Regulation of DNA Replication during Meiosis in Fission yeast

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Contents

Abstract

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The interval between meiotic nuclear divisions can be regarded as a modified mitotic cell cycle where DNA replication is blocked. Mechanisms regulating this critical aspect of meiosis that allows haploid cells to be generated from a diploid progenitor were investigated in this project. Licensing is restricted after meiosis I due to down-regulation of Cdc18 and Cdt1. Late meiotic expression of Cdc18 and Cdt1, which load the MCM helicase onto replication origins, can lead to partial DNA replication after meiosis I. This implies that block to initiation via licensing forms an important component of this regulation. As detecting any minor DNA re-replication after meiosis I requires a technique more sensitive than flow cytometry for detection of total cell DNA contents, I also investigated a procedure to allow incorporation and detection of 5-ethynyl-2'-deoxyuridine (EdU) in fission yeast.

Additional inactivation of Spd1 or stabilization of Dfp1 after MI when Cdc18 and Cdt1 are also expressed does not enhance re-replication, but cyclin-dependent kinase Cdc2 plays a role in preventing re-replication during the MI-MII interval. Unexpectedly, when the licensing block is subverted, replication forks only move a short distance in the interval between meiosis I and II, implying that the elongation step of DNA replication is also inefficient. In addition, I show that the regulation of entry into meiosis II is not delayed by a partial round of DNA replication or DNA damage, indicating that replication and DNA damage checkpoints do not operate in late meiosis.

List of publications arising from this thesis

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Abbreviations

ATM: ataxia-telangiectasia mutated

ATR: ATM and Rad3-related

BrdU: bromodeoxyuridine

Cdc: cell division cycle/control

Cdt: Cdc10 dependent transcript

CFP : cyan fluorescent protein

CHX: cycloheximide

DAPI: 4',6-diamidino-2-phenylindole

DDB: DNA damage binding protein

DTT: dithiothreitol

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphate

DSB: double strand break

EDTA: ethylenediamine tetra-acetic acid, disodium salt

EdU: 5-ethynyl-2'-deoxyuridine

EMM: Edinburgh minimal medium

EMM-N: Edinburgh minimal medium without nitrogen

EtBr: Ethidium bromide

FEN-1: flap endonuclease and 5'-exonuclease

G-418: geneticin

GFP: green fluorescent protein

HU: hydroxyurea

IR: ionizing radiation

kb: kilo base

LB: medium Luria-Bertani medium

log: logarithmic

MCM: Mini-Chromosome Maintenance

MI: meiosis I

MII: meiosis II

MLH: MutL homolog

mM: millimolar/L

MMR: mismatch repair

MSH: MutS homolog

MTHF: methenyltetrahydrofolate

NER: nucleotide excision repair

OD: optical density

ORC: origin recognition complex

PCNA: proliferating cell nuclear antigen

PCR: Polymerase Chain Reaction

pre-RC: pre-replication complex

RFC: replication factor C

RNA: ribonucleic acid

RNaseA: ribonuclease A

RNR: ribonucleotide reductase

RPA: replication protein A

SCF: skp1-cullin 1-F-box

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

ssDNA: single-stranded DNA

SDW: sterile distilled water

TAE: tris acetate ethylene diamine tetra acetic acid

TAP: tandem affinity purification

TCA: trichloroacetic acid

TE: tris EDTA

ts: temperature-sensitive

UV: ultraviolet

wt: wild-type

YE3S: yeast extract medium with supplements

YFP: yellow fluorescent protein

Chapter 1

Introduction

Meiosis is a specialized form of cell division in sexually reproducing organisms, by which gametes are generated and the cell's ploidy is reduced. This process of haploidisation (for a diploid organism) is essential for sexual reproduction, and together with meiotic recombination (a meiosis-specific event), it contributes to broaden genetic diversity in sexually reproducing organisms. To achieve this, a single round of DNA replication (pre-meiotic S phase) is followed by two rounds of chromosome segregation (meiosis I and II), which is one of the unique features of meiosis. Precisely implementing this program is important for establishing the correct genetic constitution of the meiotic products, and problems in this process may result in genomic instability of gametes (Marston and Amon 2004). Therefore, it is of interest to investigate the special mechanisms regulating DNA synthesis during meiosis, especially, how DNA replication is repressed during the MI and MII interval. Currently, such regulation is thought to mainly involve regulation of pre-replicative complex (pre-RC) components and control of cyclin dependent kinases (CDKs). Therefore, in the following content, I would first like to make an introduction about how DNA replication is carried out in eukaryotes, and then give a brief discussion about general features of meiosis and how it differs

from mitosis. As my project is focused on meiosis in fission yeast, I will discuss features of *S. pombe* meiosis. Finally, I will review the findings regarding mechanisms preventing replication between the two meiosis divisions.

1.1 Mechanisms of Eukaryotic DNA replication

The increased complexity of higher organisms requires more genes, thus the great increase in genome size during evolution becomes a challenge for DNA replication. For example, the largest chromosome in *Drosophila* is 6.5 x 10^7 bps (Adams *et al*. 2000). If *Drosophila* followed the mechanism bacteria (e.g. *E.coli*) use to replicate their DNA (bidirectional replication from a single origin at a rate approaching 1000 nucleotides per second), it would take about 9 hours to finish replication. But in fact, this genome is replicated in several minutes in early development (Garrett 2004). In addition, the replication fork rate in eukaryotes is much lower than in bacteria, ranging from 0.6 kb/min to 6 kb/min (in humans it is about 5 kb/min and, in *S. pombe*, 3 kb/min) (Garrett 2004, Heichinger *et al*. 2006). Therefore, replication of eukaryotic chromosomes must be initiated from multiple origins. Basically, in all eukaryotes, DNA replication involves three steps: pre-RC formation (i.e. licensing), initiation and elongation (Fig. 1.1).

1.1.1 Origin choice

A.

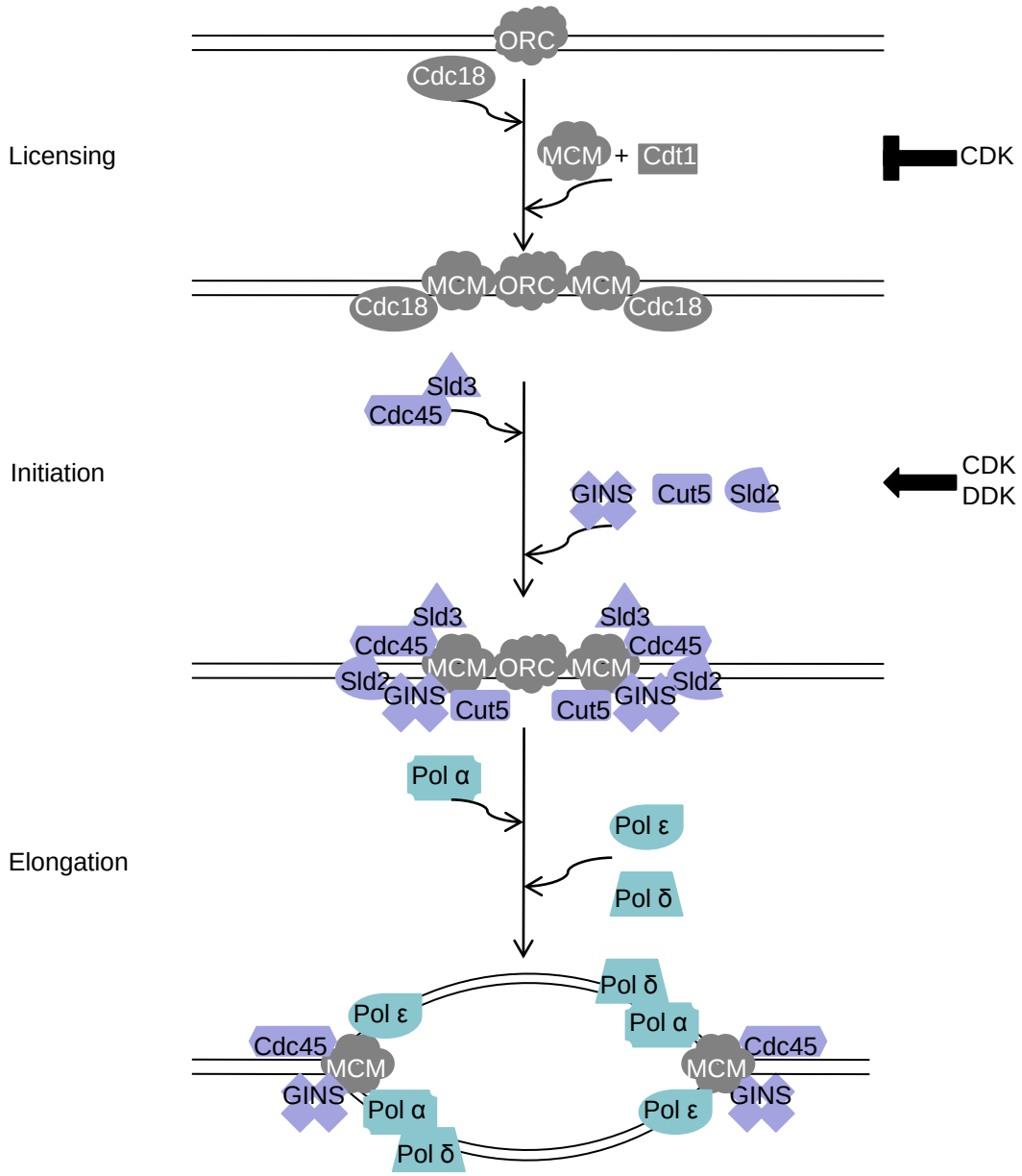


Figure 1.1. A simplified summary of steps involved in eukaryotic DNA replication. For details see section 1.1.2 - 1.1.4.

In *S. cerevisiae*, replication origins contain a special DNA sequence – ACS (ARS consensus sequences), whereas in other eukaryotes, including *S. pombe* and metazoans, less or no sequence specificity of origins has been identified (reviewed in Bell and Dutta 2002). In *S. cerevisiae* the origin was originally termed an autonomously replicating sequence (ARS) as at first it was identified as a DNA fragment that was necessary for maintenance of the exogenous plasmids, i.e. it allowed the replication of these extrachromosomal DNAs (Stinchcomb *et al*. 1979). All known ARSs have a 11bp consensus DNA sequences – (A/T)TTTA(T/C)(A/G)TTT(A/T) – named ACS, which can be specifically recognised by ORC (Newlon and Theis 1993). Meanwhile, though ACS plays a central role, several additional elements (such as B1, B2 and B3 elements in *S. cerevisiae* ARS1) are also required for origin function (Gilbert 2001). In fission yeast, the situation is different in that the only common characteristic of origins is that they usually are located at AT-rich areas, but no sequence similarity was identified (Segurado *et al*. 2003). Interestingly, *S. pombe* Orc4 contains a domain consisting of multiple HMG-I (Y)-related "AT-hooks" at its N-terminus, which is not found in other orthologues (Chuang and Kelly 1999). Evolutionarily, this may be derived from fusion of the progenitor ORC subunit and a DNA binding protein that assists origin recognition, just like some DNA viruses (such as Epstein Bar virus, EBV) use their own DNA binding protein (e.g. EBNA1 in EBV) to localize the cellular ORC to the viral origin (Ritzi *et al*. 2003). In addition, regarding to the timing of

replication origin firing, a recent report suggested that in *S. pombe*, Cds1 (a S phase checkpoint kinase) may play a role in preventing origins in a “late factory”, which are destined to fire late, from firing early during S phase (Hayashi *et al*, 2007).

In metazoans, there is no consensus sequence at origins as well (reviewed in Cvetic and Walter 2005). Some initiation sites (in human and *Xenopus*) have asymmetric A+T rich stretches (one strand is polydA whist the complementary strand is polydT), and in human and mouse cells some CpG islands are associated with nascent DNA strands in during early S phase (Cvetic and Walter 2005). It seems, however, that *Drosophila*, *Xenopus* and human ORC can bind to DNA with little sequence specificity (Kohzaki and Murakami 2005, Delgado *et al*. 1998). Despite the fact that DNA sequence per se seems to be insufficient to direct origin recognition in these organisms, origin distribution should still be strictly regulated as well. Random placement of origins will lead to the risk that some inter-origin spacings are too large to be replicated in the time available for S phase, and because licensing can only occur before S phase, there may be problems completing genome replication in this situation (Hyrien *et al*. 2003). To get around this “random completion problem”, the number of potential licensed origins may be much greater than those actually used – the ‘‘origin redundancy’’ model (Edwards *et al*. 2002, and reviewed in Goldar *et al*. 2008). Evidence supports the notion that in higher eukaryotes,

epigenetic controls or chromatin dynamics rather than DNA sequence itself affect the origin choices (reviewed in Cvetic and Walter 2005, Méchali 2010). This mechanism may provide more flexibility so that DNA synthesis can adapt to various conditions during differentiation and development (Méchali 2010).

1.1.2 Licensing

During the cell cycle, to faithfully pass genetic information to the daughter cells, re-replication is strictly prevented, i.e. it is important to make sure that initiation occurs once and only once per origin per cell cycle. One critical mechanism to prevent re-initiation is to regulate pre-RC formation, which refers to the stepwise assembly of Cdc6, Cdt1 and MCM (Mini-Chromosome Maintenance) proteins onto sites in the chromosome where the origin recognition complex (ORC) is bound (Drury *et al.* 2000, Mendez and Stillman 2000).

1.1.2.1 ORC

ORC is a highly conserved heterohexameric protein complex composed of six subunits, which was originally identified in *S. cerevisiae* as being responsible for origin recognition (Bell and Stillman 1992). Subsequently, ORC homologues have been found in various eukaryotes (reviewed in Dutta and Bell 1997). Most ORC subunits except Orc6 are AAA+ ATPases (ATPases

associated with various cellular activities, with conserved Walker-A and -B motifs) (Koonin 1993, Klemm *et al*. 1997). In *S. cerevisiae*, ATP-binding activity of the ORC is required for its DNA binding and also formation of the pre-RC (Sclafani and Holzen 2007). Association of ORC and DNA is dependent on ATP-ORC binding but not ATP hydrolysis, and then ORC hydrolyzes ATP in later replication events, which can be stimulated by binding of Cdc6 or single stranded DNA that are produced by origin unwinding (Klemm *et al*. 1997, Lee *et al*. 2000). So ATP hydrolysis by ORC may act as a molecular switch between different replicative states (Sclafani and Holzen 2007). In addition, ORC is always chromosome bound during all phases of the cell cycle in *S. cerevisiae*, though a transient displacement by RPA after initiation occurs (Lodish *et al*. 1999). In human cells, ATP stimulates the DNA-binding activity of ORC, and mutations in the Walker-A motif of Orc1, Orc4, or Orc5 reduced the DNA binding efficiency of ORC (Giordano-Coltart *et al*. 2005). *S. pombe* Orc4 seems to be the most important subunit for origin recognition as it can bind to origins on its own (Kong and DePamphilis, 2001). The role of ORC in MCM loading also depends on its ATPase activity but also relies on assistance of Cdc6/Cdc18, so it will be discussed in the section 1.1.2.2.

ORC is phosphorylated by CDKs during the S to M period, and this contributes to preventing re-replication (reviewed in DePamphilis 2003). In mammalian cells, Orc1 is a substrate of Cdk2/cyclin A (Méndez *et al*. 2002). When cells

enter into S phase, the selective release of Orc1 from chromatin accounts for the inactivation of the whole ORC complex (DePamphilis 2003). Afterwards, it is degraded in an ubiquitin-dependent manner (HeLa cells) or rebinds to DNA during the next M to G1 transition after deubiquitylation (hamster cells) (Li and DePamphilis, 2002). In *Xenopus* somatic cells, ORC disassociates from the chromatin entirely during S phase, although whether if this process is CDK/cyclin dependent remains unclear (Sun *et al*., 2002). Nevertheless, the rebinding to chromatin is inhibited in *Xenopus* cells at least because some subunits (Orc1 and Orc2) are hyperphosphorylated by CDK/Cyclin A (Findeisen *et al*. 1999).

Besides the roles in licensing, recent reports suggest that ORC is also associated with other cell process, such as transcriptional regulation, centrosome duplication and chromosome segregation. In *S. cerevisiae*, ORC is involved in silencing the transcriptionally inactive mating type loci (*HMR* or *HML*). Silencing requires cooperation of multiple elements such as cis-acting regulatory sequences (E and I silencers), ORC, Rap1 and Abf1 (Micklem *et al*. 1993, Loo and Rine 1994, Cheng and Gartenberg 2000). In mammalian cells, ORC may have an analogous role in heterochromatin formation, and Orc2 was shown to tightly associate with heterochromatin and heterochromatin proteins 1a (HP1a) and HP1b during G1 and early S phase (Sasaki and Gilbert 2007, Prasanth *et al*. 2004). In addition, in human cells during late S, G2 and M

phase, heterochromatin association of Orc2 was restricted to centromeric region. More specifically, human Orc2 and Orc3 formed a complex with CENP-A at centromeres during mitosis. Also, similar centromeric association of Orc2 was identified in *C. elegans* as well (Sasaki and Gilbert 2007, Prasanth *et al*. 2004). Orc6 also localized to kinetochores during mitosis in human cells, as do Orc1 and Orc4 in *S. pombe* (Hayashi *et al*. 2007).

Orc6 may also have non-replication related function. A fraction of Orc6 formed a reticular-like structure around the cell periphery in early mitosis in human cells; later on (in metaphase and anaphase) this reticular-like structure was close to chromatin; finally before cytokinesis it moved to a position align along the plane of cell division and Orc6 proteins concentrated to midbody (Prasanth *et al*. 2002). *Drosophila* Orc6 also localized to cleavage furrow between dividing cells and interacted with a septin protein pnut, mediated by a C-terminal domain in Orc6. Disrupting the interaction between Orc6 and pnut does not affect DNA replication but leads to defective cell division (multinucleate cells), indicating a role of Orc6 in cytokinesis (Chesnokov *et al*. 2003).

