Chapter 27

Using the DHFR Heat-Inducible Degron for Protein Inactivation in *Schizosaccharomyces pombe*

Stephen E. Kearsey and Juraj Gregan

Summary

Inactivating a specific protein *in vivo* can yield important information about its function. One strategy previously developed in *Saccharomyces cerevisiae* by the Varshavsky group involves fusing a degron, derived from mouse dihydrofolate reductase, to the N-terminus of the target protein, which thereby confers temperature-sensitive degradation at the restrictive temperature. We describe here the application of this technique in the fission yeast, *Schizosaccharomyces pombe*.

Key words: Fission yeast, *Schizosaccharomyces pombe*, Degron, Conditional mutant, Proteolysis.

1. Introduction

A good way of investigating the function of a protein is to knock it out and see what the effect is. For essential proteins, some conditional method is needed to inactivate the protein, and the most widespread genetic method is to use temperature-sensitive mutants. Although this method has been extensively used, a disadvantage is that it is not possible to work out from the protein sequence which amino acid substitution(s) is needed to make the protein inactive at high or low temperatures. Random mutagenesis followed by screening is normally required to obtain suitable alleles.

A number of other methods have been developed for use in fission yeast which involve either replacing the native promoter with a regulatable one, or fusion of the protein with a module which can allow conditional inactivation. The *nmt1* promoter (and variants with lower expression levels), which is repressed by

Sonya Vengrova and Jacob Z. Dalgaard (eds.), *Methods in Molecular Biology, DNA Replication, vol. 521*

DOI: 10.1007/978-1-60327-817-5_27

thiamine, has been widely used to shut off gene expression *(1)*, although it is less useful with stable proteins as transcriptional shut off may not lead to rapid inactivation of the protein. Proteins can be modified with a cleavage site for the specific TEV protease, so that inactivation via cleavage can be achieved on TEV induction *(2)*. Fusion to an estradiol-binding domain (hormone binding domain, HBD) can also be used to regulate protein function; in the absence of estradiol, the HBD forms a complex with Hsp90 and this can inactivate the protein *(3)*.

A final method, which is dealt with here in detail, involves the heat-inducible degron derived from dihydrofolate reductase. This method, originally developed by Varshavsky and colleagues in *Saccharomyces cerevisiae*, involves fusion of the target protein with an amino-terminal module consisting of ubiquitin fused to the degron via an arginine residue *(4)*. Expression of the fusion protein in yeast leads to removal of the ubiquitin, leaving the target protein with N-terminal arginine (**Fig. 1**). Normally this is a destabilizing N-terminal amino acid and N-Arg proteins have a half-life of only a few minutes *(5)*, but at the permissive temperature the N-degron is not recognized by the relevant E3 ubiquitin ligase (Ubr1). Upon shifting to the restrictive temperature (37°C), ubiquitylation of internal lysine residues takes place, presumably due to

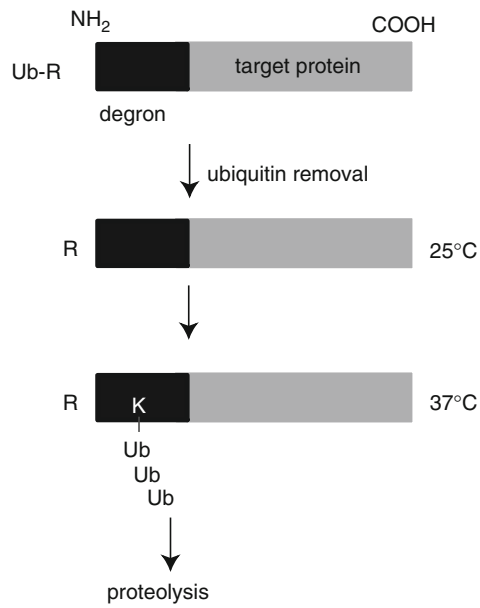

Fig. 1. Principle of heat-inducible degron method. The target protein is expressed with an N-terminal ubiquitin (Ub) joined via an arginine residue to the temperature-sensitive degron (derived from dihydrofolate reductase). Upon expression in fission yeast, the N-terminal ubiquitin is removed leaving the protein with a destabilizing N-terminal arginine residue. At the permissive temperature the protein is stable but upon shifting to 37°C, the degron is recognized by the Ubr11 ubiquitin ligase (presumably owing to a conformational change in the degron), and ubiquitylation of lysine residues in the degron ensues, resulting in proteolysis of the tagged protein.

a conformational change in the degron which allows recognition by the ubiquitin ligase, and proteolysis ensues. The utility of this method in *S. cerevisiae* has been extended by Labib, who used overexpression of Ubr1 to enhance ubiquitylation of the degron at the restrictive temperature *(6)*. Using this approach, a large number of proteins can be modified so that they become temperature sensitive. A major advantage of using this approach compared to other methods is that the protein is not present under the restrictive condition, rather than being present but inactive. This can make the interpretation of phenotypes easier.

