
Revision: 1st Pass

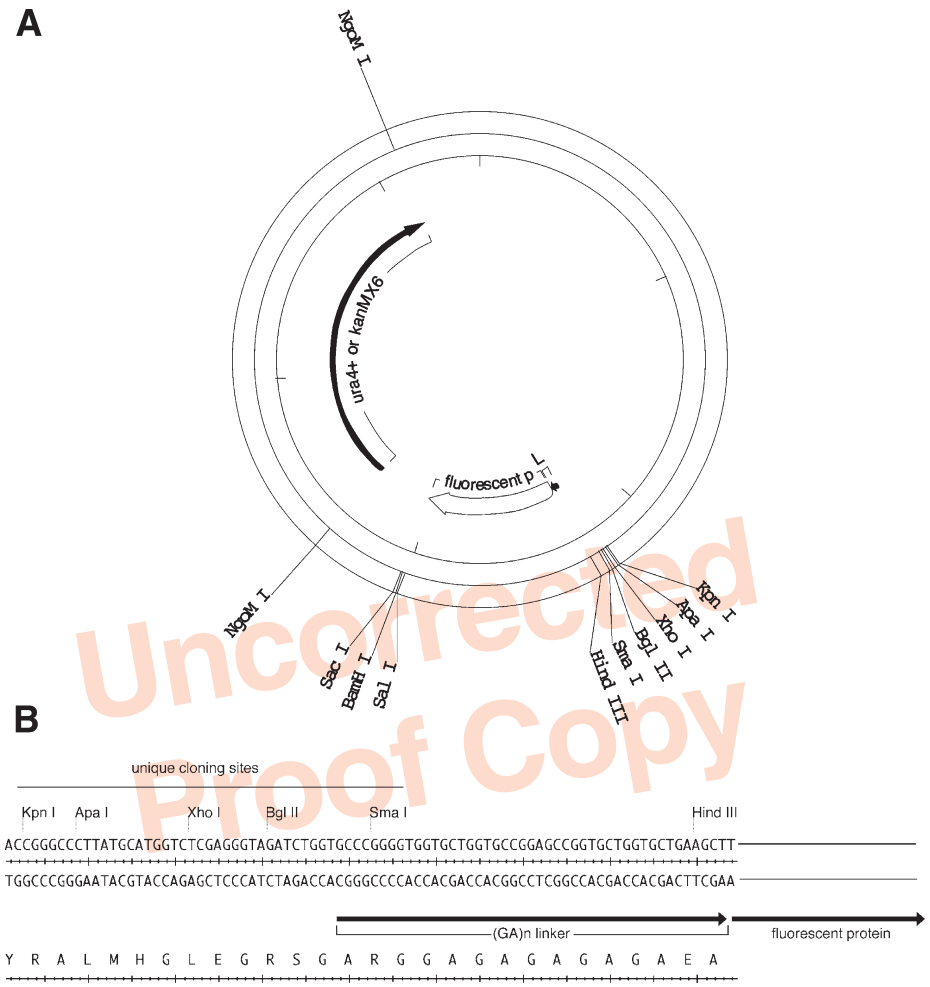

Fig.2. **(A)** Generic structure of pSM vectors. **(B)** Sequence of pSM vectors in region of cloning sites.

7. Ethanol, 100%, (at 0°C) may be used as an alternative to methanol/acetone fixation, although the fixation procedure may have to be modified depending on the protein being examined.
8. Cells that were not adequately digested during the zymolyase digestion step appear phase bright and should be ignored in subsequent analysis. Suitable filter sets for GFP and its derivatives are: 41017 (eGFP), 31044v2 (CFP), and 41028 (YFP), from Chroma (Brattleboro, VT).
9. In flow cytometric analysis, samples extracted with Triton X-100 will give Sytox green histograms shifted to the left compared with nondetergent-extracted cells, due to lower cytoplasmic fluorescence. Samples compared should all have had the same extraction and fixation treatment.

10. Propidium iodide (4 μ*M*) can also be used instead for DNA staining, but Sytox green gives tighter histograms. The GFP fluorescence from proteins expressed at native levels is generally too faint to complicate the analysis of DNA content using Sytox green.

References

1. Gregan, J., Lindner, K., Brimage, L., et al. (2003) Fission yeast Cdc23/Mcm10 functions after pre-replicative complex formation to promote Cdc45 chromatin binding. *Mol. Biol. Cell* **14,** 3876–3887.
2. Donovan, S., Drury, L., and Diffley, J. (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Natl. Acad. Sci. USA* **94,** 5611–5616.
3. Tanaka, T., Knapp, D., and Nasmyth, K. (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90,** 649–660.
4. Todorov, I. T., Attaran, A., and Kearsey, S. E. (1995) BM28, a human member of the MCM2-3-5 family, is displaced from chromatin during DNA replication. *J. Cell Biol.* **129,** 1433–1446.
5. Klein, F., Laroche, T., Cardenas, M. E., Hofmann, J. F., Schweizer, D., and Gasser, S. M. (1992) Localization of RAP1 and topoisomerase II in nuclei and meiotic chromosomes of yeast. *J. Cell Biol.* **117,** 935–948.
6. Kearsey, S. E., Montgomery, S., Labib, K., and Lindner, K. (2000) Chromatin binding of the fission yeast replication factor mcm4 occurs during anaphase and requires ORC and cdc18. *EMBO J.* **19,** 1681–1690.
7. Lindner, K., Gregan, J., Montgomery, S., and Kearsey, S. (2002) Essential role of MCM proteins in pre-meiotic DNA replication. *Mol. Biol. Cell* **13,** 435–444.
8. Gomez, E. B., Catlett, M. G., and Forsburg, S. L. (2002) Different phenotypes in vivo are associated with ATPase motif mutations in *Schizosaccharomyces pombe* minichromosome maintenance proteins. *Genetics* **160,** 1305–1318.
9. Wolkow, T. and Enoch, T. (2003) Fission yeast Rad26 responds to DNA damage independently of Rad3. *BMC Genet.* **4,** 6.
10. Williams, D. R. and McIntosh, J. R. (2002) mcl1+, the *Schizosaccharomyces pombe* homologue of CTF4, is important for chromosome replication, cohesion, and segregation. *Eukaryot. Cell* **1,** 758–773.
11. Tomonaga, T., Nagao, K., Kawasaki, Y., et al. (2000) Characterization of fission yeast cohesin: essential anaphase proteolysis of Rad21 phosphorylated in the S phase. *Genes Dev.* **14,** 2757–2770.
12. Moreno, S., Klar, A., and Nurse, P. (1991) Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194,** 795–823.
13. Wu, L. C., Fisher, P. A., and Broach, J. R. (1987) A yeast plasmid partitioning protein is a karyoskeletal component. *J. Biol. Chem.* **262,** 883–891.
14. Bahler, J., Wu, J. Q., Longtine, M. S., et al. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14,** 943–951.