1.1.2.2 Cdc18/Cdc6

During licensing, Cdc6 (known as Cdc18 in *S. pombe*) firstly associates with

ORC in an ATP dependent way. Cdc6 is also an AAA+ ATPase and shows sequence similarity to Orc1 (Bell and Dutta 2002). The latter may be an ancestral form of ORC subunits since among others, only Orc1/Cdc6 has a related protein in Archaea and these proteins appear to be also involved in DNA replication (Stillman 2005). ATP binding and hydrolysis is essential for Cdc6 function.

Recruitment of Cdc6 to origin requires ORC binding and is required for the subsequent MCM assembly (Bell and Dutta 2002). ORC-Cdc6 forms a ring shaped structure that DNA is wrapped around in the presence of ATP (Speck *et al*. 2005). This ORC-Cdc6 ring-shaped complex is similar to the MCM ring in size and shape, so it may bind to the torus of MCM complex to help its chromatin loading (Kubota *et al*. 2003). Cdc6 and Orc1 shares significant sequence similarity with AAA+ subunits in clamp loader protein complexes, which is found in eukaryotes, Archaea, *E. coli* and bacteriophage T4 (reviewed in Sclafani and Holzen 2007). So presumably, Cdc6-ORC-Cdt1 forms a “clamp loader”: the loading of MCM complex to chromatin may be similar to loading of PCNA by the RF-C clamp loader in an ATP dependent way (Davey *et al*. 2002). The clamp loader binds to the clamp (e.g. MCM or PCNA), opens it a bit by using energy released from hydrolyzing ATP, to allow DNA strand get into the channel inside the clamp, and finally the clamp closes (Bell and Dutta 2002). In addition, in *S. cerevisiae*, association of Cdc6 to ORC (that is already on DNA)

may enhance the specificity and stability of ORC-DNA association, restricting ORC binding to specific origin sequences (Mizushima *et al*. 2000).

Cdc18/Cdc6 is a key regulatory target for pre-RC formation. For example, in *S. pombe*, merely over-expressing Cdc18 could induce multiple rounds of DNA synthesis in the absence of mitosis (Yanow *et al*. 2001). In yeasts, Cdc18/Cdc6 is subjected to both transcriptional regulation and cell cycle dependent degradation. First, it is only synthesized before S phase (Baum *et al*. 1998, Piatti *et al*. 1995). Secondly, it is a substrate of CDK. Elevated CDK activity during G1/S transition results in Cdc18/Cdc6 phosphorylation, followed by its ubiquitylation by SCF then proteolysis (Drury *et al*. 2000; Jallepalli *et al*., 1998). In budding yeast, this first wave of extremely rapid Cdc6 proteolysis requires the SCF complex and Cdc28-Cln, whilst subsequent Cdc6 destruction from S phase is dependent on SCF and Cdc28-Clb (Drury *et al*. 2000). In mammalian cells, compartmentalization rather than proteolysis may regulate Cdc6 function but some Cdc6 remains associated with chromatin during S phase (Petersen *et al*. 1999, reviewed in Kearsey and Cotterill 2003,). Phosphorylated Cdc6 is exported from the nucleus after entry into S phase (Delmolino *et al*. 2001, Sclafani and Holzen 2007). The rate of Cdc6 synthesis was nearly constant during G1 and S phase in human cells and newly produced Cdc6 could bind to chromatin after initiation (Mendez and Stillman 2000, Piatti *et al*. 1995, Biermann *et al*. 2002).

1.1.2.3 Cdt1

To complete pre-RC formation, Cdt1 and MCM complexes associate with the origin bound ORC and Cdc18/Cdc6 (Randell *et al*. 2006). In *S. cerevisiae*, electron microscopy analysis suggested that Cdt1 and Mcm2–7 formed a single heptamer and the MCM complex may be loaded onto DNA as a double hexamer (Remus *et al*. 2009). Presumably, Cdt1 associates with MCM complex, brings it to chromatin, and Cdt1-Cdc18-ORC forms a "helicase clamp loader", which opens the MCM and facilitates DNA strand entry into the central channel of MCM complex (Sclafani and Holzen 2007).

In *S. cerevisiae*, Cdt1 localization is controlled by CDK activity, starting to accumulate in the nucleus at the end of previous round of mitosis and being transported to the cytoplasm upon replication onset (Tanaka and Diffley 2002). In *S. pombe*, Cdt1 is expressed only during G1, due to its expression being controlled by the same transcriptional regulation as Cdc18, in a Cdc10 (a component of DSC1) dependent way (Nishitani *et al*. 2000, Hofmann and Beach 1994). In addition, S phase proteolysis of Cdt1 is regulated by CRL4(Cdt2)-mediated ubiquitylation. This Cdt1 proteolysis is dependent on forming a complex with DNA–associated PCNA and two PIP (PCNA-interacting peptide) motifs in Cdt1 are both required (Guarino *et al*.

2011). Human Cdt1 is also present only during G1 and regulated by CRL4(Cdt2)-mediated proteolysis as well (Nishitani *et al*. 2001).

In metazoans, there is a unique inhibitor of Cdt1, geminin, whose levels in the cell cycle show an inverse relationship to the Cdt1 levels (Wohlschlegel *et al*. 2000, Nishitani *et al*. 2001). Geminin is absent during the period from metaphase-anaphase transition to G1 and its destruction is APC (Anaphase Promoting Complex) mediated (McGarry and Kirschner 1998). Cdt1 and geminin directly interact and this interaction prevents MCM loading (Tada *et al*. 2001). Research in *Xenopus* cells suggests a model explaining how geminin is functioning: a Cdt1-geminin complex instead of Cdt1 alone assists MCM chromatin binding, and when excess geminin exists, the additional association of geminin inactivates the Cdt1-geminin complex (Lutzmann *et al*. 2006).

Geminin also plays a role in centrosome duplication in mammalian cells. It is localized at the centrosomes in M phase and late G1, S and G2 phase, via actin-related protein Arp1 (Lu *et al*. 2009). Depletion of geminin leads not only to re-replication but also centrosome over-duplication, with checkpoint activation and cell cycle arrest (Tachibana *et al*. 2005). If the the checkpoint is deficient, cells enter into defective mitosis with multipolar spindles (Tachibana *et al*. 2005).

1.1.2.4 MCM

The MCM complex, consisting of six subunits (Mcm2-7), is thought to be a heterohexameric complex functioning as a DNA helicase (reviewed in Sclafani and Holzen 2007 and Labib and Diffley 2001). Its structure is proposed to be a ring with a central hole, through which DNA may pass (Lodish *et al*. 1999, Kearsey and Labib 1998). All eukaryotes appear to have at least six MCM protein paralogues (Bell and Dutta 2002). There are MCM homologues in Archaea as well, reflecting the fact that Archaea and eukaryotes share basic similarities in their replication apparatus (Sclafani and Holzen 2007, Smith *et al*. 1997). The MCM complex has ATPase activity. In fact, all six MCM subunits are AAA+ ATPases with walker A, B motifs, and their simultaneous association with chromatin is required for completing licensing (Schwach *et al*. 2001). The roles of MCM in initiation and elongation will be discussed in the following section.

In the budding yeast cell cycle, Mcm2-7 is subject to regulation by cell compartmentalization in a CDK-dependent way (Labib *et al*. 1999). In other organisms (mammals, *Xenopus*, *Drosophila*, fission yeast), MCM proteins are constitutively located in nucleus and probably are relatively stable (Lodish and Berk *et al*. 1999, Kearsey and Labib 1998, Pasion and Forsburg 1999). In *S. pombe*, assembly of the intact MCM complex is essential for nuclear

localization of the complex, which seems to depend on an NLS (nuclear localization signal) in Mcm2 (Bell and Dutta 2002, Pasion and Forsburg 1999).

1.1.3 Initiation

The licensing reaction, where loading of the MCM complex onto ORC occurs confers competence for the initiation step (Randell *et al*. 2006). Initiation is triggered by CDK and DDK (Dbf4-Dependent Kinase, Hsk1 in *S. pombe* and Cdc7 in *S. cerevisiae*), and involves the assembly of more replication factors (including Sld2/Drc1, Cdc45, Sld3, Cut5/Dpb11, GINS – composed of four subunits, Sld5, Psf1, Psf2, and Psf3,) onto origins. In *S. cerevisiae*, Sld3 and Cdc45 firstly join to the pre-RC complex (reviewed in Botchan 2007). Afterwards, Sld3 and free Sld2 are phosphorylated by S-CDK, which facilitates the formation of a Sld2-Sld3-Dpb11 (11-3-2) complex on chromatin (Tanaka *et al*. 2007, Zegerman and Diffley 2007) (Fig. 1.1). The role of this 11-3-2 complex is possibly to mediate the association of other replication proteins, as a matchmaker. Cdc45 association to chromatin becomes stable when Dpb11, Sld2 and GINS are recruited to origins (Yabuuchi *et al*. 2006). In *S. pombe*, same factors are assembled onto chromatin during initiation, but in a slightly different order: first Sld3 associates to origins in a process dependent on DDK but not CDK. Then Cut5, Sld2 and GINS complex are recruited in a mutually dependent manner, requiring CDK. Finally, Cdc45 binds to chromatin in a

process dependent on Sld3, GINS, DDK and CDK (Yabuuchi *et al*. 2006) (Fig. 1.1). So Sld2, Sld3 and Dpb11 facilities the recruitment of other factors, including GINS and Cdc45, which may be involved in DNA unwinding together with the MCM complex. The 11-3-2 complex only functions in initiation but not elongation, so it will not travel along chromatin with elongation factors like DNA polymerase during elongation (Tanaka *et al*. 2007).

In initiation, the Mcm complex plays a role as well, via activation of its helicase activity, triggered by assembly of other factors (e.g. GINS) (reviewed in Botchan 2007). In *S. cerevisiae*, the free MCM complex is a ring-shaped single hexamer; whilst after loading onto chromatin, two Mcm2-7 complexes form a double hexamer connected via their N-terminal rings, which can slide on dsDNA (Evrin *et al*. 2010, Remus *et al*. 2009). As substrates of DDK, N-terminal phosphorylation of MCM2, 4 and 6 may facilitate activation of its helicase activity (Sclafani and Holzen 2007, Masai *et al*. 2006). In addition, MCM and GINS co-immunoprecipitate with Cdc45, and this CMG (Cdc45/Mcm2-7/GINS) complex possesses ATP-dependent helicase activity (Moyer *et al*. 2006). A recent study suggests a model as to how the CMG complex unwinds the DNA helix. First, the double-strand bound MCM dimer splits and a gate between Mcm2 and Mcm5 opens, mediated by DDK-dependent MCM phosphorylation. Then one DNA strand is extruded from the central channel thus now MCM circles single strand DNA. Finally the gate

is reclosed, and helicase activity of MCM complex is activated by allosteric changes in MCM2-7 affected by GINS and Cdc45 (Ilves *et al*. 2010, Fu *et al*. 2011).

1.1.4 Elongation

Elongation, i.e. the DNA synthesis process, is carried out by DNA polymerases with the assistance of other factors such as PCNA (proliferating cell nuclear antigen), RF-C (replication factor C) and RPA (replication protein A). The polymerases responsible for nuclear DNA replication are Pol α, ε and δ. Pol α has associated primase activity and firstly synthesizes a short RNA-DNA primer in the presence of RPA on the single-strand template DNA. RPA is a heterotrimeric protein consisted of three subunits (RPA1, RPA2 and RPA3). It non-specifically binds to single-stranded DNA and may prevent ssDNA from winding back or forming other secondary structure by destabilizing the helix (Wold 1997).

Afterwards, RF-C and PCNA replace Pol α at the 3’ end of the RNA-DNA primer, and introduce Pol ε (on the leading strand) or Pol δ (on the lagging strand) to continue DNA synthesis. First, RF-C inhibits Pol α and reduces affinity of Pol α to DNA. Then RF-C loads PCNA onto chromatin in a process dependent on ATP, a process similar to ORC-Cdc18-Cdt1 loading of the MCM

complex, i.e. RF-C opens PCNA and allows the DNA strand to go through the central channel in PCNA. Finally, Pol α is released from chromatin and the primer 3'OH-bound PCNA clamp recruits Pol ε or Pol δ (Maga *et al*. 2000). Another function of PCNA is to increase processivity of Pol ε or Pol δ during replication fork movement, i.e. enhancing their affinity to DNA (Hubscher *et al*. 2002). The oligonucleotides synthesized by Pol α are short RNA/DNA hybrid of 8~12 RNA nucleotides followed by a stretch of 20 to 30 DNA nucleotides, which is necessary as Pol ε or Pol δ cannot initiate synthesis de novo (Waga *et al*. 1994). Both Pol δ and Pol ε are highly processive and have 3' to 5' exonuclease activity (proofreading ability) that allows incorrectly incorporated nucleotides to be excised. In contrast, Pol α does not have a proof reading activity (Hubscher *et al*. 2002). So the short patch synthesized by the error-prone Pol α should be removed to ensure genome stability, and these mechanisms will be discussed in the following paragraph about Okazaki fragment maturation (Balakrishnan *et al*. 2010).

On the lagging strand, replication is discontinuous because DNA can only be synthesized in the 5' to 3' direction. The short DNA patches (150~200 bps) synthesized by Pol δ and Pol α on the lagging strand are termed Okazaki fragments. There are two pathways for Okazaki fragment maturation: Dna2 or FEN1 excises the RNA-DNA primer between each Okazaki fragments and the gap is filled by DNA polymerase and ligase (Lig I). Basically, when two Okazaki

fragments meet each other, a flap with the 5' end containing the RNA-DNA primer is extruded from DNA helix. Fen1 can digest a short flap up to 10 nucleotides, and if for some reason, the flap escapes Fen1 excision and becomes long enough to bind RPA (>22 nucleotides), Fen1 cannot excise it anymore due to RPA association (Rossi *et al*. 2008). In this case, Dna2 replaces RPA and cleaves the long flap, finally leaving a short extruding length about 5 or 6 nucleotides, which can be digested by Fen1 (reviewed in Balakrishnan *et al*. 2010).

The proteins, being assembled in the previous steps and travelling with DNA polymerases, including Cdc45, GINS and MCM (CMG complex), provide the helicase activity (Botchan 2007). The processes of packaging DNA into nucleosomes and activating cohesins that link sister chromatids are coupled with replication fork movement (Khorasanizadeh 2004).

1.1.5 CDK activity in cell cycle

As described above, CDK plays an important role in regulating replication by phosphorylating multiple replication factors (especially pre-RC components and initiation proteins) and thus regulating their functions. Therefore, it will be useful to have a brief discussion about oscillation of CDK activities during the cell cycle. Generally, during late anaphase of mitosis, APC polyubiquitinates

the mitotic cyclins thus leading to their proteolysis. Together, with CDK inhibitory phosphorylation by Wee1 and Mik1, CDK activity is reduced to a low level at the end of M-phase and in G1, which produces a permissive environment for the formation of pre-RCs. Subsequently, CDK is reactivated by the degradation of CDK inhibitors and re-synthesis of cyclins. The elevated CDK activity promotes initiation of DNA synthesis from pre-RCs and, at the same time, inhibiting pre-RC formation, which ensures that DNA replication occurs just once per cell cycle. Following S phase and G2, mitosis is triggered by M-CDK, and this may involve the expression of mitotic specific B type cyclins (reviewed in John *et al*. 2001). In yeasts only one CDK regulates all the events. For instance, in fission yeast, Cdc2 and a single mitotic cyclin (Cdc13/Cyclin B) can allow a viable cell cycle when G1 cyclin (Cig2), the cyclin that normally is involved in S phase activation, or other cyclins with minor roles in G1 such as Cig1 and Puc1, are deleted (Fisher and Nurse 1996, Coudreuse and Nurse 2010). In higher organisms, more regulators (e.g. Emi1) and multiple CDKs are involved in cell cycle control, but there is still a basic pattern of CDK activity oscillation (Porter 2008).

1.2 A general discussion about meiosis

The purpose of meiosis is to increase genetic variation and broaden genetic diversity in reproduction, which is mainly achieved by two mechanisms:

production of haploid gametes and meiotic recombination. By conducting two rounds of cell division without DNA replication, the number of chromosomes is halved in gametes. Ploidy will be restored via fusion of gametes. Compared to mitosis – when the progeny are identical to the parental cells, in meiosis and the following fertilization, nuclear genetic material in zygotes is a mixture of the maternal and paternal homologues. Meiotic-specific recombination facilitates exchange of information between the maternal and paternal chromosomes within the gamete precursor cells, and produces new genetic combinations in each gamete (Inagaki *et al*. 2010). Meanwhile meiotic recombination is also critical for proper segregation of homologous chromosomes during the first meiotic division.

1.2.1 Induction of meiosis in fission yeast

Normally *S. pombe* grows in a haploid state, in either of the two mating types h^- and h^+. Upon the depletion of environmental nutrition (mainly nitrogen), cells are prone to exit from the mitotic cell cycle, and either enter G0 phase (quiescent state) or meiosis. The other prerequisite of meiosis is the availability of cell with an opposite mating type. Two cells with different mating types conjugate to form a zygote. An ascus containing four spores is produced finally, after pre-meiotic S phase and two rounds of nuclear division. *S. pombe* meiosis can also start from a diploid h^+/h^- cell without conjugation under

nutritional limitation. However, the normal h^+/h^- cell is prone to conduct meiosis under all growth conditions, thus it is slightly difficult to maintain its diploid state.

