# The Regulation of Competence to Replicate in Meiosis by Cdc6 Is Conserved During Evolution

JEAN-MARC LEMAÎTRE,<sup>1</sup> STÉPHANE BOCQUET,<sup>1</sup> MARIE-EMILIE TERRET,<sup>2</sup> MANDANA NAMDAR,<sup>3</sup> OUNISSA AÏT-AHMED,<sup>4</sup> STEPHEN KEARSEY,<sup>3</sup> MARIE-HÉLÈNE VERLHAC,<sup>2</sup> and MARCEL MÉCHALI<sup>1</sup>\*

<sup>1</sup>Institute of Human Genetics, CNRS, Genome Dynamics and Development, 141, rue de la, Cardonille, Montpellier, France

<sup>2</sup>Biologie Cellulaire et Moleculaire du Developpement, UMR 7622 CNRS, Universite Pierre, et Marie Curie, Paris, France
<sup>3</sup>Department of Zoology, University of Oxford, South Parks Road, Oxford, England
<sup>4</sup>Institute of Human Genetics, CNRS, 141, rue de la Cardonille, Montpellier, France

ABSTRACT DNA replication licensing is an important step in the cell cycle at which cells become competent for DNA replication. When the cell cycle is arrested for long periods of time, this competence is lost. This is the case for somatic cells arrested in GO or vertebrate oocytes arrested in G2. CDC6 is a factor involved in replication initiation competence which is necessary for the recruitment of the MCM helicase complex to DNA replication origins. In Xenopus, we have previously shown that CDC6 is the only missing replication factor in the oocyte whose translation during meiotic maturation is necessary and sufficient to confer DNA replication competence to the egg before fertilization (Lemaitre et al., 2002: Mol Biol Cell 13:435-444; Whitmire et al., 2002: Nature 419:722-725). Here, we report that this oogenesis control has been acquired by metazoans during evolution and conserved up to mammals. We also show that, contrary to eukaryotic metazoans, in S. pombe cdc18 (the S. pombe CDC6 homologue), CDC6 protein synthesis is down regulated during meiosis. As such, the lack of cdc18 prevents DNA replication from occurring in spores, whereas the presence of cdc6 makes eggs competent for DNA replication. Mol. Reprod. Dev. 69: 94-100, 2004. © 2004 Wiley-Liss, Inc.

Key Words: meiosis; Cdc6

# INTRODUCTION

Cells have developed mechanisms to replicate their genome once and only once during S phase. This is achieved by mechanisms that allow the DNA to become competent to replicate just before S phase and prevent re-initiation of DNA replication during the same S phase and in G2. DNA replication initiation requires the regulated assembly of pre-replicative complexes (pre-RCs) onto DNA during G1 phase (Bell and Dutta, 2002). Cdc18 in the yeast *Schizosaccharomyces pombe* was identified as a temperature-sensitive cell-cycle mutant showing defects in the initiation of replication (Kelly et al., 1993). Cdc18 protein was later shown to be essential for the initiation of DNA replication, for loading of the MCM putative replication helicase onto chromatin

(Nishitani and Nurse, 1997; Nishitani et al., 2000). Cdc6 binding to mammalian and Xenopus chromatin in in vitro replication systems has also been shown to be a crucial early step in higher-eukaryotic DNA replication. Once the DNA has been licensed, S phase promoting factor, consisting of kinase activities including Cdc7 and cyclin-dependent kinases (CDKs), triggers the prereplication complexes to fire (Bell and Dutta, 2002). Recent studies on control of DNA replication in different organisms have underlined the importance of carefully regulating the function of the replication protein CDC6, not only during cell proliferation but also during cell death. Reduction in levels of CDC6 is commonly associated with loss of proliferative capacity in human cells. Meiotic maturation, the final step of oogenesis, is a crucial stage of development in which an immature oocyte becomes a fertilizable egg. During meiosis, oocytes lose the ability to replicate during a period whose length depend on the organism (2 years in Xenopus oocyte and 15-50 years in women). After a single round of premeiotic S-phase, oocytes enter meiosis and rapidly arrest at prophase of meiosis I (MI). Upon hormonal stimulation, arrested oocytes resume meiosis, and reestablish DNA replication competence but maintain repression of DNA replication until fertilization. We previously showed that in *Xenopus*, the acquisition of the ability to replicate DNA during maturation at the end of MI was due to accumulation of CDC6, a factor essential for recruiting the MCM complex to the prereplication complex. We showed that CDC6 protein is synthesized during maturation from mRNA stored in the oocyte and that CDC6 was the only missing replication factor whose translation was necessary and sufficient to confer DNA replication competence to the egg before fertilization. In this report, we show that this