The DHFR degron can be used to aid protein inactivation in fission yeast *(7–9)*. Deletion of the fission yeast ***ubr11*** gene (one of two fission yeast genes related to Sc***UBR1***) abolishes the temperature sensitivity of a degron-Bir1 fusion, suggesting conservation of the degradation pathway and that Ubr11 is orthologous to budding yeast Ubr1 *(7)*.

This chapter describes use of degron vectors that we have developed for tagging fission yeast genes. As well as tagging wild-type genes, this approach can be used to make existing temperature-sensitive alleles "tighter" in terms of effective protein inactivation at the restrictive temperature *(8, 10)*. One restriction, compared to use of the degron in *S. cerevisiae*, is that cells overexpressing *S. pombe* Ubr11 from the ***nmt1*** promoter are sick in the absence of degron modification, and degron-modified strains are not markedly more temperature sensitive under these conditions (unpublished observations and *see* **ref. 7***)*.

2. Materials

2.1. Constructing a Degron-Tagging Plasmid

1. *LB + Ampicillin plates.* 1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar. Autoclave and add ampicillin to 50 mg/L after cooling.
2. *SOC medium.* 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. Autoclave.
3. Plasmids are described in **Table 1** (available on request from stephen.kearsey@zoo.ox.ac.uk).
4. Primers are given in **Table 2**.
5. Megamix blue ready PCR mix (Microzone, Haywards Heath, UK http://www.microzone.co.uk/).
6. QIAquick PCR purification Kit (QIAGEN).
7. QIAquick gel extraction kit (QIAGEN).
8. QIAquick nucleotide removal kit (QIAGEN).

Table 1
Degron plasmids

Plasmid	Plasmid abbreviation	Promoter	Selectable marker
pSMUG2-mcm4-degronHA	v1	*mcm4*	*ura4*$^+$
pSMRG2-mcm4-degronHA	v2	*mcm4*	*kanMX6*
pSMRG2-nmt41-degronHA	v3	*nmt1* (medium strength)	*kanMX6*
pSMUG2-nmt41-degronHA	v4	*nmt1* (medium strength)	*ura4*$^+$
pSMRG2-nmt1-degronHA	v5	*nmt1* (full strength)	*kanMX6*
pSMUG2-nmt1-degronHA	v6	*nmt1* (full strength)	*ura4*$^+$
pSMRG2-nmt81-degronHA	v7	*nmt1* (low strength)	*kanMX6*
pSMUG2-nmt81-degronHA	v8	*nmt1* (low strength)	*ura4*$^+$

Plasmid sequences are available from: http://users.ox.ac.uk/~kearsey/plasmids/

Table 2
PCR primers

Primer	Label in Fig. 2	Sequence
XhoI-target (forward)	p1	5′-tttC **TCG AG**X ATG-(gene-specific sequence)-3′[a]
BglII-target (reverse) XmaI-target (reverse)	p2	5′-tttAGATCT-(gene-specific sequence)-3′ 5′-tttCCCGGG-(gene-specific sequence)-3′
Colony screen/sequence (forward)	p3	5′-GGCAAGTAAAGTAGACATGG-3′
Colony screen (reverse)	p4	5′ -(gene-specific sequence)-3′
Sequence (reverse)	p5	5′-TAACATCACCATCTAATTCAAC-3′

[a]The reading frame of the degron is indicated. ATG should be the initiator codon of the target gene and X is any nucleotide

9. Restriction enzymes (XhoI, BglII) and buffer (#3) (New England Biolabs).
10. T4 DNA ligase and buffer (New England Biolabs).
11. Competent *E. coli* cells (5-alpha competent *E. coli*, subcloning efficiency C2988J, New England Biolabs).
12. Wizard plus midiprep DNA purification system (A7640, Promega Corporation).