DNA microarray analysis has been used to analyze the transcriptional profile of *S. pombe* meiosis and this identifies hundreds of genes being up or down regulated in successive waves (Mata *et al*. 2002). Several meiotic-specific proteins are involved in regulating this process, including transcription factors, kinases, binding proteins, etc (eg Ste11, Pat1, Mei2, Mei4, Mes1). These proteins function at different time points during meiosis, making sure that each meiotic event occurs at the correct time. Pat1 is a negative regulator of meiosis. It phosphorylates Mei2 (a RNA-binding protein responsible for the initiation of meiosis) on residues Ser438 and Thr527, thus inhibiting Mei2 activity (Harigaya and Yamamoto 2007). Inactivating Pat1 permits commencement of meiosis by activating Mei2 via releasing the inhibitory phosphorylation. Taking advantage of a *pat1* temperature-sensitive mutant (*pat1-114*) to inactivate Pat1 at the restrictive temperature, G1 (or G2)-arrested cells can leave cell cycle and enter into meiosis with high synchronization even if the nutrition, ploidy and mating-type gene configuration requirements are not fulfilled (Iino and Yamamoto, 1985). It is noteworthy that even a diploid *pat-114* meiosis appears to have abnormal features compared to a normal meiosis (Bahler *et al*. 1991). Meiotic landmark events of *pat1-114* cells occur at the same time after meiosis

induction compared with the *pat1*$^{+}$ cells, but recombination frequency was reduced in the *pat1-114* meiosis, and that might be due to (i) high temperature, (ii) azygotic meiosis (compared to mating of haploids), and (iii) the *pat1-114* mutation itself (Bahler *et al*. 1991).

Initiation of meiosis by Mei2 is related to expression of meiosis-specific proteins, such as Mei4, Rec8, Ssm4, and Spo5. Messenger RNAs encoding these proteins contain a consensus signal sequence termed DSR (determinant of selective removal), which can be recognised by an YTH-family protein Mmi1 and then selectively degraded during vegetative growth (Harigaya *et al*. 2006). Mmi1 protein only has a putative RNA-binding motif and no other characteristic domains, indicating the requirement of other factors involved in this proteolysis process (Yamamoto 2010). Indeed, a poly(A) tail is firstly added to the 3' terminus of the mRNA by a canonical polyadenylation polymerase composed of Pla1 and Rna15, promoted by association of Mmi1 to DSR. Then Pab2, a poly(A)-binding protein is recruited, possibly also interacting with Mmi1. Finally, the exosome is recruited, to eliminate the transcripts (Yamanaka *et al*. 2010). During initiation of meiosis, Mei2 and meiRNA binds to Mmi1, inhibiting Mmi1 by forming a nuclear dot, thus Mmi1 cannot exert its binding function and mRNAs containing DSR are stabilized (Harigaya *et al*. 2006, Harigaya and Yamamoto 2007).

Genes containing MCB (M *lu* 1 cell cycle box) motifs in their promoter regions, such as *cdc18* and *cdc22*, are subject to DSC1 (DNA synthesis control-like transcription factor complex) mediated transcriptional regulation during both S phase and pre-meiotic S phase. The difference is that during vegetative growth, the DSC1 complex consists of Cdc10, Res1, Res2, and Rep2 whilst in meiosis Res1 is not required (Cunliffe *et al*. 2004). Instead a meiosis-specific protein Rep1 is required for the meiotic pattern of gene expression, including (i) genes whose expression is also essential in mitotic G1/S transition, such as *cdt1*, *cdc18*, and (ii) genes specially required for meiotic events such as recombination, e.g. *rec8*, *rec11* (Harigaya and Yamamoto 2007).

1.2.2 Pre-meiotic S phase

Pre-meiotic S phase is generally longer than mitotic S phase in the same organism, for example, in *C. elegans* meiotic S phase is at least twice as long as mitotic S phase (Jaramillo-Lambert *et al*. 2007). In *S. pombe*, pre-meiotic S-phase is about 30% longer compared to the time of S phase in mitotic cells (Heichinger *et al*. 2006). In *S. cerevisiae*, pre-meiotic S phase is two to three times as long as mitotic S phase (Cha *et al*. 2000). This might indicate that different mechanisms are used in mitotic and meiotic DNA replication (Forsburg and Hodson 2000). On the other hand, meiotic-specific factors like Mum2 and Ime2 (their functions will be discussed in the following section) are

dispensable for mitosis (Davis *et al*. 2001, Foiani *et al*. 1996). However, it is currently well acknowledged that the process of DNA replication is similar in meiosis and mitosis; the same replication factors and same origins are used (Murakami and Nurse 2001). The replication fork velocity is also similar (~3 kb/min in *S. pombe*) (Heichinger *et al*. 2006); but the origin utilization efficiency is lower in pre-meiotic S phase than in mitotic S phase, which may be one reason for the longer time of DNA synthesis in meiosis (Heichinger *et al*. 2006). The other reason accounting for the longer S phase in meiosis is possibly the preparation for meiotic-specific events such as recombination. For example, generation of double strand breaks are found to be related to the DNA replication event, and DSBs are generated immediately after DNA synthesis in pre-meiotic S phase in *S. cerevisiae* (Marston and Amon 2004, Cha *et al*. 2000).

1.2.2.1 Origin usage and DNA replication machinery in pre-meiotic S phase

In most situations, origin usage in pre-meiotic S phase is similar to that in mitotic S phase. For example, in *S. cerevisiae*, where origins are defined by sequence-specific elements, a well-characterized origin *ARS1* functions in pre-meiotic DNA replication as well (Hollingsworth and Sclafani 1993). Two-dimensional agarose gel electrophoresis also revealed that five known

mitotic replication origins on chromosome III also actively used as origins during pre-meiotic S phase (Collins and Newlon 1994). In *S. pombe*, genome-wide analysis of replication origins by microarray (comparative genomic hybridization) indentified almost complete overlapping of origin usage between meiotic and mitotic S phases - only 1% mitotic origins are not functional in meiotic DNA replication, and all the origins identified in pre-meiotic S phase are also origins in the mitotic cell cycle (Heichinger *et al*. 2006). However, origin efficiency in pre-meiotic S phase (15%) is only half of that in mitotic S phase (29%) (Heichinger *et al*. 2006).

Transcriptional regulation can be an explanation for why some mitotic origins are not operative in meiotic DNA replication and why there are differences of origin efficiency between mitosis and meiosis. In *S. cerevisiae*, *ARS605*, one of the most efficient origins in DNA replication during the mitotic cell cycle, is completely silenced in pre-meiotic S-phase. The reason is that *ARS605* located in gene *MSH4* (Msh4 protein is required for formation of synaptonemal complex and regulation of crossover distribution) (Novak *et al*. 2001) and transcription of *MSH4* in early meiosis removes ORC from ARS605 (Mori and Shirahige 2007). If transcription of *MSH4* was activated in the mitotic cell cycle, origin activity of *ARS605* was inhibited as well (Mori and Shirahige 2007).

The same DNA replication machinery is used in S phase of both meiosis and

mitosis. In *S. pombe*, absence of pre-RC components Cdc18, Mcm2, Mcm4 or Orc1 during pre-meiotic S phase blocked completion of pre-meiotic DNA replication (Murakami and Nurse 2001; Lindner *et al*. 2002). In addition, the Mcm2–7 complex associated with chromatin only during early pre-meiotic S phase but not thereafter (Lindner *et al*. 2002). This behaviour of MCM complex is similar to how it behaves in the mitotic cell cycle, i.e. it is only chromatin-bound during G1 and S phase, indicating that the MCM complex has similar roles in both meiosis and mitosis (Lindner *et al*. 2002). Other replication factors, including Cdc22 (subunit of ribonucleotide reductase), RPA and DNA polymerases are also essential for normal progress of pre-meiotic S phase, and inactivation of DNA ligase (Cdc17) or Cdc24 has similar effects on pre-meiotic S phase as on mitotic S phase (Forsburg and Hodson 2000). In *S. cerevisiae*, it has also been shown that Cdc6 (Cdc18), Mcm4, DNA polymerase I and DNA ligase (Cdc9) are required for pre-meiotic DNA replication (Schild and Byers 1978, Budd *et al*. 1989, Johnston *et al*. 1982).

The roles of cell cycle kinases CDK and DDK in pre-meiotic DNA replication are similar to their roles in mitotic replication i.e. stimulating initiation by activating helicase (MCM complex) and loading replisome components (reviewed in section 1.1.3) (Sclafani and Holzen 2007). In addition, DDK (Hsk1-Dfp1) and CDK have some additional functions in meiosis that are related to recombination and biorientation of homologous chromosomes

during meiosis I, which will be discussed in section 1.2.3.

In spite of the fact that the same replication factors are used in meiosis compared to mitosis, there seem to be meiosis-specific regulators for DNA synthesis in pre-meiotic S phase, such as Mum2 and Ime2 in *S. cerevisiae*. Cells with deficient Mum2 have no problems in vegetative growth but are defective in completing meiotic DNA replication and recombination (Davis *et al*. 2001). *Mum2* genetically interacts with *orc2* and *pol1* (Pol1 is the catalytic subunit of Polα-primase holoenzyme) in meiosis, however, exactly how Mum2 regulates meiotic DNA replication remains unknown (Davis *et al*. 2001). Ime2 is a meiosis-specific CDK-like kinase in *S. cerevisiae*, playing multiple roles in meiosis – one of which is ensuring correct timing of meiotic replication (Foiani *et al*. 1996). In pre-meiotic S phase, Ime2 is required for RPA phosphorylation that is essential for DNA replication (Clifford *et al*. 2004).

1.2.2.2 Meiotic cohesin and connections between pre-meiotic replication and recombination

Precise recognition of sister chromatids generated after replication guides their correct segregation during the following nuclear division, which is of primary importance in maintaining genome stability. This is achieved by pairing sister chromatids together via cohesions between them, established during

replication. More specifically, a protein complex named cohesin that contains Smc1, Smc3, Scc3/Irr1 and Mcd1/Scc1/Rad21 is responsible for maintenance of cohesion between sister chromatids (Skibbens 2009). Cohesin has to be released from chromosomes to permit chromosome segregation during anaphase and it seems to be able to re-load on the chromosome during the following telophase and G1 phase (Shintomi and Hirano 2009). However, association of cohesin onto chromatin is different from establishment of cohesion. The competence of cohesin to connect sister chromatids is gained along with passage of replication fork, mediated by Eco1 that may be recruited to replication forks through binding to PCNA (Nasmyth and Haering 2009). Crystal structure of the Smc1–Smc3 interface indicated that Smc1 and Smc3 can form a V-shaped heterodimer with a small channel between, and α−kleisin subunit Scc1/Rad21 binds to Smc1 and Smc3 at either end to close the ring (Nasmyth 2011). However, how exactly cohesin complex entraps sister chromatids remains largely unknown, i.e. if both sister chromatids pass through the same channel between Smc1 and Smc3, or each of them is encircled by a cohesin ring and then two cohesin rings are interacted (Nasmyth 2011).

Establishment of cohesion between sister chromatids during replication is also essential for meiosis, where chromosome segregations are more complicated than in mitosis. The cohesin complex in meiosis is composed of Smc1, Smc3,

Scc3/Irr1, but with a meiosis-specific α -kleisin Rec8 instead of Mcd1/Scc1/Rad21 (Nasmyth and Haering 2009). In addition to preventing segregation of sister chromatids during MI with the assistance of Shugoshin (will be discussed in section 1.2.4), meiotic cohesin also coordinates interactions between homologous chromosomes, required for formation of synaptonemal complexes (SC) and axial elements (AE), and involved in meiotic recombination. *S. cerevisae* cells with *rec8Δ* or *smc3-ts* mutations have lower meiotic recombination frequencies and fail to form SCs and AEs (Klein *et al*. 1999). In other organisms, including *C. elegans*, maize and Arabidopsis, Rec8 is involved in chromosome axis formation as well. In *S. pombe*, which lacks SC but has linear element (LinE) instead, Rec8 is required for assembly of LinE (Bardhan 2010b). Additionally, in *S. cerevisae*, pre-meiotic S phase in *rec8Δ* cells was 10% extended compared with the wild type (Cha *et al*. 2000). As far as has been experimentally verified, in *S. cerevisae* and *S. pombe*, initiation of recombination (i.e. formation of double strand breaks) is directly coupled to DNA replication (Borde *et al*. 2000, Tonami, Murakami *et al*. 2005). In *S. pombe*, pre-meiotic S phase is linked to reductional chromosome segregation; meiosis I induced from G2 phase without pre-meiotic S phase exhibits equational chromosome segregation (Watanabe *et al*, 2001). As cohesion activation is related to replication fork passage, it is very likely that connections between replication and recombination are, at least partially, mediated by meiotic cohesins.

1.2.3 Meiotic recombination

The first meiotic division is termed “reductional”, in which homologous chromosomes rather than sister chromatids are separated. To ensure the equal distribution of genetic material and correct segregation of homologues, the first important thing is to accurately recognize homologues and pair them – in fact, as mentioned above, meiotic recombination plays a crucial role in this process. All the complicated events of meiotic recombination (Fig. 1.2) occurs in the meiotic G2/M transition, i.e. the end of the extended G2 phase of meiosis and meiotic prophase I. This period is further subdivided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis according to chromosome morphologies (reviewed in Tsai and McKee 2011, MacQueen and Hochwagen 2011).

1.2.3.1 Deliberately generated double strand breaks

First, double strand breaks (DSBs) are deliberately introduced by Spo11 (sporulation-specific protein 11, named Rec12 in *S. pombe*), a widely conserved topoisomerase-like enzyme. Activation of Spo11 requires passage through pre-meiotic S phase (Watanabe *et al*. 2001, Forsburg 2002). At least in *S. cerevisiae*, Spo11 generated meiotic-specific DSBs seems to be able to be

A

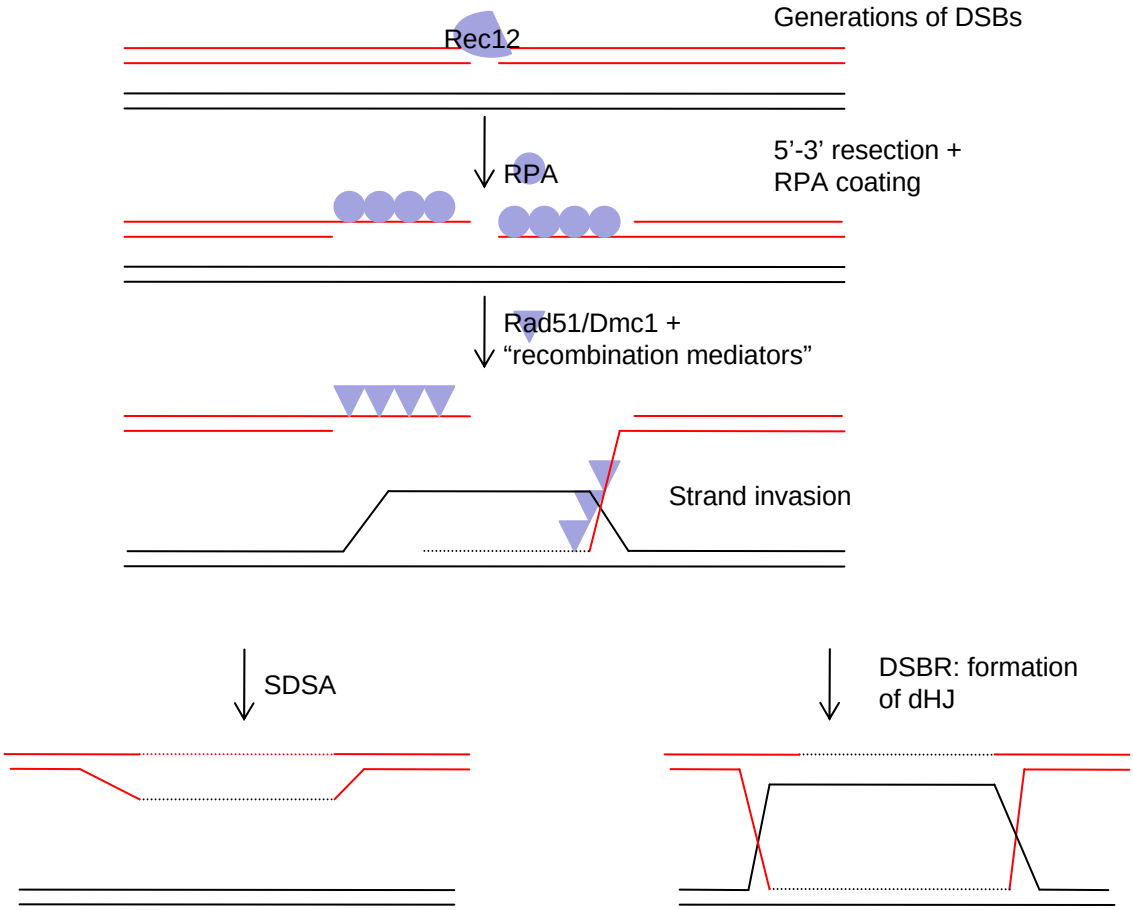


Figure 1.2. A simplified illustration of homologous recombination in meiosis. SDSA: synthesis-dependent strand annealing, DSBR: double-strand break repair. For details see section 1.2.3.

distinguished from the exogenous DSBs, and lead to somewhat different downstream responses (MacQueen and Hochwagen 2011). DNA strands at the break point are resected, leaving two long single-stranded tails with 3'-OH ends, mediated by the MRN (Mre11/Rad50/Nbs1) complex (Nakamura *et al*. 2010, and reviewed in Inagaki *et al*. 2010, Jazayeri *et al*. 2008, Filippo *et al*. 2008).

1.2.3.2 Strand invasion and "inter-homologue bias"

After end resection, the single-stranded DNA tails are coated by RPA. Then RPA is replaced by the recombinases – Rad51 (SpRhp51) (the general one) and Dmc1 (the meiosis specific recombinase, meiotic homologue of Rad51) with help of the "recombination mediators", including Rad52 (SpRad22), BRCA2, Rad55 (SpRhp55) - Rad57 (SpRhp57) complex (Filippo *et al*. 2008). Rad51/Dmc1 cannot bind to the RPA-coated ssDNA on its own, so role of the mediators is to overcome the inhibitory effect of RPA on the Rad51/Dmc1-ssDNA association.