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<sup>\*</sup>Correspondence to: Marcel Méchali, Institute of Human Genetics, CNRS, Genome Dynamics and Development, 141, rue de la, Cardonille, 34396 Montpellier, France. E-mail: mechali@igh.cnrs.fr

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oogenesis control has been acquired by metazoans during evolution and conserved up to mammals to ensure rapid development. We also show that in contrast to metazoans, in *S. pombe*, protein synthesis of the CDC6 homologue cdc18 is down regulated during meiosis to prevent DNA replication from occurring in spores, whereas the presence of cdc6 makes eggs competent for DNA replication, activated by fertilization.

## RESULTS

Vertebrates oocytes are arrested at the prophase stage of the first meiotic division (GV stage for germinal vesicle) and are not fertile at this stage. Various stimuli induce the resumption of meiotic maturation which starts with nuclear envelope breakdown (GVBD for germinal vesicle breakdown) then completion of meiosis I (MI) with extrusion of the first polar body followed by an arrest in M-phase of meiosis II (MII) at metaphase (Fig. 1A). Figure 1B shows that in the mouse, as well as in Xenopus, MCM2 protein is already present in the fully grown immature oocyte, but CDC6, a factor involved in MCM loading at replication origins, is absent. CDC6 is synthesized during mouse oocyte maturation (Fig. 1B) as previously observed in Xenopus (Lemaitre et al., 2002; Whitmire et al., 2002). Since no transcription is detectable during meiotic maturation in vertebrates, we conclude that, like in Xenopus, translation of CDC6 in mouse oocyte occurs from messenger RNA already stored in the prophase I oocyte. The amount of protein accumulated at Metaphase I suggests that translation of CDC6 during maturation in mouse starts soon after GVBD, as in *Xenopus*. Immunolocalization analysis reveals that MCM2 is already stored in the GV in mouse oocytes, as previously observed for Xenopus, and released into the cytoplasm at GVBD (Fig. 1C). No staining was detected for CDC6 at the prophase I, either in cytoplasm or in GV, whereas CDC6 protein is detected at Metaphase I. Although CDC6 is synthesized at the end of MI, no replication occurs between the two meiotic divisions and oocytes arrest at Metaphase II due to CSF (cytostatic factor). Repression of replication between MI and MII requires the mos/MAP kinase pathway for stabilization of cdc2 kinase activity which inhibits licensing (Colledge et al., 1994; Furuno et al., 1994; Hashimoto et al., 1994; Verlhac et al., 1996; Dupre et al., 2002). This negative regulation of the license to replicate by CDK activity and (or) by the mos/MAPK pathway before fertilization can be generalized to vertebrates. Moreover a shift in electrophoretic mobility of CDC6 protein also suggests a posttranslationnal modification potentially involved in the regulation of its activity (Fig. 1B).

We then investigated whether this regulation of CDC6 also applies to invertebrate organisms. Of the established model organisms, *Drosophila melanogaster* provides a particularly tractable system to study oogenesis and ovulation. As in mammals, female *D. melanogaster* generate oocytes arrested at the prophase I stage (from stage 2 to 10) during growth (Fig. 2A). Oocytes then progress to metaphase I (stage 14) and are sur-

rounded by a vitelline envelope and a chorion. Activation permits resumption of meiosis with the progression to metaphase II and the release of mature eggs from the ovaries (Heifetz et al., 2001; Bloch Qazi et al., 2003).

We first identified a CDC6-related protein in the Drosophila genome/EST database (Flybase and supplementary information). In vitro translation of the corresponding cDNA produces a 75 kDa protein that is present in embryos and recognized by Xenopus CDC6 antibody (supplementary Figure) as well as human CDC6 antibody (data not shown). The increased molecular weight when compared to mouse, human, or *Xenopus* is explained by five blocks of supplementary sequences in the N-terminal of the protein (see supplementary Figure). Sequence analysis indicates that previously identified structural motifs are also conserved in the Drosophila homologue (Williams et al., 1997). The degree of sequence identity between Mouse, S. Pombe, and Drosophila CDC6-related protein is highest in the mid portion of the protein that contains the nucleotide binding/ATPase domains (Walker A, Walker B), sensor I, sensor II motifs that are hallmarks of AAA+ proteins (Takahashi et al., 2002). Drosophila CDC6-related also includes a conserved consensus site for phosphorylation by CDK at the N-terminus (Jans et al., 1995).