2.2. Fission Yeast Transformation

1. Minimal medium (EMM *(11)*) for selection of Ura$^+$ transformants, use premix (US Biological, Swampscott, MA), plus 0.075% adenine, 0.075% leucine, 2% Bacto agar.
2. YES. 0.5% yeast extract, 3% glucose, 0.075% adenine, 0.075% uracil, 0.75 g/l leucine (add 2.2% agar for plates).
3. KsnoT *plates.* 1% Bacto peptone, 3% glucose, 0.075% adenine, 0.075% uracil, 0.075% leucine, 2.2% Bacto agar. If required, add G418 to 100 μg/mL after autoclaving and cooling media. Thiamine is used at 15 μg/mL.
4. *S. pombe* strain, for tagging protein of interest, should contain the *ura4* auxotrophic marker for use with *ura4*$^+$-containing plasmids.
5. 1.2 M sorbitol. Autoclave. Store at 4°C.
6. G418 (Sigma). 100mg/mL in water. Store at –20°C.
7. Phloxin B (Sigma). 10 mg/mL in water. Filter sterilize. Store at room temperature. Add to plates at 3 μg/mL final concentration.
8. *Electroporation cuvettes.* Gene pulser, 0.2-cm gap (Bio-Rad, Hemel Hempstead, UK).

3. Methods

Figure 2 gives an overview of the tagging approach. A fragment expressing the N-terminal region of the target protein is amplified by PCR and cloned in a degron plasmid. For the example given here, the fragment is amplified as an XhoI-BglII fragment, but other restriction enzymes can be used (*see* **Fig. 3**). The degron plasmid is subsequently linearized by using a restriction enzyme which makes a unique cut within the target gene fragment, to direct integration into the homologous gene upon yeast transformation. Transformants are screened by PCR to identify those where integration has occurred at the homologous locus. If the target gene is essential for mitotic growth, transformants are analyzed to see if they are temperature sensitive.

The degron vectors described here have either *kanMX6* (conferring resistance to G418) or *ura4*$^+$ markers. Two types of promoter, *mcm4* and *nmt1,* are used to express the degron fusion protein, i.e., the native promoter is not used. The *mcm4* promoter is constitutive, while the *nmt1* promoter can be down regulated by growth in medium containing 15 μg/mL thiamine. Thus constructs using the *nmt1* promoter have the advantage that transcriptional shut off can be combined with the temperature shift to inactivate the degron-tagged protein. Note that the

nmt1 promoter (particularly the full and medium strength versions) is leaky after thiamine shut off, and some expression of the tagged protein will occur in the presence of thiamine.

3.1. Constructing a Degron-Tagging Plasmid

1. Design forward and reverse primers p1 and p2 (**Table 2**) to amplify the 5′ end of the target gene. Gene-specific sequences should be 25 nucleotides or longer and have a T_m of 65°C. The fragment amplified should include the initiator codon which needs to be in frame with the degron sequence (**Fig. 3**). The fragment should ideally be 300–600 bp in length and must contain a unique restriction site (not found in the vector) which has at least 100 bp of flanking DNA.
2. Amplify the gene fragment by mixing 100 µL megamix blue PCR mix, 1 µL of p1 (100 µM), 1 µL of p2 (100 µM),