The next step is strand invasion. Here the most different aspect between mitotic homologous recombination (HR) and meiotic HR comes, which is so-called "inter-homolog bias", i.e. choice of the repair template. In budding yeast meiosis, Goldfarb and Lichten (Goldfarb and Lichten 2010) estimated

that only one-third of all recombination events occur between sister-chromatids, i.e. during meiosis, most DSBs are repaired by using the homologous chromosome as template. Though this ratio could be expected by chance since there are two homologous chromatids for one sister, considering that during the mitotic cell cycle, almost all recombination events are between sister-chromatids, there must be certain mechanism in meiosis for repressing sister-chromatid recombination (Pradillo and Santos 2011). First, Rad54 may be involved in such a mechanism. In the mitotic cell cycle, Rad54 interacts with Rad51 directly and promotes strand invasion to the homologous region of the sister chromatid. However, in meiosis Rad54 is inhibited by phosphorylation by a CHK2-like meiosis-specific kinase Mek1. Meanwhile interaction between Rad54 and Rad51 is blocked by Hed1 (reviewed in MacQueen and Hochwagen 2011, Inagaki *et al*. 2010). Meiosis-specific recombinase Dmc1 promotes inter-homologue recombination as well, assisted by Hop2-Mnd1 and Rdh54, a meiotic homologue of Rad54 (Filippo *et al*. 2008).

In most organisms, including *S. pombe*, formation of a “bouquet configuration” at zygotene after generation of DSBs, i.e. attaching telomeres to the nuclear membrane, facilitates searching for homologues by concentrating chromosomes within a limited region. Therefore, it provides a chance for recognition of homologous chromosomes via chromosome movements aided by the cytoskeleton (Tsai and McKee 2011).

1.2.3.3 Synaptonemal complex and linear element

In meiosis, after homologous recognition and during DSB repair, a structure named the synaptonemal complex (SC) forms and links the two aligned homologous chromosomes, until recombination is completed and then the homologous chromosomes are connected by a tighter structure called “chiasma” at diplotene (Tsai and McKee 2011). Basically, SC is constituted with lateral filaments along the chromosome axis and central elements forming zipper-like structure between the two homologues (Tsai and McKee 2011, Inagaki *et al*. 2010). SC seems to be able to stabilize alignment of the homologous chromosomes, and mediate chromosome interaction and recombination. Disturbance of SC formation by knocking out the genes of SC components blocks double strand break repair in mouse meiosis (de Vries *et al*. 2005, Bolcun-Filas *et al*. 2007).

In *S. pombe*, where no SC is formed in meiosis, a structure similar to the lateral elements of SC in other organisms, called linear elements (linE), are correlated to meiotic homologous pairing (Loidl 2006). One reason for the simplified structure of synaptonemal complex is the low number of chromosomes (three) in *S. pombe*. In addition, the nuclear oscillation of the “horse-tail” elongated nucleus in meiotic prophase may help recognition of

homologues by increasing encounters between chromosomes (Yamamoto and Hiraoka 2001).

1.2.3.4 Homologous recombination in meiosis

There are usually two ways to complete homologous recombination: synthesis-dependent strand annealing (SDSA) and DSB repair (DSBR). In SDSA, the invading strand leaves the template after repair synthesis fills in the gap, and it ligates to the other end of the break point of the original strand, thus it can only lead to non-crossover (NCO) events (Pradillo and Santos 2011) (Fig. 1.2). In DSBR, the invading strand remains between the duplex template, and the other end of the break point of the original strand engages in the homologous chromosome, forming an intermediate structure termed a “double Holliday junction (dHJ)” (Fig. 1.2). DSBR produces both crossover (CO) and non-crossover (NCO) events, depending on how the dHJ is resolved (reviewed in Pradillo and Santos 2011).

Crossing-over leads to formation of a chiasma, and leads to reciprocal exchange of genetic materials. In mitosis, to maintain genome stability and avoid chromosome rearrangement, SDSA is the main (if not only) pathway for HR and NCOs are the major result. In meiosis, production of crossovers are more frequent (though still less than NCOs). Most importantly, formation of

COs is carefully regulated during meiosis to make sure that at least one CO is formed per homologous pair, because chiasmata (as well as the sister chromatid cohesion) are critical for maintaining connections and alignments of homologous chromosomes thus are essential for the “reductional” division of meiosis I (Inagaki *et al*. 2010).

1.2.4 Meiosis I – reductional division

As mentioned in section 1.2.3, during meiosis I homologous chromosomes rather than sister chromatids are separated. To precisely implement this, meiotic recombination ensures recognition of homologous chromosome by homologue pairing, and then by formation of chiasmata. Bi-orientation of homologues, i.e monopolar attachment of sister kinetochores, is also essential for their correct segregation. Cohesin plays a central role in this process (Watanabe 2005).

In yeast mitosis, cohesin is retained on chromosomes until establishment of the bipolar attachment of sister chromatids, and then it is removed by separase to trigger sister chromatid separation in anaphase; whilst in vertebrate cells, cohesin is released from chromosome arms during mitotic prophase-metaphase and only centromeric cohesin is preserved until onset of anaphase, protected from prophase dissociation by Shugoshin (McGuinness

et al. 2005, Watanabe 2005). In contrast, during meiosis, cohesin on sister chromatid arms is removed in meta-anaphase I to resolve chiasmata, but centromeric cohesin remains thoughout meiosis I until anaphase II, which is an evolutionary conserved feature, from yeast to humans (Watanabe 2005). Failure to preserve centromeric cohesin in meiosis I causes a defect in reductional division due to deficient monopolar attachment of sister chromatids.

In *S. pombe*, deleting *rec8* or replacing Rec8 by Rad21 in meiosis leaded to sister chromatid segregation at meiosis I, presumably via loss of centromeric cohesion during MI (Watanabe 2005, Yokobayashi *et al*. 2003, Watanabe 1999). Shugoshin protein Sgo1 protects meiotic centromeric cohesin containing Rec8 from separase during meiosis I, and this protection only acts on Rec8 but not Rad21 (Kitajima *et al*. 2004). The special role of Rec8 but not Scc1/Rad21 in establishing sister kinetochore mono-orientation during meiosis I is conserved in maize and *Arabidopsis* (Yokobayashi and Watanabe 2005, Chelysheva *et al*. 2005). In addition, a meiosis-specific protein Moa1 interacts with Rec8 and assists meiotic cohesin containing Rec8 to establish or maintain cohesion at the “central core” region of centromere, where Rad21 cohesin complexes normally do not associate (Yokobayashi and Watanabe 2005). Deletion of Moa1 caused disrupted monopolar attachment and equational segregation in meiosis I as well (Yokobayashi and Watanabe 2005).

In *S. cerevisiae*, roles of the protector Shugoshin protein Sgo1 in directing reductional division by maintaining centromeric cohesion during meiosis I is similar (Watanabe 2005). In addition, like Moa1 in *S. pombe*, a protein complex monopolin consisting of Hrr25, Mam1, Lrs4 and Csm1 directs sister chromatid mono-orientation in meiosis I via kinetochore modification (Bardhan 2010a), and DDK is essential for kinetochore localization of monopolin (Matos *et al*. 2008).

1.2.5 The Interval between MI and MII

1.2.5.1 Repressing replication in MI-MII interval in other organisms

In the interval between meiosis I and II, pre-RC components should be tightly controlled to prevent any re-replication of DNA. Studies with *Xenopus* eggs depict a picture of the regulatory networks. *Xenopus* stage VI oocytes are arrested at the G2/M transition (prophase) and lose ability to replicate DNA due to the absence of Cdc6, which is also the only missing replication protein during this period (Lemaître *et al*. 2002). At the same time, Orc1 and Orc2 are excluded from the nucleus (Lemaître *et al*. 2004). Although oocytes regain replication competence shortly after germinal vesicle breakdown (GVBD) because of synthesis of Cdc6 protein, from onset of GVBD to meiosis II

metaphase arrest, phosphorylation of Orc2, Mcm4 and Cdt1 by the MAPK pathway and reactivated Cdc2/Cyclin B contribute to prevention of untimely DNA replication (Frank-Vaillant *et al*. 2001, Lemaître *et al*. 2004, Whitmire *et al*. 2002).

So intermediate CDK activity maintained after meiosis I is essential to prevent origin licensing by repressing pre-RC formation (Nakajo *et al*. 2000, Furuno *et al*. 1994, Taieb *et al*. 2001, Hochegger *et al*. 2001, Iwabuchi *et al*. 2000). A protein kinase Mos (product of the c-mos proto-oncogene) involved in the MAPK pathway is proposed to prematurely reactivate CDK (Furuno *et al*. 1994). During the MI/MII interval, if the phosphorylation process catalyzed by Mos is compromised either by depletion of Mos or by the presence of a dominant negative form of Cdc2, cells seem to enter into interphase again and DNA replication could be induced after meiosis I (Furuno *et al*. 1994). Also, Wee1 kinase is identified to be almost non-functioning following meiosis I, since only low activity of Wee1 is present during the MI/MII transition, thus Cdc2 cannot be phosphorylated (Nakajo *et al*. 2000, Iwabuchi *et al*. 2000). High Wee1 protein level, achieved either by injection of *wee1* RNAs or exogenous Wee1 protein after meiosis I, leads to DNA re-replication (Nakajo *et al*. 2000, Iwabuchi *et al*. 2000). Partial destruction of Cyclin B is another reason for maintenance of residual CDK activity (Nakajo *et al*. 2000, Iwabuchi, *et al*. 2000).

In contrast to the situation in *Xenopus* meiosis, in mouse oocytes, the CDK level abruptly drops after the meiosis I (Choi *et al*. 1991), which leads to a hypothesis that, rather than CDK, an alternative kinase performs phosphorylation of the essential licensing components and thus prevents pre-RCs formation and DNA replication during the MI/MII transition, similar to the situation in budding yeast.

In budding yeast, a meiotic-specific protein kinase Ime2 was firstly identified as being required for early meiotic events but later found to possibly also play a role in late meiosis. Ime2 shares substrates with CDK, including Sic1 and multiple pre-RC components (Cdc18, Mcm6 and Orc6). Ime2 substrates are phosphorylated at different sites compared to CDK, however, and these phosphorylations are resistant to dephosphorylation by the Cdc14 phosphatase (Holt *et al*. 2007). Ime2 protein is undetectable during vegetative growth, and if Ime2 was ectopically expressed when cells are released from G1 arrest, DNA replication was repressed, possibly due to inhibition of licensing (Holt *et al*. 2007). During meiosis, transcription of Ime2 starts before pre-meiotic S phase and is maintained at a constant level until the MI/MII transition. Its activity peaks twice during meiosis: on entry to pre-meiotic S phase and on the onset of meiosis I (Dirick *et al*. 1998, Benjamin *et al*. 2003). In agreement with this, proteolysis of Cdc6 happens upon pre-meiotic S phase

onset, but in an Ime2, not Cdc28, dependent manner (Ofir *et al*. 2004).

In *S cerevisiae*, Mcm2 dissociates from chromatin after the completion of pre-meiotic S phase and does not rebind before the completion of meiosis II (Holt *et al*. 2007, Ofir *et al*. 2004). Inactivation of Ime2 after pre-meiotic S phase prevents cells from proceeding into MI, and only if both Ime2 and Cdk1 are inactivated, MCM subunits could re-enter into the nucleus during this period, which indicates that Ime2 can help to prevent nuclear importation of Mcm2-7 proteins after pre-meiotic S phase (Holt *et al*. 2007). During the MI and MII interval, the duplication of spindle pole body is ensured by activated Cdc14 phosphatase but licensing may be restricted by Ime2 phosphorylation of pre-RC components since the phosphates added by Ime2 may not be removed by Cdc14 (Holt *et al*. 2007).

In the human primary oocyte, expression of Cdc6 is identified as a rate-limiting step for establishment of replication competence (Eward *et al*. 2004). The fact that Cdc6 plays a role as “master regulator” during human female gametogenesis whereas high levels of Cdc6 and geminin are detectable in primary spermatocytes, and synthesis of Cdt1, instead of Cdc6, becomes rate-limiting in spermatogenesis during late prophase reflects a striking sexual dimorphism in human meiotic DNA replication regulation (Eward *et al*. 2004).

1.2.5.2 MI-MII interval in *S. pombe*

In contrast to the studies of meiotic DNA synthesis control in *Xenopus* oocytes and budding yeast, in *S. pombe*, regulation of DNA replication in meiosis has been little studied. First, regulation of pre-RC components, such as Cdc18 and Cdt1, may play a crucial role. In fission yeast, Cdc18's transcription during meiosis is controlled by the meiotic-specific DSC1 complex, which is slightly different (lacking Res1 but having Rep1 instead) from the mitotic DSC1 (DNA synthesis control-like transcription factor complex) (Cunliffe *et al*. 2004). However, similarly to the mitotic cycle, Cdc18 is subject to the Cdc2 mediated degradation and becomes undetectable after pre-meiotic S phase (Lemaître, *et al*. 2004). Cdt1 is also down regulated following pre-meiotic S phase (Namdar 2006). Chromatin association of Mcm4 has also been shown not to occur during fission yeast meiotic divisions (Lindner *et al*. 2002).

In addition, some progress has been made in terms of understanding how two consecutive nuclear divisions can occur during meiosis. One protein important for this progress is Mes1, which is absent during vegetative growth and the gene is only expressed from late meiosis I. Mes1 was firstly identified in a screen of meiotic deficient mutants and deletion of *mes1* will trap cells in the MI/MII transition (Shimoda *et al*. 1985). Mes1 can prevent APC from ubiquitinating Cdc13 by binding to an APC activator (Izawa *et al*. 2005).

Deleting *mes1* induces a reduction in CDK activity, which becomes too low to promote the second nuclear division. Therefore, in *S. pombe* residual CDK activity is maintained, as degradation of cyclin B is partially inhibited, similar to the mechanism in *Xenopus* eggs. However, in the *mes1* deletion mutant, although CDK activity declines upon the completion of meiosis I, and meiosis II does not occur, DNA re-replication cannot be induced, which is quite different from the results in *Xenopus* oocytes (Izawa *et al*. 2005).

1.2.6 Meiosis II – equational division

Meiosis II is similar to mitosis, where sister chromatids segregate. Centromeric cohesion persists until anaphase II and then is destroyed by separase (Nasmyth 2001). Activity oscillation of APC/C^{Cdc20} is required for completion of nuclear division in both MI and MI. In addition, a meiosis-specific activator of APC/C, Ama1 (in *S. cerevisiae*), coordinates the meiosis exit and mediates destruction of Cdc20 during anaphase II (Cooper and Strich 2011). In yeast, meiosis II is followed immediately by sporulation that requires co-operation of multiple proteins and expression of sporulation-specific genes.

1.2.7 Checkpoints in meiosis

Checkpoint mechanisms operate in the meiotic cell cycle as well. Some are

similar to those operating in the mitotic cycle, and some are meiosis specific (reviewed in (Sun and Kim 2012, MacQueen and Hochwagen 2011, Carr 2002, Murakami and Nurse 2000). In this section, I will focus on meiotic checkpoints operating in *S. pombe* because the checkpoint mechanisms may not be conserved, e.g. *S. pombe* cells do not form synaptonemal complexes prior to meiosis I so there is no synapsis checkpoint.

1.2.7.1 DNA replication/ damage checkpoint

The DNA replication checkpoint in meiosis is similar to the mitotic one. When DNA replication was blocked by hydroxyurea (HU) in pre-meiotic S phase, both checkpoint Rad proteins (9-1-1 complex, Rad17, Rad3, Rad26 - the sensors) and Cds1 (acts downstream of the Rad proteins to communicate checkpoint signals) were required to prevent entry into meiosis I (Murakami and Nurse 1999). Cells were arrested with decondensed chromosomes, unseparated SPBs without spindles, and low CDK/Cdc2-Cdc13 activity with Cdc2-tyrosine-15 phosphorylation (Murakami and Nurse 1999, Murakami and Nurse 2000). However, in contrast to the mitotic S phase cells, preventing Cdc2-tyrosine phosphorylation by inactivating Wee1 and Mik1 when incomplete replication was induced by HU treatment could not promote meiosis I entry. Instead cells arrested at metaphase I with separated SPBs and spindle formation, indicating a second DNA replication checkpoint operating in

early meiosis (Murakami and Nurse 1999, Murakami and Nurse 2000). In addition, if DNA replication is inhibited by HU, DSB formation is down regulated via a checkpoint mechanism, i.e. mediated by checkpoint proteins including the 9-1-1 complex (Rad9-Rad1-Hus1), and Rad3-26. A target of this mechanism is presumably Rec12 (Tonami *et al*. 2005, Ogino and Masai 2006). It represents a meiotic-specific checkpoint linking onset of recombination to completion of DNA replication.

In contract to the mitotic cell cycle, the DNA damage checkpoint seems not to operate during early meiosis at least in fission yeast, i.e. DNA damage before meiosis I cannot induce meiotic arrest. Instead, DNA damage is repaired in prophase I together with the Rec12-generated DSBs (Pankratz and Forsburg 2005).