Immunolocalization experiments indicate that, CDC6 is present in the cytoplasm of nurse cells and follicle cells which surround the oocyte during growth (stage 2-10), but not in the oocyte blocked at the prophase I stage (Fig. 2B). However, MCM2 is already present and localized in the nucleus of *Drosophila* oocytes, as observed in Xenopus and mouse oocytes, as well as in nuclei from nurse cells and follicle cells (Fig. 2B). Figure 2C(a), shows that CDC6 protein is present in eggs laid from both virgin and fertilized females. Whereas MCM2 is already present in stage 14 oocytes at metaphase I, CDC6 is absent and is only synthesized during ovulation in Drosophila, between metaphase I and metaphase II (Fig. 2C(b)). Therefore, in the invertebrate D. melanogaster, as in Xenopus and the Mouse, CDC6 is absent from prophase I oocyte and translated between Metaphase I and Metaphase II.

Is CDC6 also synthesized during meiosis in a unicellular organism that does not couple meiosis to early embryogenesis? S. Pombe Cdc18 is the CDC6 homologue. In this species of yeast, meiosis is induced by nutrient deprivation, which promotes conjugation between haploid cells of opposite mating type, followed by premeiotic S phase and nuclear divisions leading to four spores (Fig. 3A). Cdc18 expression was analyzed in a strain (P1279) where the protein was tagged with cyan fluorescent protein (CFP) and which also contained a conditional pat1temperatures mutation. Pat1 encodes a negative regulator of meiosis, and the pat1ts strain can be induced to enter meiosis synchronously by using a temperature shift from 25 to 34°C (Bahler et al., 1991). In the experiment shown in Figure 3, cells were arrested in G1 by nitrogen starvation, then released from the block after the temperature shift to induce meiosis.

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Fig. 1. CDC6 control in meiosis in vertebrates. A: Scheme for vertebrate meiosis. B: Extracts from five *Xenopus* maturing oocytes or 85 maturing mouse oocytes were separated by SDS-PAGE and examined by immunoblotting with anti CDC6 and anti MCM2 antibodies as described in Materials and Methods. C: Immunolocalization of CDC6 and MCM2 proteins in mouse maturing oocytes. The very weak signal detected for CDC6 in prophaseI is due to background as nonimmune IgG used as control give similarly weak signal.

Progress through meiosis was monitored by flow cytometry (Fig. 3C) and DAPI staining of cells to monitor nuclear divisions (Fig. 3B(b) "Dapi," Fig. 3B(d)). This showed that pre-meiotic S phase took place about 1.5-2 hr after the temperature shift, MI around 4-5 hr, and MII around 5-7 hr. Western blotting (Fig. 3B(a)) and analysis of CFP by fluorescence microscopy (Fig. 3B(b) "Cdc18") showed that Cdc18 is not detectable in G1, but levels increase prior to pre-meiotic S phase, as expected. After this step in oocytes, a block of variable length occurs (a few days in Drosophila, and 12-50 years in women) without synthesis of CDC6. In S. pombe, no prophase block occurs and Cdc18 is absent after premeiotic S phase, as in oocytes (Fig. 3B). During S. pombe meiotic nuclear divisions, Cdc18 remains undetectable (Fig. 3B), whereas it is synthesized, but is presumably not active in the presence of CDK activity, in maturing metazoan oocytes (Figs. 1 and 2).



Fig. 2. CDC6 control in meiosis in *D. melanogaster*. A: Schematic drawing of *Drosophila* meiosis. B: Immunolocalization of CDC6 and MCM2 during *D. melanogaster* oogenesis. **Right panels** are enlargements of the covering cells at the posterior end of the stage 14 oocyte. C: Western blot analysis. In (a) the proteins were prepared from eggs newly laid by 25 virgin (1), and fertilized female (2). In (b) the protein extracts were prepared from 25 stage 14 oocytes directly taken from ovaries (1), or 25 newly laid eggs from non fertilize females (2). The egg chorion and vitelline membrane were removed prior to protein extraction.