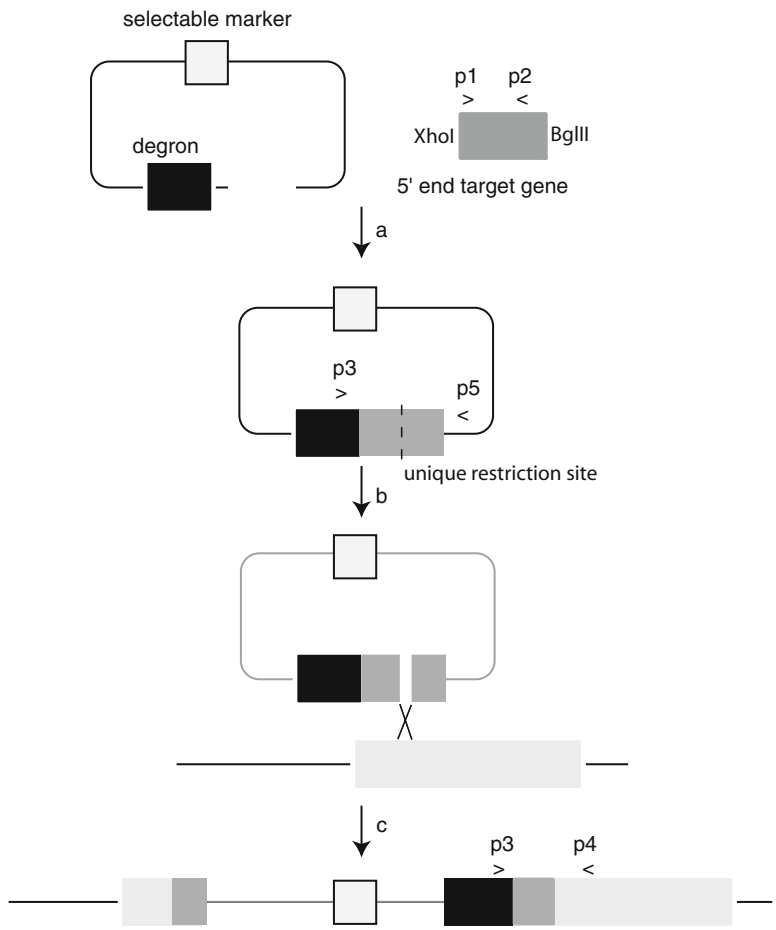

Fig. 2. Strategy for degron tagging the target gene. (**a**) DNA fragment of the target gene expressing the N-terminal region of the protein is amplified by PCR and inserted into a degron vector, in frame and downstream of the degron cassette (which consists of a promoter expressing the ubiquitin-degron fusion). (**b** and **c**) cleavage of the plasmid at a unique restriction site in the target gene directs integration at the homologous site upon yeast transformation, resulting in a short truncated gene fragment and the full-length degron-tagged gene. p1–p5 are PCR primers as described in **Table 2**.

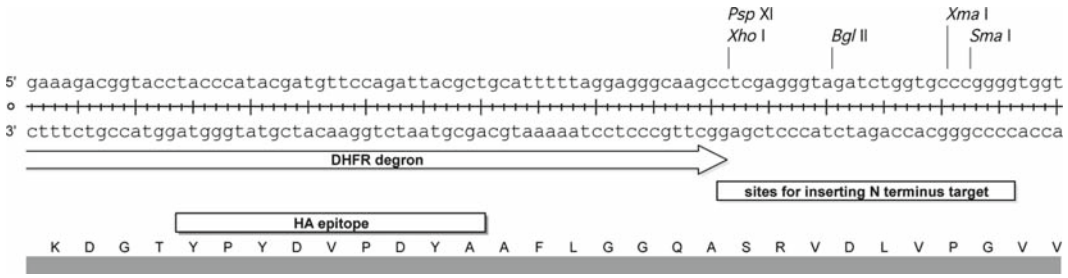

Fig. 3. Sequence of the pSM degron vectors around restriction sites used for insertion of gene specific fragments. The reading frame extending from the DHFR degron is shown.

1 µL of 1 mg/mL total *S. pombe* DNA (*see* **Note 1**). Amplify for 25–30 cycles of 94°C for 10 s, 55°C for 30 s, and 68°C for 2 min. Check amplification by electrophoresis of 1 µL of the amplified fragment on a 1% TAE agarose gel. Purify rest of PCR-amplified DNA using QIAquick PCR purification kit, according to the manufacturer's instructions. Elute DNA at the final step from the spin column with 50 µL water.

3. Digest PCR fragment by adding 6 µL of 10× buffer #3 (NE Biolabs), 50 units of BglII, and 50 units of XhoI. Digest at 37°C for 5 h. Purify fragment on a 1.5% agarose gel using QIAquick gel extraction kit, according to the manufacturer's instructions.
4. Prepare degron vector by digesting 5 µg of DNA with 30 units of Xho I and 30 units of Bgl II in buffer #3 (NE Biolabs) in a total volume of 50 µL for 5 h at 37°C. Heat inactivate at 70°C for 5 min; then purify fragment on 1% TAE agarose gel using QIAquick gel extraction kit, according to the manufacturer's instructions.
5. Set up 20 µL ligation mix containing 0.5 µg linearized vector, 0.1 µg insert, 2 µL of 10× ligation buffer (NE Biolabs), 1 µL T4 DNA ligase. Incubate at room temperature for 10 min-2 h.
6. Transform ligation mix into competent *E. coli*. Thaw competent cells on ice and add 2 µL of ligation mix to 50 µL of cells. Incubate on ice for 30 min, then heat shock in thin-walled tube for 30 s at 42°C. Add 1 mL of SOC and incubate for 1 h at 37°C. Plate out onto prewarmed LB plates containing 50 µg/mL ampicillin and incubate overnight at 37°C.
7. Screen ampicillin-resistant colonies by PCR using forward and reverse gene specific primers to determine whether they contain inserts. Mix 100 µL of megamix blue with 1 µL of p1 primer (100 µM) and 1 µL of p2 primer (100 µM). Aliquot 10 µL into PCR tubes and add small quantity of *E. coli* cells from an ampicillin-resistant colony using a toothpick. Amplify using the following program: 5 min at 95°C followed by 30 cycles of 94°C for