1.2.7.2 Meiotic recombination checkpoint

The pachytene checkpoint (or meiotic recombination checkpoint) monitors progress of recombination and delays entry into MI if recombination is not complete (Roeder and Bailis 2000). Generally, both ATM and ATR are activated when DSBs are produced in meiotic G2, similar to the DNA damage checkpoint in somatic cells, but roles of the DNA-damage checkpoint proteins may be more complicated in meiosis, and other meiosis specific effectors are

involved. For example, in budding yeast, preventing accumulation and phosphorylation of Ndt80, a meiotic transcription factor, contributes to arresting cells in pachytene stage (Tung *et al*. 2000). In addition, Mek1, the meiosis-specific Chk2 homologue, is not only responsible for inhibiting the Cdc25 phosphatase thus blocking Cdc2 activation when meiotic recombination is defective, but also takes part in suppressing DSB repair using sister chromatid as template, i.e. creating the so-called “inter-homolog bias” (MacQueen and Hochwagen 2011, Perez-Hidalgo *et al*. 2003). Activation of Mek1 depends on phosphorylation of Hop1 by ATM/Tel1 and ATR/Mec1 (MacQueen and Hochwagen 2011).

Similarly, in fission yeast, incomplete recombination, i.e. persistent DSBs, triggers phosphoarylation of Mek1 by Rad3 (ATR) and/or Tel1 (ATM) and then Mek1 autophosphorylation (Tougan *et al*. 2010). Activated Mek1 phosphorylates Cdc25 and promotes its cytoplasmic accumulation, resulting in prolonged phosphorylation of Cdc2 at tyrosine 15, thus delaying entry into meiosis I (Perez-Hidalgo *et al*. 2008). However, in fission yeast, no meiotic recombination-deficient mutants can induce meiosis I arrest (Shimada *et al*. 2002, Murakami and Nurse 2000). For example, incomplete recombination can be induced by repair defects resulted from *meu13Δ*, but in such *meu13Δ* cells, after a delay, cells would finally enter meiosis I with fragmented chromosome if DSBs persisted, even though the recombination checkpoint is operating

(Shimada *et al*. 2002). This is similar to what has been observed in Arabidopsis where unrepaired DSBs activated ATR/ATM but no obvious cell cycle delay was observed (MacQueen and Hochwagen 2011), probably indicating insensitivity to DNA damage in early meiotic cells.

In addition, *S. pombe* Cut5 is also involved in the meiotic recombination checkpoint, apart from its role in initiation of DNA replication. As mentioned above, inactivating Meu13 causes delay of meiosis I which is a consequence of activating the meiotic recombination checkpoint, but additionally inactivating Cut5 partially recues this meiotic delay due to a defective checkpoint response (Perera *et al*. 2004). The mammalian homologue of Cut5, TopBP1 co-localizes with ATR, to γ-H2AX domains that indicate double strand breaks (DSB) during prophase I (Germann *et al*. 2011).

1.2.7.3 Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) monitors kinetochore-microtubule attachments and prevents anaphase onset if kinetochores are improperly attached in mitosis (Sun and Kim 2012). Unlike mitotic cells that arrest in a pro-metaphase like stage when treated with microtubule poisons, meiotic cells treated with similar drugs will arrest in G2 (in *S. cerevisiae*) or a pachytene stage related to the synapsis checkpoint (during mice spermatogenesis); both

of these arrests are independent of SAC (Hochwagen *et al*. 2005, Malmanche *et al*. 2006). However, budding yeast cells with deficient SAC proteins (*mad1Δ* and *mad2Δ*) exhibited a higher frequency of non-disjuction during meiosis I (Shonn *et al*. 2000). The similar results were observed in mouse oocytes as well (Homer *et al*. 2005). In addition, precocious destruction of cyclin B and securin in meiosis I was found in both situations, similar to what happened when the mitotic SAC is defective (Shonn *et al*. 2000, Homer *et al*. 2005). It seems that during meiosis I, at least in *S. cerevisiae*, spindle checkpoint proteins have meiotic-specific roles to make sure that bivalents are properly separated. Mad1 and Mad2 contribute to bi-orientation of homologous chromosomes, correcting the wrong attachments if these occur, thus preventing non-disjunction, and Mad3 controls the timing of meiosis I thus permiting enough time to correct wrong attachments (Lacefield and Murray 2007, Cheslock *et al*. 2005). As for meiosis II, vertebrate eggs are arrested in metaphase II by cytostatic factor activity (CSF) and activation of this metaphase II arrest in *Xenopus* eggs is dependent on SAC proteins Mad1, Mad2 and Bub1 (Malmanche *et al*. 2006). Mouse oocytes released from CSF arrest would still be arrested in metaphase II if treated with nocodazole that disrupts the MI spindle, and inactivating Bub1 or Mad2 led to loss of this nocodazole-induced metaphase II arrest (Tsurumi *et al*. 2004).

In fission yeast meiosis I, incorrect chromosome-spindle attachment (that can

be induced by *rec12Δ*) delayed anaphase I, in a manner dependent on Mad2 (Yamamoto *et al*. 2008). In addition, SAC proteins are also involved in segregation of homologues in MI in fission yeast cells. Bub1 is required in maintaining centromeric localization of Rec8 that is essential for mono-polar orientation of sister chromatids (Yamaguchi *et al*. 2003). Mad2 also delayed onset of anaphase II when sister kinetochores did not properly attach to the spindle during MII in *rec8Δ* or *clr4Δ* mutants, where centromeric cohesion could not be maintained until MII (Yamamoto *et al*. 2008).

1.3 Aims of this project

A reasonable hypothesis to explain how replication is suppressed between MI and MII in *S. pombe* is that availability of certain essential components for DNA replication may be restricted during late meiosis, which prevents DNA synthesis in addition to the CDK activity control.

Previous to my project, a survey of the representative replication factors reveals that Cdc18 and Cdt1 are absent following meiosis I, while the protein levels of Orc subunits, Mcm2-7, Cut5, Cdc45, Psf2, Cdc23, and Hsk1 either remain at the same level after pre-meiotic S phase or are up regulated (Namdar 2006). Thus presumably absence of licensing is at least part of the explanation for lack of DNA replication during this interval.

I am interested in the mechanism regulating DNA replication during late meiosis in fission yeast. First, what is the minimum condition needed to evoke re-replication between the two meiotic nuclear divisions; i.e. if Cdc18 and Cdt1 are present after meiosis I and the ectopic expression levels are comparable to their physiological levels in pre-meiotic S phase, is it enough to induce some re-replication? Second, is control of CDK activity also playing a role in repressing replication after meiosis I, or are there any other factors that are relevant, such as meiotic-specific kinases? Third, as the previous survey did not include all replication factors, I extended the analysis to more replication-related proteins, to see if they also play critical roles in repressing replication during MI-MII interval.

As detecting any minor DNA re-replication after meiosis I requires a technique more sensitive than flow cytometry for detection of total cell DNA contents, I also investigated a procedure to allow incorporation and detection of 5-ethynyl-2'-deoxyuridine (EdU) in fission yeast, a thymidine analogue which has some technical advantages over use of bromodeoxyuridine (Salic and Mitchison 2008).

Chapter 2

Materials and Methods

2.1 *S. pombe* strain construction and culture

Strains were constructed by transformations or crosses. The strains used in this project are listed in Table 2.2. Strain maintenance was as described previously, following standard procedures (Moreno *et al*. 1991). Cells were grown in YE3S rich medium (0.5% yeast extract, 3% glucose, supplemented with 250 mg/l adenine, uracil and leucine) or Edinburgh Minimal Medium (EMM, see Table 2.3 for recipe) (Moreno *et al*. 1991).

2.1.1 Transformation of *S. pombe* cells

10OD units (OD= 0.2~0.8) of fission yeast cells in log phase were washed sequentially with equal volumes of SDW (sterile deionized water), 1ml SDW and 1ml LiAc/TE (10mM Tris, 1 mM EDTA, 0.1M LiAc), finally resuspended in 200µl LiAc/TE. Then cells in LiAc/TE were mixed with 2µl carrier DNA (sheared /sonicated salmon testis DNA) and 2~3µg linear transforming DNA (for plasmid transformation, using 0.5~1ug DNA) and left at room temperature for 10

Table 2.2 Strains used in this study

No.	Genotype	Reference
3007	*h- pat1-114 ade+ adh1prom-TK-kanMX6::adh1 adh1prom-hENT1-leu1+::leu1-32 ura4+*	This study
3008	*h- pat1-114// h- pat1-114*	This study
3010	*leu- [nmt1Xmde3-TAP leu+] ade+ ura+*	This study
3011	*h- pat1-114 ade+ leu+ mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294 mes1pr-dfp1M12(kanMX6)::mes1pr spo4Δ::NAT*	This study
3012	*leu1-32 [nmt1X leu+] ade+ ura+*	This study
3013	*leu1-32 [nmt1Xmde3-TAP leu+] ade+ ura+*	This study
3014	*h- pat1-114 ade+ leu- ura4- [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] dfp1M12(hyg)::dfp1*	This study
3015	*h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294 mde3Δ::KanMX6*	This study
3016	*h- pat1-114 ade+ leu1-32 ura4-D18 [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] spo4Δ::NAT cdc2as(kanMX6)::cdc2*	This study
3017	*pat1-114 suc22-GFP*	AM Carr
3018	*pat1-114 cdc22-GFP*	AM Carr
3019	*pat1-114 sld3-5FLAG*	M. Masakata
3020	*h- pat1-114 ade+ leu+ mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294 mes1pr-dfp1M12(kanMX6)::mes1pr*	This study
3021	*h- pat1-114 ade+ leu- ura4- [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] spd1Δ::NAT*	This study
3022	*pat1-114 sld2-3FLAG*	M. Masakata
3023	*h+ pat1-114 adh1prom-TK-kanMX6::adh1 adh1prom-hENT1-leu1+::leu1-32 rec12Δ::NAT ade+ leu- ura4+*	This study
3024	*h- pat1-114 ade+ leu+ mes1pr-cdc18-tap(hyg)::mes1pr spd1Δ::NAT mes1pr-cdt1-myc(ura4+)::ura4-294*	This study
3025	*h- pat1-114 ade+ leu+ ura4+ dfp1M12(hyg)-CFP::dfp1*	This study
3026	*h- pat1-114 dfp1-CFP ade+ leu+ ura+*	Lab stock

3027	*h- pat1-114 ade+ leu+ ura4+ spo4Δ::nat cdc2as(kanMX6)::cdc2*	This study
3028	*h- pat1-114 ade+ leu+ mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294 spo4Δ::NAT cdc2as(kanMX6)::cdc2*	This study
3029	*h- pat1-114 ade+ leu+ ura4+ spo4Δ:: NAT*	This study
3030	*h- pat1-114 ade+ leu+ mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294 spo4Δ::NAT*	This study
3031	*h- pat1-114 ade+ adh1prom-TK-kanMX6::adh1 adh1prom-hENT1-leu1+::leu1-32 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294 // h- pat1-114 ade+ adh1prom-TK-kanMX6::adh1 adh1prom-hENT1-leu1+::leu1-32 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4-294*	This study
3032	*h- pat1-114 ade+ adh1prom-TK-kanMX6::adh1 adh1prom-hENT1-leu1+::leu1-32 ura4+// h- pat1-114 ade+ adh1prom-TK-kanMX6::adh1 adh1prom-hENT1-leu1+::leu1-32 ura4+*	This study
2450	*h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 ade+ leu+*	This study
2451	*h- pat1-114 cdc18::mes1pr-cdc18-cfp ura4+ ade+ leu+*	This study
2452	*h+ pat1-114 [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu-*	This study
2453	*h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 ade+ leu+//h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 ade+ leu+*	This study
2454	*h- pat1-114 ura4+ ade+ leu+//h- pat1-114 ura4+ ade+ leu+*	This study
2455	*h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 rec12Δ::nat ade+ leu+//h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 rec12Δ::nat ade+ leu+*	This study
2456	*h- pat1-114 rec12Δ::nat ura4+ ade+ leu+//h- pat1-114 rec12Δ ura4+ ade+ leu+*	This study
2457	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(leu1+)::leu1 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1(ura4+)::cdt1 ade+ leu- ura4-*	This study
2458	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(leu1+)::leu1 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1(ura4+)::cdt1 rec12Δ::nat ade+ leu- ura4-*	This study

2459	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(leu1+)::leu1 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1(ura4+)::cdt1 rec12Δ::nat ade+ leu- ura4-//h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(leu1+)::leu1 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1(ura4+)::cdt1 rec12Δ::nat ade+ leu- ura4-*	This study
2460	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(leu1+)::leu1 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1(ura4+)::cdt1 mes1Δ::nat ade+ leu- ura4-*	This study
2461	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(hyg)::leu1 [mes1pr-cdc18-tap(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu- ura4-*	This study
2462	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(hyg)::leu1 [mes1pr-cdc18-tap(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu- ura4-//h- pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(hyg)::leu1 [mes1pr-cdc18-tap(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu- ura4-*	This study
2463	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(hyg)::leu1 [mes1pr-cdc18-tap(leu1+)] [mes1pr-cdt1-myc(ura4+)] rec12Δ::nat ade+ leu- ura4-*	Thisstudy
2464	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(hyg)::leu1 [mes1pr-cdc18-tap(leu1+)] [mes1pr-cdt1-myc(ura4+)] mes1del ade+ leu- ura4-*	Thisstudy
2465	*h+ pat1-114 adh1pr-TK(kanMX6)::adh1pr adh1pr-hENT1(hyg)::leu1-32 [rRep1(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu- ura4-*	Thisstudy
2466	*h- pat1-114 mcm2-cfp::hyg [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu- ura4-*	This study
2467	*h- pat1-114 mcm2-cfp::hyg [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] mes1Δ::nat ade+ leu- ura4-*	This study
2468	*h- cdc25-22 [rRep-mei4] mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 leu1- ura4- ade+*	This study
2469	*h- [rRep-mei4] mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 leu1- ura4- ade+*	This study
2470	*h- adh1prom::adh1prom-TK-kanMX6 leu1-32::adh1prom-hENT1-leu+ ade+ ura4+ leu1-32*	This study
2471	*h- adh1prom::adh1prom-TK-kanMX6 ade+ ura4+ leu1-32*	Thisstudy
2472	*h- adh1prom::adh1prom-TK-kanMX6 leu1-32::adh1prom-hENT1-leu+ rad3del::ura4 ade+ ura4-*	This study
2473	*h- pat1-114 cdt1-mPIPn(hyg)::cdt1 ade+ leu+ ura4+*	This study
2474	*h- pat1-114 ddb1Δ::kan spd1Δ::ura4+ [mes1pr-cdc18 leu+] ura4- ade+ leu-*	This study

2475	*h+ pat1-114 [mes1pr-cdc18(leu1+)] [mes1pr-cdt1-myc(ura4+)] ade+ leu- ura4-*	This study
2476	*h- pat1-114 mes1pr-cdc18-tap(hyg)::mes1pr mes1pr-cdt1-myc(ura4+)::ura4 mes1pr-wee1::wee1 leu1- ura4- ade+*	This study
2086	*h- ade- ura- leu- [nmt3x-cdc18 leu+]*	This study
2087	*h- ade- ura- leu- [nmt41x-cdc18 leu+]*	This study
2088	*h- Ade- Ura- Leu- [nmt81X-Cdc18 Leu+]*	This study
2089	*h- ade- ura- leu- [nmt3x leu+]*	This study
2090	*h- ade- ura- leu- ddb1Δ spd1Δ [nmt3x-cdc18 leu+]*	This study
2091	*h- ade- ura- leu- ddb1Δ spd1Δ [nmt41x-cdc18 leu+]*	This study
2092	*h- ade- ura- leu- ddb1Δ spd1Δ [nmt81x-cdc18 leu+]*	This study
2093	*h- ade- ura- leu- ddb1Δ spd1Δ [nmt3x leu+]*	This study
2027	*h- pat1-114 ddb1Δ::kan spd1Δ::ura4+ mes1pr-cdc18-tap(hyg)::mes1pr ura4- ade+ leu+*	Thisstudy
2383	*h- pat1-114 spd1-yfp::ura4 ura4- leu+ ade+*	Lab stock
1416	*h- pat1-114 cdt1-myc*	Lab stock
1424	*pat1-114 cdc18-tap*	Lab stock
1756	*h- pat1-114 mcm2-cfp::hyg cdc21pr-cdc18-tap::ura4 mes1pr-cdt1-myc::kan+ mes1del::nat ade+ leu+ ura4-*	Lab stock
1505	*cdc25-22*	Lab stock

Table 2.3 Media recipes

A. EMM (for 1 litre)

KH Phthallate	3g
Na_2HPO_4	2.2g
NH_4Cl	5g
Salts stock	20ml
vitamins stock	1ml
mineral stock	0.1ml
10M NaOH	0.1ml
glucose	20g

B. EMM-N (for 1 litre)

KH Phthallate	3g
Na_2HPO_4	2.2g
Salts stock	20ml
vitamins stock	1ml
mineral stock	0.1ml
10M NaOH	0.1ml
glucose	20g

C. Salts stock (for 1 litre)

$MgCl_2.6H_2O$	52.5g
$CaCl_2$.2H2O	0.74g
KCl	50g
Na_2SO_4	2g

D. Vitamin stock (for 1 litre)

Na Pantothenate	1g
Nicotinic Acid	10g
myo-inositol	10g
Biotin	10mg

E. Mineral stock (for 1 litre)

H_3BO_3	5g
$MnSO_4$	4g
$ZnSO_4.7H_2O$	4g
Biotin	10mg
KI	1g
CuSO4.5H2O	0.4g

$H_2MBO_4.H_2O$	0.4g
Citric Acid	10g
$FeCl_2.6H_2O$	2g

minutes. 260µl of 40% PEG/LiAc/TE (LiAc/TE + 40% PEG4000) was gently mixed with the cells and then cells were incubated at 30ºC (or 28ºC for temperature sensitive strains) for 30~60 minutes, then heat shocked at 42ºC for 5 minutes after addition of 43µl DMSO. Cells were washed once with SDW and finally resuspended in SDW and plated out. For selection of auxotrophic markers, cells were direct plated out on the EMM plate containing appropriate supplements. For antibiotics markers, cells were firstly plated out on YE3S plates; then after 12~24 hours incubation, replica plate onto YES + antibiotic plates.