Therefore, two different modes of cdc6 regulation lead to a similar result: repression of CDC6 activity during meiosis, with the difference that eggs are competent to replicate whereas spores are not.

## DISCUSSION

Under certain conditions, the cell cycle can be arrested for a long period of time. Vertebrate oocytes are arrested at G2 phase, while somatic cells arrest at G0 phase. In both cells, nuclei have lost the ability to initiate DNA synthesis due to the absence of CDC6. CDC6 is absent in the nuclei of quiescent cells rending them unable to form preRCs (Williams et al., 1998). In rat fibroblasts, Cdc6 expression has been shut off both transcriptionally and post-transcriptionally, when the cell ceases proliferation in the absence of anchorage (Jinno et al., 2002). Quiescent NIH 3T3 nuclei, devoid of CDC6, are incompetent to replicate their DNA in S-phase cytosol



Fig. 3. Cdc18 regulation in meiosis in S. pombe, showing Cdc18 is repressed after pre-meiotic S phase. A: Scheme for meiosis in S. pombe. B: Cdc18 levels during meiosis. a: Western analysis of Cdc18 levels during meiosis. A pat1ts cdc18-CFP strain (P1279) was arrested in G1 by transferring to EMM medium lacking nitrogen for 16 hr at 25°C, after which cells were re-fed and shifted to 34°C to induce meiosis. The blot shows Cdc18 levels (assessed using an anti-GFP antibody) at different times after the shift. The "nda3" lane is a control sample from an nda3 cdc18-CFP strain(P1280) arrested in mitosis when Cdc18 level is high, a-tubulin is shown as a loading control, **b**: Analysis of Cdc18-CFP fluorescence during meiosis. Cells were fixed with ethanol before imaging for CFP (top panel) and DNA (DAPI staining). The lower DAPI-staining panels are merged with the phase images to show the positions of cells. The levels of fluorescence seen at 0, 3.5, 5, and 7 hr are not higher than background levels seen in a non tagged strain (not shown). c: Flow cytometric analysis showing execution of premeiotic S phase around 1.5-2 hr after the shift. "exp" is an exponential mitotic culture. d: The average number of nuclei per cell during the time course of meiosis was counted to indicate the timing of the meiosis I (MI) and II nuclear divisions.

of HeLa cells (Stoeber et al., 1998). We recently reported that *Xenopus* oocytes prevent DNA synthesis during the long G2 arrest by preventing CDC6 translation (Lemaitre et al., 2002) and we show here that the regulation of cdc6 synthesis appears to be a common strategy used in the control of fertilization in both *D. melanogaster*, *Xenopus*, and the mouse. CDC6 protein is absent from prophase I oocytes in all these species. Cdc6 mRNA, accumulated during the prophaseI-block in oocytes is

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only translated during oocyte meiotic maturation in Xenopus (Lemaitre et al., 2002), and similarly, translation of Cdc6 mRNA occurs only after GVBD in the Mouse. In D. melamogaster, as in vertebrates, transcription is prevented during ovulation, indicating that the regulation of the acquisition of the competence to replicate by CDC6 synthesis before fertilization is under translational control. Strikingly, similar strategies for preventing the untimely replication in cells blocked in G2 for a long period of time or cells arrested in G0 suggest that the suppression of replication licensing due to lack of CDC6 could be a universal mechanism for securing a prolonged arrest of the cell cycle (Kubota and Takisawa, 2003). When a G0 cell is released from guiescence to G1, replication competence is acquired, before the cell enters S phase, by accumulating Cdc6 and MCM onto chromatin. In quiescent REF52 fibroblasts, overexpression of Cdc6 induces MCM binding to chromatin. Furthermore, coexpression of CyclinE/Cdk2 with Cdc6 is sufficient to initiate DNA replication in these cells (Cook et al., 2002). The absence or presence of CDC6 could thus be viewed as a switch for the competence to replicate.

During ovulation, CDC6 is synthesized conferring on the egg the competence to replicate before fertilization. Consequently, a second mechanism for preventing replication licensing before fertilization must be activated to prevent DNA replication before sperm entry. Phosphorylation, might mediate this second level of regulation of CDC6 activity. CDC6 contains several CDK phosphorylation sites involved in the regulation of its activity (Pelizon et al., 2000; Weinreich et al., 2001). In the Mouse and in *Xenopus*, we observed a shift in the electrophoretic mobility at Metaphase II (Fig. 1), that could be due to phosphorylation by a CDK. This shift could not be experimentally detectable between metaphaseI and II during Drosophila ovulation, although among potential sites located in the N terminal of mouse and human CDC6, Ser/thr 54, involved in the regulation of subcellular localization in the mouse is conserved in Xenopus and D. melanogaster suggesting a similar regulation.