10 s, 55°C for 30 s, and 68°C for 2 min. Analyze PCR reactions by running on 1.5% TAE agarose gel to determine if clones contain inserts of the appropriate size.

8. Make plasmid preparation from a positive clone using midiprep kit, according to the manufacturer's instructions. The insert should be sequenced using primers p3 and p5 to determine whether the PCR has introduced any mutations.
9. Linearize plasmid with a restriction enzyme that makes a unique cut in the gene fragment and check an aliquot of digest by agarose gel electrophoresis. Desalt DNA using QIAquick nucleotide removal kit and proceed to yeast transformation **(Subheading 3.2)**.

3.2. Tagging Yeast Gene with N-Degron

This transformation method is taken from reference *(12)*.

1. Grow *S. pombe* cells in YES (YE + supplements) to 10^7 cells/mL (ca. OD_{600} = 0.5) at 32°C; 20 mL culture per transformation.
2. Pellet the cells at 3,000 × *g*, discard supernatant, and resuspend in 50 mL of 1.2 M sorbitol. Repeat this washing step twice. After the final centrifugation, resuspend cells so that final concentration is 10^9 cells/mL.
3. Mix, on ice, 0.2 mL of cells with 0.1–1 μg of DNA (prepared as described under **Subheading 3.1**). Immediately transfer to ice-cold 0.2 cm electroporation cuvette (Bio-Rad) and electroporate at 2 kV, 200 Ω, 25 μF (*see* **Note 2**). Add 0.5 mL of 1.2 M sorbitol and plate 0.35 mL on two plates as follows: EMM-uracil plates for vectors v1, v4, v6, & v8; YES plates for vector v2; KsnoT for vectors v3, v5, v7 (*see* **Note 3**). Grow plates at 25°C.
4. After 1 day, replica plate YES plates to YES + 100 μg/mL G418 and KsnoT plates to KsnoT + 100 μg/mL G418. It may also help to replica plate EMM-uracil plates to fresh selective plates after 2–3 days, as the lawn of nontransformed cells can inhibit the growth of Ura^+ transformants. Incubate plates at 25°C for 3–4 days.
5. Restreak or replica plate any G418-resistant colonies on YES + 200 μg/mL G418, or KsnoT + 200 μg/mL G418 to distinguish transformants from G418-resistant mutants (transformants should form colonies, while spontaneous mutants are unable to form colonies on medium containing a high concentration of G418).
6. Screen transformants by colony PCR. Mix 100 μL of megamix blue with 1 μL of p3 primer (100 μM) and 1 μL of p4 primer (100 μM). Note that the p4 sequence should correspond to a region of the target gene that is downstream of the fragment amplified by primers p1 and p2. Aliquot 10–20 μL into PCR tubes and add small quantity of *S. pombe* cells from Ura^+ or G418-resistant colonies using a toothpick (10^5–10^6 cells). Amplify using

the following program: 5 min at 95°C followed by 30 cycles of 94°C for 10 s, 55°C for 30 s, and 68°C for 6 min. Analyze PCR reactions by running on 1.5% TAE agarose gel to see if a product of the predicted size is generated, indicating that integration at the correct site has occurred (*see* **Note 4**).