2.1.2 Genetic cross and random spore analysis

Freshly growing h^+ and h^- cells were mixed on an EMMG3S (EMM + glucose + supplements – leucine, Adenine and Uracil) plate and incubated at 32ºC (26ºC for temperature sensitive strains) for 2~3 days until formation of asci. Then a loopful of the cross, 5µl of Helicase (Helix pomatia juice, Industrie Biologique Franis), and 1ml SDW were mixed, incubated overnight at 25-29°C or for at least 6 hours at 29°C. Cells were spun down afterwards, washed by 30% EtOH and gently sonicated to break up spore clumps, then plated out on YE3S plate after appropriate dilution (about 200~500 spores per plate). After about 3 days, when colonies appeared, the plate was replicated on to selective plates.

2.1.3 Making diploids by centrifugation

1ml log phase haploid cell culture was centrifuged in an Eppendorf tube for 15 min (13000 rpm) at room temperature, and then plated on to a YE3S/phloxin B plate after appropriate dilution. After about 3~4 days when the colonies appeared, the red colonies were picked and ploidy was checked by flow cytometry.

2.1.4 Details about construction of some strains

Primers used were listed in Table 2.1.

Plasmids padh1prom-TK-kanMX6/pFS255, expressing thymidine kinase from the *adh1* promoter, and padh1prom-hENT1-leu1/pFS181, expressing hENT1 also from the *adh1* promoter, were obtained from Addgene Inc (plasmids 12 382 and 12 536, respectively). *adh1-TK* was integrated into the *adh1* locus by linearizing with MfeI; integration of the plasmid at *adh1* was confirmed by colony PCR using the primers: 871 and 872. adh1-hENT1 was integrated into the *leu1-32* locus by linearizing with NruI; integration at *leu1* was confirmed by colony PCR using the primers 1039 and 827.

Plasmid pSMU-mes1pr-cdt1-myc/y825, expressing Cdt1 from the *mes1*

Table 2.1 Primers used in this study

No.	Sequence 5'-3'	Reference
692	CTAAGTAAGACATAAACTGATTAGTCGG	*mes1pr-cdt1*
802	TTAGCGGCCGCCTGTCGGCA	*mes1pr-cdt1*
626	CCTAAAGGGAGCCCCCGATTTAGAGC	*mes1pr-cdt1*
627	TGATTCCATACATTAAATGGATAAAACTCCTGAAGC	*mes1pr-cdt1*
630	CGTTACAAGATATCGATTTATCATCCCAAATGGTG	*mes1prcdc18*
673	GCATCTTCGAGGTGTATGACAACCTATTGGA	*mes1prcdc18*
726	ctattgatttttacactgtaaccatacgcgctttgcataattatctagacaatgcaaagaatgctgaacgcgtccagaaagtaaaaaaaGGAGGCCCAGAATACCCTCCTTGACAG	*rec12Δ*
727	tttatcgtaaaaattgaaatctagcaagtcatccatgttggaggctttattatgggagtgtagtgaaagtaggttgaactatccaagaacCACTGGATGGCGGCGTTAGTATCGAAT	*rec12Δ*
1025	GACTATTAATGAATCAAGCAAACACACTACATA	*rec12Δ*
1026	ACGGTGTCGGTGGTGAAGGACCC	*NAT insert*
644	aaaacatggtaaacaccgacaacaaggaaaatgagcctccaaacatggagaaggcccatatggattcgtccaatgctcttGGAGGCCCAGAATACCCTCCTTGACAG	*mes1Δ*
645	ggatatacaagtttcgagtacaaatgaagttaagaggtattacattcagtaagaagacttgatgaacggacgagaaacgcCACTGGATGGCGGCGTTAGTATCGAAT	*mes1Δ*
646	CTGTCAAGGAGGGTATTCTGGGCCT	*mes1Δ*
647	CTCAATTGTCAATGCATGTTTACTGAGTTGCC	*mes1Δ*
1027	acaaacaaactattaatgaaaattgtgcctcatctaacagcccgcactttaccaaacaaaaggcgactaaaataaaaattaaaacaggaaGGAGGCCCAGAATACCCTCCTTGACAG	*spo4Δ*
1028	aagagttaatcgatacaataaataaaactttgaatgatattatggaattccgacgtgtggaaatttctccatctttggcagatacacgtcCACTGGATGGCGGCGTTAGTATCGAAT	*spo4Δ*
1029	CTAGCCCATCGAGAATCTTAAACAAAAGTCAGTT	*spo4Δ*

1030	ccagacaaatttgactgctttaaacgtcacaaagccgagtagcaaacgctcccacaaccaaacactacacaaaggttcgtcgacaGGAGGCCCAGAATACCCTCCTTGACAG	*spd1⊿*
1031	tcaacaaagtcaaagactaatgaacgaaggaataattggaactatgaggattggtgtcaaattatccttaaagaagagatatacgCACTGGATGGCGGCGTTAGTATCGAAT	*spd1⊿*
1032	AACGAATATTTTGAGTGAGGCAGTCAGCGTG	*spd1⊿*
1033	CATTCGGGCCCTTGATCAACCACTAAAACAACAGCT	*dfp1M*
1034	CGGGAGTACTATATTCATGTGGTACAGCCACCTTACT	*dfp1M*
1035	ACCACATGAATATAGTACTCCCGAAACATGATGCCG	*dfp1M*
1036	TCCAGGGATCCCGGTGTTGGATATATCAG	*dfp1M*
1050	CAAGGCGAGCTGATAATTCGCATCTTAAG	*dfp1M*
1051	CGAGGGCAAAGGAATAATCAGTACTGAC	*dfp1M*
1052	TGGCAGTGTTCCTGCGCCGGTT	*cdc2as*
1053	GGCGGTGGTGTATTGCCTCATATCAAT	*cdc2as*
1037	tttatttttcttttaaataaaaaaaaatgttttgttttctttgcgagcaggtggagaagtctttgacagtttttacaatttcacatttatc	*cdc2as*
1038	caaaggataagagtaagtaagaaaaagggagattaaaaggcttcagttttatcaggaatgaaagaaaaatagatttaaacactcagagccAAGCCGGCGAGCTCGTTTAAACTGGAT	*cdc2as*
871	CTACGACAAAAGAAGGCTAAAGGAATAGG	*TK*
872	GCGGTCGAAGATGAGGGTGAGG	*TK*
1039	CAGACAAGCCCGTCAGGGCGCG	*hENT1*
827	CAAAATACTTGCGAACCATCAAAGTGGAAAGG	*hENT1*
826	ATTCGATACTAACGCCGCCATCCAG	*hENT1*
1040	gccaagcgcgtaatatgttggaatgtaaacaaacataaacaaacccattctcagcttctatttctaccactatacatattTCTTCCCTTCCTTTCTCGCCACGTTCG	*mde3⊿*
1041	gaaataacttcagatggaaacatgaatgccgataaaataattagtgtccaagtttctcttcatttgcattttactttcacAAGCCGGCGAGCTCGTTTAAACTGGAT	*mde3⊿*
1042	TGCGACCAAAGGCAGCAAGAGAGAT	*mde3⊿*
1043	TGCGACCAAAGGCAGCAAGAGAGAT	*mde3⊿*

promoter, was integrated into the *ura4-294* or *cdt1* locus by linearizing with Pme1 or HpaI. Integration of the plasmid at *ura4-294* was confirmed by colony PCR using the primers 692 and 802; Integration of the plasmid at the *cdt1* locus was confirmed by colony PCR using the primers 626 and 627.

Plasmid pSMH-mes1pr-cdc18-TAP/y757, expressingCdc18 also from the *mes1* promoter, was integrated into the *mes1*-promoter locus by linearizing with HindIII; integration at *mes1* was confirmed by colony PCR using the primers 630 and 673.

To construct the deletion mutant of *dfp1*, first two partially complementary fragments were amplified by PCR, using primers containing the mutation (primers 1033, 1034, 1035 and 1036, see Table 2.1). Then the two fragments were annealed on the overlapping ends; the full length fragment with the desired deletion was produced by PCR (Fig. 2.1). This fragment was cloned into a pSMH vector. The resulting Plasmid pSMH-dfp1pr-dfpM/y991, containing the *dfp1* promoter (1000 kb sequence upstream of the *dfp1* gene) linked to the N-terminal half of the *dfp1* gene with a deletion corresponding to the second to twelfth amino acids, was integrated into the *dfp1* locus by linearizing with SpeI. Integration at *dfp1* was confirmed by colony PCR using the primers 1050 and 1051. Introduction of the mutation was confirmed by sequencing from the integrated gene after colony PCR.

A

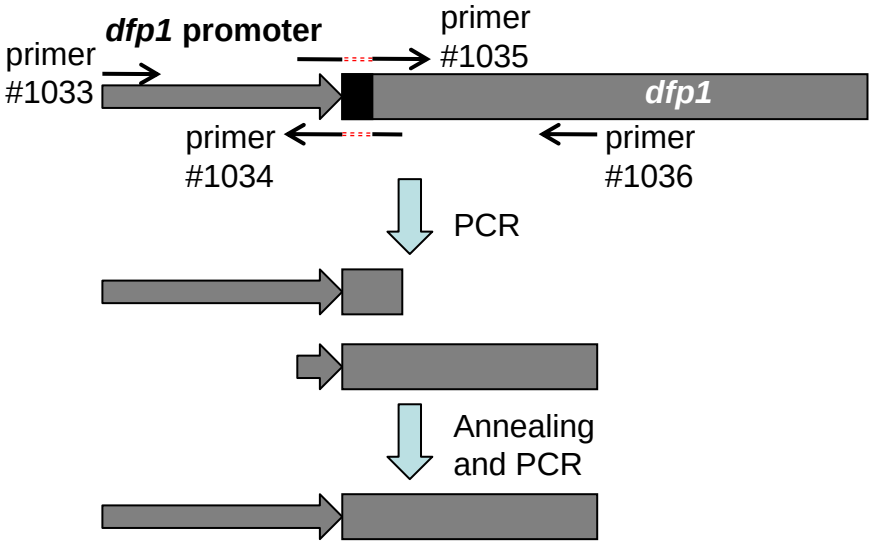


Figure 2.1 A simple illustration of how the deletion mutation of *dfp1* is introduced. Black box: area required to be deleted, including the N-terminal APC destruction box.

Deletion of a gene (*mde3Δ*, *mes1Δ*, *rec12Δ*, *spd1Δ*, *spo4Δ*) was done by the homologous recombination, and deletions were confirmed by colony PCR. Primers used were listed in Table 2.1.

The original plasmid containing *cdc-as* was obtained from J. Gregan (Cipak *et al*., 2011). The *cdc-as* gene was amplified from the original plasmid, and cloned into another plasmid, where the *cdc2-as* was put next to a *kanMX6* marker. The *cdc2as-kanMX6* fragment was amplified from this resulting plasmid pSMR-cdc2as/y992 using long oligos 1037 and 1038. Thus sequences homologous to upstream or downstream of the *cdc2* gene were added the ends of the *cdc2as-kanMX6* fragment, respectively. Then the genomic *cdc2-wt* gene was replaced by *cdc2-as* via homologuous recombination. Mutants were selected on G418 plates, and constructs was confirmed by colony PCR using primers 1052 and 1053. Expression of *cdc2-as* and deletion of *cdc2* (wt) was also confirmed by growth sensitivity in YE3S medium containing 1uM 1-NM-PP1.

2.2 Plasmid cloning

2.2.1 Plasmid construction

Standard PCR programme (an initial denaturation at 94°C for 5 minutes, then 30 cycles of (94°C for 10 seconds, 55°C for 30 seconds, and 68°C for 6 minutes) and an indefinite hold at 4°C) or High-Fidelity Polymerase PCR programme (an initial denaturation at 97°C for 2.5 minutes, then 30 cycles of (97°C for 20 seconds,55°C for 30 seconds, and 72°C for 6 minutes) and an indefinite hold at 4°C) was used to amplify target genes/ DNA fragments, with *Taq* polymerase or Phusion® High-Fidelity DNA Polymerase (NEB) as required. Primers used in this study are listed in Table 2.1. PCR products were purified using QIAquick® PCR purification kit (QIAGEN), then digested with the appropriate restriction endonuclease. The digested DNA fragments and plasmid backbones were cleaned up using the QIAquick® gel extraction kit (QIAGEN) after running in 1% TAE gel and visualized by SYBR Gold® staining (Invitrogen). Ligation was catalysed by T4 ligase (NEB) at 16ºC overnight or 23ºC for 2 hours.

2.2.2 Transforming *E.coli* cells

Escherichia coli XL-blue strain was used for cloning of plasmids. Competent *E. coli* cells were thawed on ice and then mixed gently with 20µl of ligation products. The tube was left on ice for 30 minutes. Afterwards, cells were heat shocked at 42ºC for 90 seconds, and then returned to ice and incubated for 2 minutes. 0.5ml LB medium (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl)

was added and cells were cultured at 37ºC with shaking for 45 minutes, then plated on LB plates containing the appropriate antibiotic and incubated at 37ºC overnight.

2.2.3 Plasmids preparation from *E.coli* cells

For Minipreps, 2ml saturated *E. coli* cultures was spun down and resuspended in 300µl TENS (10mM Tris, 1mM EDTA, pH 8.0, 0.5%SDS, 0.1M NaOH), then mixed with 150µl 3M sodium acetate (pH 5.4), and spun again for 2 minutes. 500µl supernatant was taken off and mixed with 0.9ml 90% EtOH. Then the mixture was spun down, and the supernatant was discarded. The pellet was washed by 70% EtOH and allowed to dry, finally resuspended in 20µl TE (10 mM Tris, 1 mM EDTA, pH 8.0) containing 20µg/ml RNaseA. PureYield™ plasmid midiprep kit (Promega) was used for midipreps, following the manufacturer's instructions.

2.3 Induction of synchronized meiosis by Pat1 inactivation

pat1-114 cells were first cultured in EMM or YE3S medium at 26°C to log phase, and then washed twice by SDW, resuspended in EMM-N medium (Table 2.3) to OD=0.25, and incubated at 26°C for 16 hours. Then the

G1-arrested cells were spun down, and transferred to EMM medium and cultured at 34°C to inactivate Pat1 (Fig. 2.2).

2.4 Protein extraction and immunoblotting

2.4.1 TCA extraction

TCA extraction was done as described previously (Ralph *et al*., 2006). Basically, 10OD cells were spun down, resuspended in 1ml 1.2M sorbitol, and then spun down again. 200µl 20% TCA and 0.5ml glass beads were added to the tube containing the cell pellet and mixed. The mixture was vortexed at 4ºC for 6 minutes. Then 900µl 5% TCA was added and the tube was vortexed briefly. 800µl of the extract was removed to a fresh Eppendorf tube and centrifuged for 10 minutes at low speed (3000 rpm). The supernatant was discarded and 200µl 1X Laemmli loading buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.025% bromphenol blue, 62.5 mM Tris-HCl, pH6.8) was added to the pellet, vortexed and boiled for 3 minutes. Finally the extractions were centrifuged again and the supernatant containing proteins was removed to a fresh tube, which was kept at -20ºC.

2.4.2 Alkaline extraction

A.

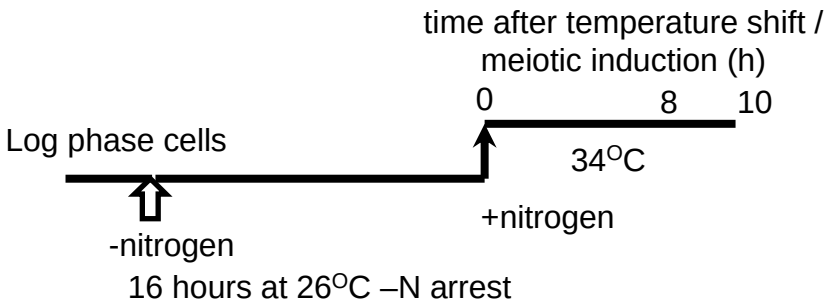


Figure 2.2. Experiment scheme illustrating how synchronized meiosis is achieved with a *pat1-114* mutant. Cells were arrested in G1 by growing in EMM-N for 16 hours at 26ºC, then released from the cell cycle block and induced to undergo meiosis in EMM+N medium at 34ºC. Samples were taken every one or two hours after meiotic induction for analysis.

Alkaline protein extraction was carried out as previously described, as an alternative method for TCA extraction (Matsuo *et al*. 2006). 5OD cells were spun down and washed in 1ml SDW. Afterwards, cells were resuspended in 0.3ml SDW. Then 0.3ml of 0.6 M NaOH was added to the tube, vortexed briefly. The tube was left at room temperature for 10 minutes, and then centrifuged to discard the supernatant. 70ul SDS sample buffer (60 mM Tris-HCl pH 6.8, 4% beta-mercaptoethanol, 4% SDS, 0.01% bromophenol blue, 5% glycerol) was mixed with the pellets. This was boiled for 3 minutes, spun down again, and the supernatant was ready for SDS-PAGE.

2.4.3 Western Blotting

SDS-PAGE was conducted to separate the proteins following the standard procedure (Sambrook and Russell 2001). Western blotting (semi-dry blotting) was carried out as previously described (Sambrook and Russell 2001), using a Immobilon-P membrane (Millipore) with semi-dry transfer buffer (25 mM Tris, 250 mM glycine, 15% methanol) in a Bio-Rad blotter. The settings were as follows: 250mA (<16V); 45 minutes. The membrane was then blocked in 1XPBS containing 5% semi-skimmed milk before hybridizing to the antibodies. Finally images were collected by using the AlphaImager HP System, following the enhanced chemiluminescence procedure (Pierce).