Recently, evidence for a similar regulation in the acquisition and repression of the competence to replicate before fertilization has been shown in echinoderm starfish *Asterina pectinifera* (Tachibana et al., 2000). Ablation of c-mos messenger RNA in starfish oocytes caused them to initiate DNA replication immediately after MI and triggers parthenogenetic activation (Tachibana et al., 2000), as previously observed in *Xenopus* (Furuno et al., 1994; Dupre et al., 2002). This indicates that the competence to replicate is present between MI and MII but is normally repressed by the Mos/MAPK pathway directly or indirectly.

The regulation of CDC6 synthesis and activity appears to be a common strategy used both for cell proliferation and the control of fertilization (Fig. 4). The lack of CDC6 in oocytes can be viewed as a safeguard strategy that will prevent any risk of recruiting the MCM helicase on DNA, thereby preventing illegitimate



**Fig. 4.** Acquisition of the competence to replicate by CDC6 during eukaryote meiosis. Synthesis of CDC6 during meiosis is described in vertebrates (*Xenopus*, mouse, and invertebrates (*Drosophila* and starfish)). In starfish, competence to replicate has been shown to be present after GVBD, implying that CDC6 is present at this stage (hatched bars). This reentry in the cell cycle is achieved at fertilization by the relief of CDC6 repression CDKs (Sagata et al., 1989; Masui, 2000), whereas in *S. pombe*, Cdc18 remains absent until gemination. Supplementary Figure: Characterisation of *D. melanogaster* CDC6 homologue. **A**: Sequence alignment of *D. melanogaster* CDC6 homo-

logue with X. laevis, mouse, and S. pombe yeast. Conserved functional motifs, the nucleotide binding/ATPase domains (Walker A, Walker B), sensor I, sensor II motifs that are hallmarks of AAA+ proteins (Takahashi et al., 2002) are underlined with dark line. A conserved consensus site in N-terminal for phosphorylation by CDK (Jans et al., 1995) is marked by a star. **B**: Immunoblot analysis of protein extracts from five Xenopus eggs (1), 25 D. melanogaster eggs and a S35 labeled in vitro translation of the Drosophila CDC6 cDNA (LD 25083 CDC6) (3).

DNA synthesis during the long period of quiescence in female ovaries. Interestingly, CDC6 synthesis appears to be essential both for the license to replicate in the egg, and for sperm binding competence to the oocyte in vertebrates (Tian et al., 1997). Immature prophase I oocytes do not bind sperm, and under normal physiological conditions sperm binding activity is acquired at meiotic maturation.

Injection of Cdc6 can induce sperm binding in immature oocytes (Tian et al., 1997). The induction of two apparently unrelated events, competence for replication and sperm-binding ability (which are both essential for fertilization), by the same protein is unexpected, and reveals a link between DNA replication controls and developmental controls, which might be important in multicellular eukaryotes.

In fission yeast, cdc18 is absent during meiosis, preventing DNA replication during the MI–II interval (Lindner et al., 2002), whereas in *Xenopus* and mouse, CDC6 is present but its activity is negatively regulated

by CDK activity and (or) the MAPK pathway. Ectopic expression or surrexpression of cdc18 between MI and MII in S. pombe could indicate whether it is the only one to be missing to induce illegitimate DNA replication during this period. In yeast, cdc18 remains absent from spores, whereas in Drosophila, Xenopus, and the mouse, CDC6 is present in the gamete prior to fertilization (Fig. 4). In these species, however, the first cell cycles after fertilization occur without transcription and a store of CDC6 is therefore essential. In fission yeast, the absence of Cdc18 may be a safeguard against inappropriate re-replication during the later stages of meiosis in a manner that does not require additional controls (such as involving c-mos) to repress licensing. Fission yeast spores subsequently re-enter the cell cycle with new transcription of Cdc18 as in somatic cells. CDC6 can be viewed therefore both as a security system for preventing unscheduled replication (Kubota and Takisawa, 2003), and as a licensing signal to compensate the lack of transcription during early development.