7. Assuming the tagged gene is essential for mitotic growth, transformants should be screened for temperature sensitivity by replica plating to phloxin plates as follows: YES + 100 μg/mL G418 + 3 μg/mL phloxin B for vector v2; EMM-uracil plates and 3 μg/mL phloxin B for vectors v1, v4, v6, and v8; KsnoT plates 100 μg/mL G418 + 3 μg/mL phloxin B for vectors v3, v5, v7. Incubate plates at 37°C (*see* **Note 5**). KsnoT and EMM-uracil plates can also be replica plated to plates containing thiamine (15 μg/mL) to determine if promoter shut off leads to lethality.
8. The design of experiments involving degron strains will to some extent depend on the gene tagged and the aim of the experiment, but some general guidelines are given here. Strains derived from plasmids containing the *mcm4* promoter can be grown on rich medium (YES) at the permissive temperature of 25°C. Inactivation of the degron-tagged protein can then be induced by shifting log phase cells to 36–37°C. Strains derived from plasmids containing the *nmt1* promoter should be grown on EMM medium at 25°C. Inactivation of the degron-tagged protein, by temperature shift to 36–37°C, should be combined with addition of thiamine to the medium (to 15 μg/mL), to shut off the *nmt1* promoter. Degradation of the target protein and the phenotypic consequences of this can then be monitored over 1–5 h.

4. Notes

1. Purified *S. pombe* DNA is preferable, but it is also possible to use *S. pombe* cells – add 10^5–10^6 cells to 100 μL reaction. In this case heat the PCR mix for 5 min at 95°C to promote cell lysis before beginning the cycling.
2. It may be important to keep the cells cold and electroporate the cells immediately after mixing cells with DNA. Use DNA from the appropriate mutant strain if the gene being tagged is a temperature-sensitive allele, and the mutation is in the fragment amplified by p1 & 2 primers.
3. A high concentration of G418 has to be used with EMM plates, so it is cheaper to use KsnoT plates if it is necessary to select for G418 on medium lacking thiamine.

4. Transformants can also be screened by Western blotting to confirm that the target protein has increased in mass by ca. 24 kDa as result of degron tagging. The HA epitope in the degron module can be used for detection by western blotting.
5. Degron-tagged proteins can also be characterized by western blotting to assess degradation at the restrictive temperature.

References

1. Maundrell, K. (1990) nmt1 of fission yeast, *J. Biol. Chem.* 265, 10857–10864.
2. Yang, X., Gregan, J., Lindner, K., Young, H., and Kearsey, S. (2005) Nuclear distribution and chromatin association of DNA polymerase-primase is affected by TEV protease cleavage of Cdc23 (Mcm10) in fission yeast, *BMC Mol Biol* 6, 13.
3. Boe, C., Garcia, J. S., Pai, C. C., Sharom, J. R., Skjolberg, H. C., Boye, E., Kearsey, S. E., MacNeill, S., Tyers, M., and Grallert, B. (2008) Rapid regulation of protein activity in fission yeast, BMC Cell Biol. 9, 23.
4. Dohman, R., Wu, P., and Varshavsky, A. (1994) Heat-inducible degron: a method for constructing temperature-sensitive mutants, *Science* 263, 1273–1276.
5. Bachmair, A., Finley, D., and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue, *Science* 234, 179–186.
6. Kanemaki, M., Sanchez Diaz, A., Gambus, A., and Labib, K. (2003) Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo, *Nature* 423, 720–724.
7. Rajagopalan, S., Liling, Z., Liu, J., and Balasubramanian, M. (2004) The N-degron approach to create temperature-sensitive mutants in *Schizosaccharomyces pombe*, *Methods* 33, 206–212.
8. 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.
9. Gregan, J., Van Laer, L., Lieto, L. D., Van Camp, G., and Kearsey, S. E. (2003) A yeast model for the study of human DFNA5, a gene mutated in nonsyndromic hearing impairment, *Biochim Biophys Acta* 1638, 179–186.
10. Gregan, J., Lindner, K., Brimage, L., Franklin, R., Namdar, M., Hart, E. A., Aves, S. J., and Kearsey, S. E. (2003) Fission yeast Cdc23/Mcm10 functions after pre-replicative complex formation to promote Cdc45 chromatin binding, *Mol. Biol. Cell* 14, 3876–3887.
11. Moreno, S., Klar, A., and Nurse, P. (1991) Molecular genetic analysis of the fission yeast, *Schizosaccharomyces pombe, Meth. Enzymol.* 194, 795–823.
12. Prentice, H. L. (1992) High efficiency transformation of *Schizosaccharomyces pombe* by electroporation, *Nucl. Acids Res.* 20, 621.