2.4.4 Antibodies used

TAP-tagged Cdc18 was detected with peroxidase–anti-peroxidase–soluble complex (P1291, Sigma). Cdc18 was detected with anti-Cdc18 antibody (a gift from O. Harris/J. Hayles). Cdt1-myc was detected using anti-myc antibody (M5546, Sigma). Proteins tagged with YFP/CFP/GFP were detected using anti-GFP antibody (11814460001, Roche). FLAG-tagged Sld2 and Sld3 were detected with anti-FLAG antibody (F3165, Sigma). α-tubulin was used as loading control and detected with antibody T5168 (Sigma).

2.5 Detection of DNA content by flow cytometry

Cells (about 2×10^6) were fixed in 200µl ice-cold 70% ethanol and then hydrated by adding 4.8ml 10mM EDTA (pH 8.0), cells were spun down and 0.5ml 0.1 mg/ml RNaseA in 10mM EDTA (pH 8.0) was added. Cells were incubated for at least 2h at 37ºC to eliminate RNA. Cells were stained with 1µ M SYTOX Green, sonicated for 30s (setting 6 of 20) using a Soniprep 150 (SANYO) to break up the cell clumps and analysed using a Coulter Epics XL-MCL.

2.6 Basic microscopy techniques

2.6.1 Fixation of cells

Cells were fixed in ice-cold 70% ethanol or methanol/acetone. For the methanol/acetone fixation, cultures were first spun down, and resuspended in 100% cold methanol. Cells were put on ice for 15 minutes and then spun down. Cell pellets were resuspended in 100% cold acetone and then stored at -20ºC.

2.6.2 Fluorescence Microscopy

The fixed cells were concentrated to 10^9/ml in acetone and then spread on slide. Cells were mounted in 0.6% low melting point agarose containing 50ng/ml DAPI (to stain DNA) or 50µg/ml methyl blue (to stain septa) as required. Slides were analyzed using a Zeiss Axioskop microscope coupled to a Hamamatsu ORCA ER camera. The cells were first examined by phase microscopy, and then by fluorescence microscopy. Open source µManager software (Edelstein *et al*. 2010) was used to control the camera and microscope.

2.7 Pulsed field gels electrophoresis

2.7.1 Preparation of agarose plugs

25OD of yeast cells were spun down, washed in 10ml ice-cold 50mM EDTA (pH 8.0). Then the cell pellets were resuspended in 200µl cell suspension buffer (10mM Tris pH 7.2, 20mM NaCl, 50mM EDTA pH8.0) containing 2mg/ml Zymolyase 20T and mixed gently but thoroughly with an equal volume of suspension buffer containing 2% low-melting agarose. The mixture was transferred the plug molds (100µl/well) and the agarose was allowed to solidify. Afterwards the plugs were pushed into 5ml lyticase buffer (10mM Tris pH 7.2, 50mM EDTA pH8.0) containing 1mg/ml Zymolyase 20T, and incubated at 37ºC for 3 hours. Then the plugs were washed once with 25ml wash buffer (20 mM Tris pH 8.0, 50 mM EDTA pH 8.0), and then transferred into the proteinase K buffer (100 mM EDTA pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine) containing 1mg/ml proteinase K, incubated at 55ºC for 48 hours, with one change of fresh enzyme solution. Finally the plugs were washed three times with 25ml wash buffer (10mM Tris-HCl, 50mM EDTA, pH8.0), each for 20~30 minutes at room temperature. Then they were stored in wash buffer at 4ºC.

2.7.2 PFGE

Pulsed field gel electrophoresis (PFGE) was carried out as previously described (Kearsey *et al*. 2007), using a 0.8% chromosomal grade agarose gel in TAE buffer (40mM Tris-acetate, 2mM EDTA, pH 8.3) in a CHEF III apparatus

(Bio-Rad, Hercules, CA, USA). The settings were as follows: 2 V/cm; switch time, 30 min; angle, 106º; 14ºC; 48 hours. To visualize DNA, the gel was stained in TAE buffer containing 0.5μg/ml ethidium bromide for 30 minutes at room temperature, before collection of images using the AlphaImager HP System.

2.8 DNA combing

2.8.1 Preparation of agarose plugs for DNA combing

This step was similar to that described in 2.8.1 with some modifications (Patel *et al*. 2006). 10OD cells were spun down and washed in 10ml ice-cold 50mM EDTA (pH 8.0). Then the cell pellets were resuspended in 200 μl SB buffer (20 mM citrate phosphate, 50 mM EDTA pH8.0, 900 mM sorbitol) and mixed with 100μl SB buffer containing 1.5% low-melting agarose. The mixture was transferred the plug molds (100μl/well) as described in 2.8.1. Afterwards the plugs were pushed into 5ml SB buffer containing 2 mg/ml Zymolase-20T and 2 mg/ml lysing enzyme, and incubated at 37ºC for 4 hours. Then the plugs were washed once by 25ml wash buffer (20 mM Tris pH 8.0, 50 mM EDTA pH 8.0), and then transferred into DB buffer (50 mM EDTA, pH 8.0, 1% SDS,) containing 0.5mg/ml proteinase K, incubated at 50ºC for 48 hours, with two changes of fresh buffer and enzymes. Finally the plugs were washed three

times with 25ml wash buffer, each for 20~30 minutes at room temperature, then stored in 0.1M EDTA (pH8.0), at 4ºC.

2.8.2 DNA combing

DNA combing was done in Marcel Mechali's lab with help from Dr. Olivier Garnier (IGH, CNRS, France), following the previously described protocol (Michalet *et al*. 1997) with some modifications. A plug was washed with 10ml TE at room temperature with gentle agitation. Then it was soaked in 100µl TE with 1.5ul YOYO (Invitrogen), incubated at room temperature for 1 hour with gentle agitation and protected from light, and then washed 3X5 minutes with 10ml TE. The plug was then soaked in 100µl buffer for beta-agarase (NEB) and incubated at 67ºC for 15mins or longer, until the plug was totally dissolved. Then temperature was reduced to 42ºC and beta-agarase (3 units per 100ul) was added and gently mixed, and incubated overnight. The next day, 5ml of 500mM MES (pH5.7) was added to the tube, which was then incubated at 65ºC for 10 minutes. Then DNA solution was carefully transferred to a 2 ml Teflon reservoir, in which the cover slide was firstly soaked in for 5 minutes, and then pulled up slowly with a constant speed. This process was repeated once. Density and integrity of DNA fibers were checked by YOYO staining under fluorescence microscopy. The cover slip was baked at 60ºC for 1 to 2 hours and stuck to a microscope slide with glue. It was then dipped

successively in 70%, 90% and 100% EtOH, each for 5 minutes. The slide was allowed dry, and then soaked in 1M NaOH for 25 minutes, then transfer in 1XPBS for 1 minutes to neutralize NaOH. Slide was then washed 5 times in PBS – each for 3 minutes, and then blocked in PBS + 0.1% triton + 1% BSA for 30 minutes before hybridizing with the antibodies. 18 µl of PBS/T (PBS + 0.1% triton) containing primary antibodies was added to the coverslip and then the coverslip was covered with another coverslip, incubated overnight at room temperature in a humid chamber. The next day, the slides were washed 5 x 2 minutes times with PBS/T. Then secondary antibodies were added and slides were incubated at 37°C in humid chamber for 1 hour. Afterwards, the slides were washed 5 times with PBS/T – each for 3 minutes. The slide was allowed dry, and finally mounted with 20 µl of Prolong Gold Antifade reagent (Molecular Probes). The mounting reagent was allowed to polymerize for 2 hours at room temperature before proceeding with microscopy.

Primary antibodies used were rat anti-BrdU (Sera Lab, clone BU1/75) and mouse anti-DNA (Chemicon MAB3034). Secondary antibodies used were goat anti-rat Alexa 488 (Molecular Probes, A11006) and goat anti-mouse Alexa 546 (Molecular Probes, A11030).

2.8.3 Measuring fibers

After visualization of DNA fibers by antibody hybridization, images were collected using a Zeiss Axioplan microscope, as in section 2.6.2. The μ Manager software with ImageJ was used to measure fiber length. The fibres were measured to determine replication track lengths (10 μm= 20kb).For the BrdU positive fibers, only the ones with DNA staining 2.5 μm longer than the BrdU patch at either ends respectively were measured. To avoid noise, only signals longer than 2.5 μm are taken into account as real incorporation patches.

2.9 Chromatin binding assay

Chromatin binding assay was performed following the previously described protocol (Kearsey *et al*. 2005). Basically, cells were first washed with 1.8ml EMM sorb + DTT buffer (15 mM KH phallate, 15 mM Na_2HPO_4, 90 mM NH_4Cl, 1.2M sorbitol, pH 7.0, 10 mM DTT) at 4ºC, and then resuspended in 450μl EMM sorb +DTT buffer. 50μl of 20mg/ml Zymolyase 20-T in EMM sorb buffer was added, allowing digestion at 32~34°C for 10~20 minutes or longer, until cells became dark under phase microscope when mixed with 1% SDS. Then cells were washed twice with ice-cold EMM sorb buffer, then washed with 2 ml extraction buffer (20 mM Pipes-KOH pH 6.8, 0.4 M Sorbitol, 1 mM EDTA, 150 mM KAc) and finally resuspended in 0.9 ml extraction buffer containing protease inhibitor cocktail (Roche). The mixture was then split into two: 50μl

extraction buffer containing 10% Triton X-100 was added to one half, remaining 0.45 ml serves as a –Triton control. Both tubes was mixed by inverting several times and then incubated at 20°C for 5 min, with periodical inverting. Finally both samples were spun down, supernatant was discarded and cells were fixed following the methanol/acetone procedure described in section 2.6.1.

2.10 EdU incorporation and detection

Cells were grown in YE3S or EMM medium containing EdU at the indicated concentration; EdU was added from a 10mM stock in water. Following incorporation, cells were fixed by resuspending in 70% ethanol and washed in TBS (50mM Tris, pH 7.4, 150mM NaCl) twice. Approximately $2X10^6$ cells were resuspended in a freshly prepared cocktail containing 50mM Tris, pH 7.4, 150mM NaCl, 2mM CuSO4, 10mM Alexa Fluor 488 azide (added from 2mM stock in DMSO, Invitrogen), 10mM sodium ascorbate (added last from 0.1M stock) and incubated in the dark for 30 minutes. Cells were washed in TBS twice, finally resuspending in 0.5ml TBS. For detection of fluorescence by flow cytometry, cells were transferred to 1.5 ml Eppendorf tubes and sonicated for 20 seconds in a water bath (Diagenode Biorupter, power setting set to low/130 W) to break up the cell doublets before analysis using a Coulter Epics XL-MCL. For analysis by fluorescence microscopy, cells were concentrated and

mounted by mixing with an equal volume of 1.2% low melting temperature agarose, containing 50ng/ml DAPI. Images were collected using the same microscope in section 2.6.2. Working concentration of EdU in all meiosis experiment is 10μM.

2.11 Determination of cell concentrations and viabilities

Cells were fixed in 80% formyl saline (0.9% NaCl, 3.7% formaldehyde) and cell concentrations were determined using a Sysmex F-820 Cell Counter (Kobe, Japan). To determine viabilities, cells were gently sonicated and around 100~1000 cells were plated out on YE3S plates, and then incubated for several days at appropriate temperature until appearance of colonies. Cell viabilities were determined by counting the numbers of colonies. To determine spore viabilities after meiosis, asci were spun down and washed by 1XSDW, then resuspended in 1ml SDW containing 15μl of Helicase, incubated at 29ºC for several hours until spores are released from the asci. Spores were washed twice with 30% EtOH to kill any remaining live vegetative cells and gently sonicated. Spore concentration was determined by using a haemocytometer and then approximately 100~1000 spores were plated out on a YE3S plate.

Chapter 3

Monitoring DNA replication in fission yeast by incorporation of EdU

3.1 Introduction

Monitoring the process of DNA synthesis can not only provide important information about this critical biological process per se, but also give clues to cell cycle regulation, cellular responses to exogenous treatments, DNA repair and DNA recombination (Sivakumar *et al*. 2004, Hodson *et al*. 2003, Sabatinos and Forsburg 2009, Terasawa *et al*. 2007, Green *et al*. 2009, Rhind 2009). A commonly used approach is to stain nucleic acids with fluorescent dyes such as SYTOX Green, Hoechst or Propidium Iodide (PI), and then distinguish cell populations with different DNA contents by flow cytometry. However, since these methods measure total DNA content, it is difficult to detect subtle changes or disturbances during DNA synthesis by this method. Directly labelling the nascent DNA chain, i.e. monitoring incorporation of deoxyribonucleotides into the newly synthesized DNA strand, is more sensitive. Currently, the most commonly used substrates are [^{3}H] thymidine or thymidine analogues, e.g. halogenated nucleotides.

Historically, attempts to use [^{3}H] thymidine labelling DNA date back to half a century ago (Okazaki *et al*. 1959, Sirlin 1958). Incorporated [^{3}H]-thymidine is usually quantitated by scintillation counting after precipitating labelled DNA by using acid (TCA) to separate it from the unincorporated nucleotides (Borjeson *et al*. 1966, Hunt and Foote 1967). If the sites of incorporation are required to be visualized, [^{3}H] thymidine is detectable by microscopic autoradiography. Using halogenated nucleotides, e.g. bromodeoxyuridine (BrdU), chlorodeoxyuridine (CldU), and iododeoxyuridine (IdU), is safer and suits both flow cytometry and fluorescent microscopy. However, use of BrdU and related nucleosides presents some technical difficulties, in that antibodies are required to detect incorporation, though using antibodies can increase the sensitivity due to “two steps” (use of first and secondary antibodies) amplification. Because the antibody is big, it is not accessible to the interior of DNA helix. So DNA has to be converted to a single-stranded form and permeabilization of fixed cells is required. The process of DNA denaturation, which can be achieved by acid (HCL) or alkaline (NaOH) treatment, destroys the natural structure of DNA double helix and also other cellular structures (Hodson *et al*. 2003, Lengronne *et al*. 2001). Though there are other ways converting dsDNA to ssDNA, e.g. using endonucleases to cut DNA and then convert dsDNA to ssDNA with exonuclease, which can preserve most cellular structure, detection of BrdU largely depends on the efficiency of exposing the epitope (in DNA) to

enable its accessibility to the large antibodies. In addition, the immune-reaction steps are time-consuming.

Recently, the use of 5-ethynyl-2'-deoxyuridine (EdU) has been described to detect DNA replication (Salic and Mitchison 2008). This thymidine analogue contains an alkyne group which can be conjugated to a fluorescently-labelled azide in a Cu(I)-catalyzed Huisgen cycloaddition reaction – also named 'click' reaction because of the fast reaction rate (Rostovtsev *et al*. 2002, Tornøe *et al*. 2002). One advantage of this method is that incorporation can be detected in double-stranded DNA. Also, as the fluorescent label is a small permeable molecule (~0.6kD vs. 150kD of an antibody), access to the DNA is less of a problem than when using antibodies (Salic and Mitchison 2008). As there is no need to melt DNA, fine cellular structures can be preserved. Also, the nature of the click reaction gives a good signal to noise ratio (Buck *et al*. 2008).

The protocol of using EdU as a molecular probe has been developed in cultured mammalian cells, and tissue sections (Salic and Mitchison 2008, Buck *et al*. 2008, Chehrehasa *et al*. 2009), but had not been described for use in fission yeast. Some specific characteristics of fission yeast, e.g. lacking thymidine salvage pathway, requires a modification of strains to allow EdU labelling of DNA. In this Chapter, I describe a simple procedure to enable labelling of fission yeast DNA with EdU and detection by flow cytometry and

fluorescence microscopy, and also describe some problems with using EdU. Data in this Chapter have also been published elsewhere (Hua and Kearsey 2011)

3.2 Results

3.2.1 Strain modifications necessary for EdU incorporation

Efficient incorporation of BrdU into fission yeast requires expression of herpes simplex thymidine kinase (TK), which phosphorylates thymidine to dTMP, as fission yeast lacks the thymidine salvage pathway (Sivakumar *et al*. 2004, Hodson *et al*. 2003). Although moderately high levels of BrdU incorporation can be achieved by expression of TK alone, labelling is more efficient if a nucleoside transporter (human equilibrative nucleoside transporter 1 - hENT1) is expressed as well (Gómez and Antequera 1999, Hodson *et al*. 2003). Construction of strains expressing TK and hENT1 was described in section 2.1.4. Detailed procedures on how to fix the cells and do the click reaction was described in 2.10.

With medium containing 10 μM EdU, after 3 hours of labelling at 32ºC, incorporation is readily detectable by flow cytometry and fluorescence microscopy with an *adh1-TK adh1-hENT1* strain (Fig. 3.1 A, and Fig. 3.2 B),

whereas no incorporation was detectable when TK was expressed alone (Fig. 3.1 A, and Fig. 3.2 A). Only when EdU was used at a considerably higher concentration (100-300 μM) was incorporation detectable with an *adh1-TK* strain (Fig. 3.1 B, and Fig. 3.2 C). Using the *adh1-TK adh1-hENT1* strain, higher concentrations of EdU did not result in an increased fluorescence signal by flow cytometry, and the concentration could be lowered to 1 μM with little reduction in sensitivity (Fig. 3.1 C).

To study EdU incorporation in meiotic cells, *pat1-114* cells expressing TK and hENT1 were induced into meiosis following the protocol described in section 2.3. EdU was added to the culture at the time of meiotic induction in different concentrations (Fig. 3.3 A for experimental scheme). Incorporation of EdU during pre-meiotic S phase confirms that 1μM EdU concentration can also be used for monitoring meiotic replication and 10μM gives only a slightly higher signal (Fig. 3.3 B). The background peak on the most left (circled on the image) of the histogram representing samples labelled with 0.2, 1 and 10μM EdU should be due to the “G2 population” after nitrogen starvation (Mochida and Yanagida 2006), which will be discussed in details in section 3.2.3. This experiment suggests that when using EdU to monitor DNA synthesis in meiosis, a concentration between 1μM to 10μM should be used.