# MATERIALS AND METHODS Collection and Culture of Mice and *Xenopus* Oocytes

Immature oocytes arrested at prophase I of meiosis were obtained by removing ovaries from 5- to 6-week-old Swiss female mice. The ovaries were placed directly into warmed (37°C) M2 medium (Whittingham, 1971), and ovarian follicles were punctured to release the enclosed oocytes. Only those immature oocytes displaying a GV were collected and cultured further in M2 medium under liquid paraffin oil at 37°C in an atmosphere of 5%  $CO_2$  in air. The resumption of meiotic maturation was typically observed 1 hr after release from the follicles. The oocytes were scored for GVBD, for the extrusion of the first polar body and for the extrusion of the second polar body. Xenopus oocytes were sorted for stage 6 and maturation was triggered by progesterone addition as previously described (Lemaitre et al., 2002). Low-speed extracts of maturing oocytes were prepared as described for egg extracts in Eppendorf tubes (Menut et al., 1999).

After the excess buffer was removed, the oocytes were centrifuged at 8,000g for 5 min at 4°C, and then at 12,000g for 2 min at 4°C. The upper phase was collected and centrifuged again at 12,000g for 2 min at 4°C. The extract was adjusted in Laemmli Buffer for SDS–PAGE analysis (Laemmli, 1970).

Ovaries from flies were hand dissected and placed in ice cold PBS. In situ hybridization to whole-mount ovaries was carried out essentially as described above. Protein extracts with stage 14 and laid eggs from fertilized or virgin female were prepared directly by resuspending in Laemmli buffer immediately after dechorionation as described in Tautz and Pfeifle (1989).

#### Immunoblotting

Oocytes at the appropriate stage of maturation were collected in sample buffer (Laemmli, 1970) and heated to  $100^{\circ}$ C for 3 min. The proteins were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. Following transfer and blocking for 1 hr in 5% skimmed milk in PBS, containing 0.1% Tween-20, the membrane was incubated overnight at 4°C with the primary antibody diluted at the appropriate dilution in 1% BSA in PBS. We then used a secondary antibody conjugated to horseradish peroxidase (Amersham) diluted 1:1,000 in 1% BSA in PBS/Tween. The membranes were washed three times in TBS/Tween and then processed using the ECL detection system (Amersham).

# Immunofluorescence

Isolation, fixation, and labeling of mouse oocytes were performed as described by Kubiak et al. (1992) and as described by Tautz and Pfeifle (1989) for *Drosophila* oocytes. In both cases, specific primary antibodies were incubated at the appropriate dilution 1 hr at room temperature in 1% BSA. We then used secondary antibodies, conjugated either to fluorescein or rhodamine (Miles). The chromatin was visualized using propidium iodide (Molecular Probes;  $1 \mu g/ml$  in PBS) because DAPI was not suitable for confocal analysis.

Samples were observed with a Bio-Rad MRC-600 confocal microscope. Antibodies CDC6 human/mouse antibody was provided by (Santa Cruz Biotechnology, Inc.) and *Xenopus* CDC6 antibody was prepared as previously described (Lemaitre et al., 2002). Both CDC6 antibodies directed against Human and *Xenopus* CDC6 protein recognized *Drosophila* CDC6 protein (supplementary datas). Antibody against MCM2 *Drosophila* protein was kindly provided by TT Su et al. (1996).

## **Yeast Strains and Methods**

Cdc18 was tagged CFP in the background of a pat1ts allele (pat1-114) using the pSMUC2+ plasmid, to generate strain P1279, as described in (Gregan et al., 2003).

Expression of Cdc18 expression during fission yeast meiosis was analyzed using G1 block and release as described in Lindner et al. (2002). Basically, a cdc18-CFP pat1ts strain (p1279) was arrested in G1 by nitrogen starvation for 16 hr at 25°C, after which cells were refed at 34°C to induce meiosis. Samples were taken every 30 min and analyzed by flow cytometry, fluorescence microscopy, and Western blotting, as described previously (Grallert et al., 2000; Lindner et al., 2002), using anti-GFP monoclonal antibody 3E1 to detect Cdc18-CFP and a-tubulin was detected with Sigma T5168 used at a dilution of 1/1,0000. DNA was stained with sytox green for flow cytometry and DAPI (4',6-diamidino-2phenylindole) for fluorescence microscopy.

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