3.2.2 Effects of EdU incorporation on checkpoint activation

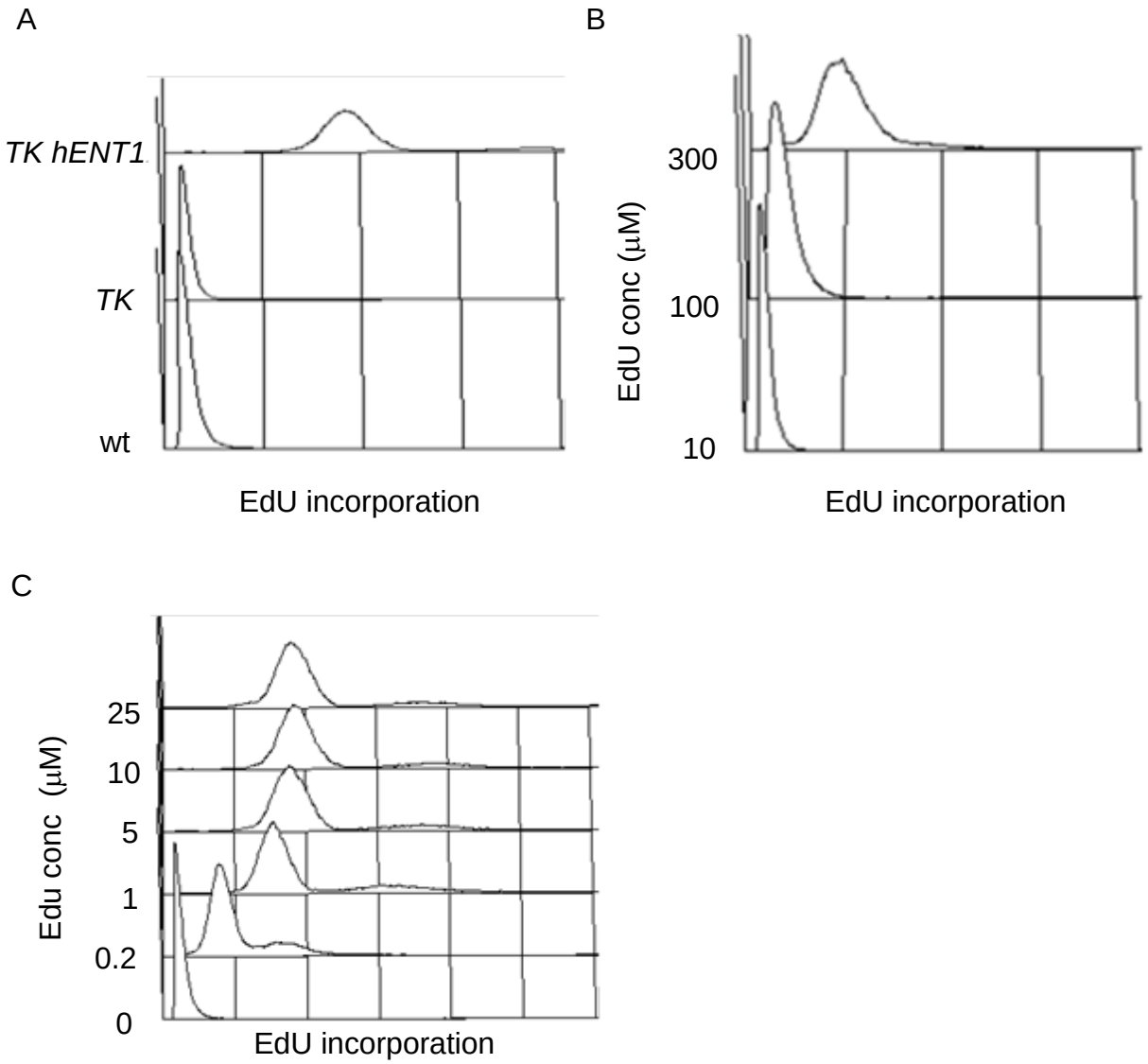


Figure 3.1 Flow cytometry analysis of EdU incorporation in fission yeast strains expressing TK and hENT1. **(A)** Wild-type (P2), TK (*adh1:tk*, P2471) and TK hENT1 (*adh1:tk adh1:hENT1*, P2470) expressing strains were grown in YE3S containing 10 μM EdU for 3 h then fixed and conjugated to Alexa Fluor 488 azide before analysis by flow cytometry, **(B)** *adh1:tk* (P2471) cells were grown in YE3S containing the indicated concentrations of EdU for 3 h then analysed as in (A), **(C)** *adh1:tk adh1:hENT1* (P2470) cells were grown in YE3S containing the indicated concentrations of EdU for 3 h then processed as in (A) and analysed by flow cytometry.
Linear x-axes are used to show EdU incorporation in all panels in this figure.

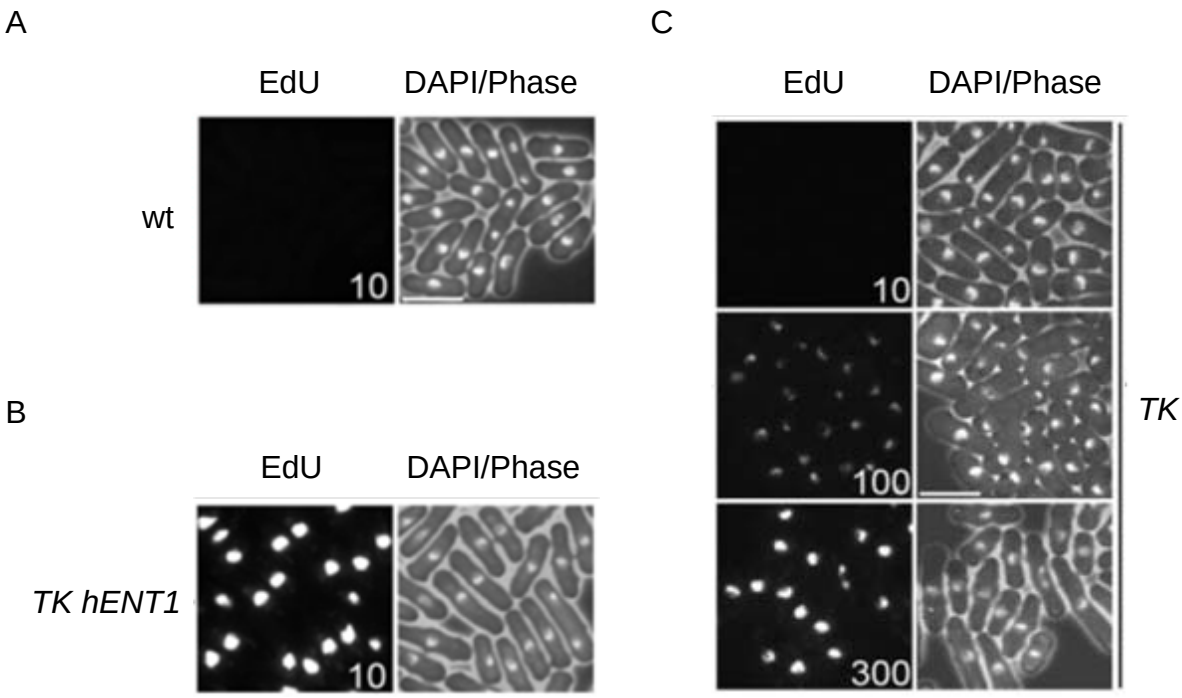
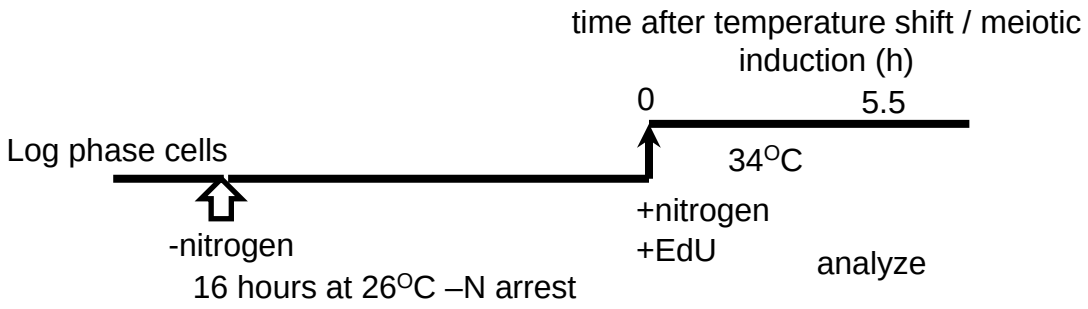


Figure 3.2 Fluorescent microscopy analysis of EdU incorporation in fission yeast strains expressing TK and hENT1. Cells from Fig. 3.1 - (A) and (B) were imaged. The numbers marked on the “EdU” images are concentrations of EdU used (unit: μM). Bar=10 μm.
(A) Wild-type cells (P2), **(B)** *TK hENT1* cells (P2470), and **(C)** *TK* cells (P2471).

A



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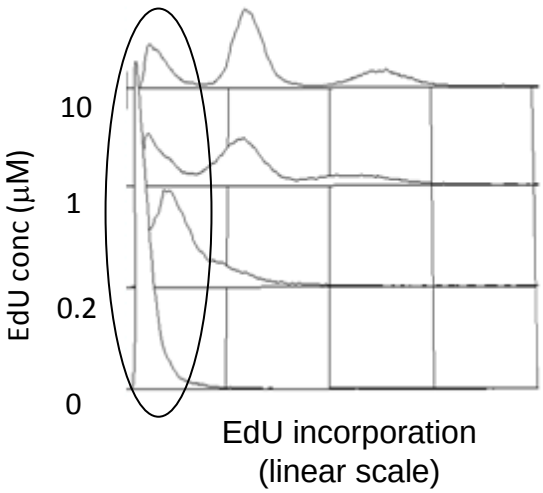


Figure 3.3 Labeling DNA replication during pre-meiotic S phase by EdU incorporation.
(A) Experiment scheme. *adh1:tk adh1:hENT1 pat1-114* (p3007) cells were released from nitrogen starvation / G1 block into medium containing nitrogen and the indicated concentrations of EdU for 5.5 hours and then processed as in Fig. 3.1 (A) and analyzed by flow cytometry. Meiosis was induced by increasing temperature to 34ºC at the time of release.
(B) Flow cytometric histograms of cells from (A).

and viability

To determine whether growth in EdU has an effect on viability, dilutions of cells were spotted onto plates containing different concentrations of the nucleoside (Fig. 3.4 A); for comparison, strains were also spotted onto plates containing BrdU (Fig. 3.4 B) or thymidine (Fig. 3.4 C). Wild-type cells, or cells expressing *adh1-TK* alone were not sensitive to EdU up to 25 mM, while cells expressing *adh1-TK adh1-hENT1* showed reduced growth at 1 μM EdU. The sensitivity of *adh1-TK adh1-hENT1* cells was exacerbated in a *rad3Δ* background, as growth was severely affected at a concentration of just 0.1 μM (Fig. 3.4 A). This suggests that EdU incorporation leads to DNA damage and a functional *rad3*-dependent checkpoint mechanism is required for viability. In contrast to EdU, the *rad3Δ* strain is not particularly sensitive to BrdU, at 0.1 μM, although at 1 μM there is a slight difference in growth compared to the *rad3+* strain and higher concentrations are similarly toxic to both *rad3Δ* and *rad3+* incorporating strains (Fig. 3.4 B). Sivakumar *et al*. have noted that high concentrations or BrdU are toxic without causing cell elongation, which they speculate is due to disturbance of major groove-protein interactions (Sivakumar *et al*. 2004). High concentrations of thymidine are also toxic to incorporating strains, as growth of the *TK hENT1* cells was somewhat affected at the concentration of 10μM and 25μM (Fig. 3.4 C), but toxicity of exogenous thymidine, which is presumably due to disturbance of cellular nucleotide pools, is less than either BrdU or EdU

(Gómez and Antequera 1999).

Consistent with these observations, *adh1-TK adh1-hENT1 rad3*$^{+}$ cells became largely elongated when grown in 1 μM EdU for 5 hours (Fig. 3.5 A). *Rad3*$^{+}$ cells released from a G1 block into EMM medium containing EdU and culturing at 32ºC (Fig. 3.5 B) were also elongated, and failed to carry out mitosis and cell division, as reflected by the low percentage of binucleated cells at 10h after release from the nitrogen starvation (Fig 3.5 C – upper panel). The block to mitotic entry was relieved in a *rad3Δ* mutant. When percentage of binucleated cells was determined, for the *rad3*$^{+}$ strain, this percentage was much less after a synchronized S phase when EdU was added before S phase, compared with the no EdU control. In contrast, whilst the *rad3Δ* cells were not affected too much by the EdU treatment (Fig. 3.6 A, left panel). However, although *rad3Δ* cells grown in EdU execute mitosis and form septa, a high proportion of cells fail to carry out later stages of cytokinesis and arrest as septated binuclear cells, with occurrence of abnormal septations ("cut phenotype") and fragmentized nuclei, indicating the defective mitosis (Fig. 3.5 C – lower panel, and D). Arrest of cell division when EdU is present is also apparent from analysis of cell numbers after release from a G1 block (Fig 3.6 B). Cells also lost viability (lowered relative survival) when they were released from G1 block into a synchronized S phase, grown in EdU for different period and then plated out on the YE3S plates without EdU (see section 2.11 for methodology).

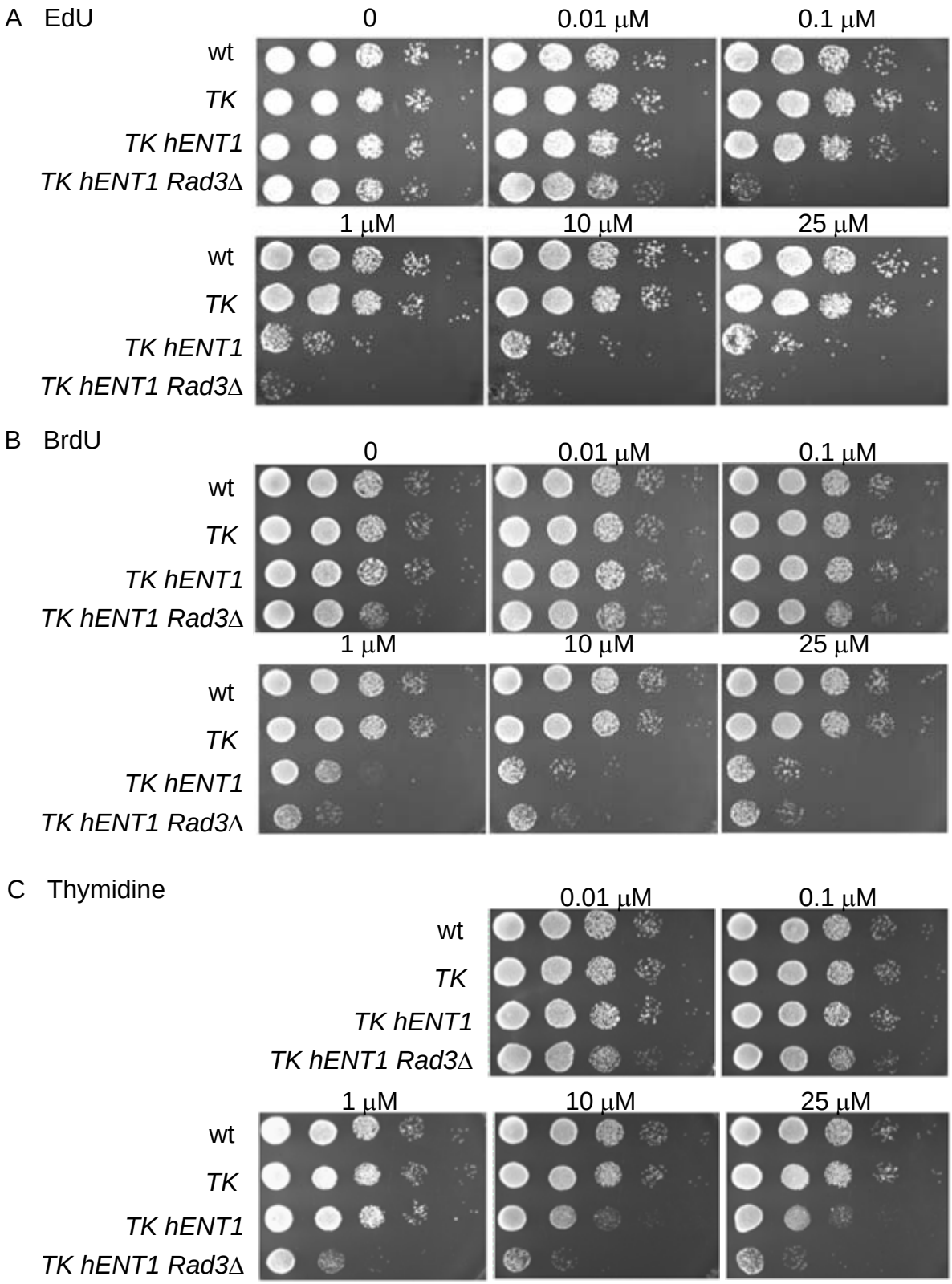


Figure 3.4 Effects of EdU incorporation on viability and cell cycle progression. **(A)** Serial dilutions (10-fold) of wild-type (P2), TK (*adh1:tk*, P2471), TK hENT1 (*adh1:tk adh1:hENT1*,P2470) and TK hENT *rad3Δ* (*adh1:tk adh1:hENT1 rad3Δ*, P2472) cells were spotted on to YE3S plates containing EdU at the indicated concentrations, **(B)** As (A) but cells were spotted onto YE3S plates containing BrdU at the indicated concentrations, **(C)** As (A) but using plates containing thymidine at the indicated concentrations.

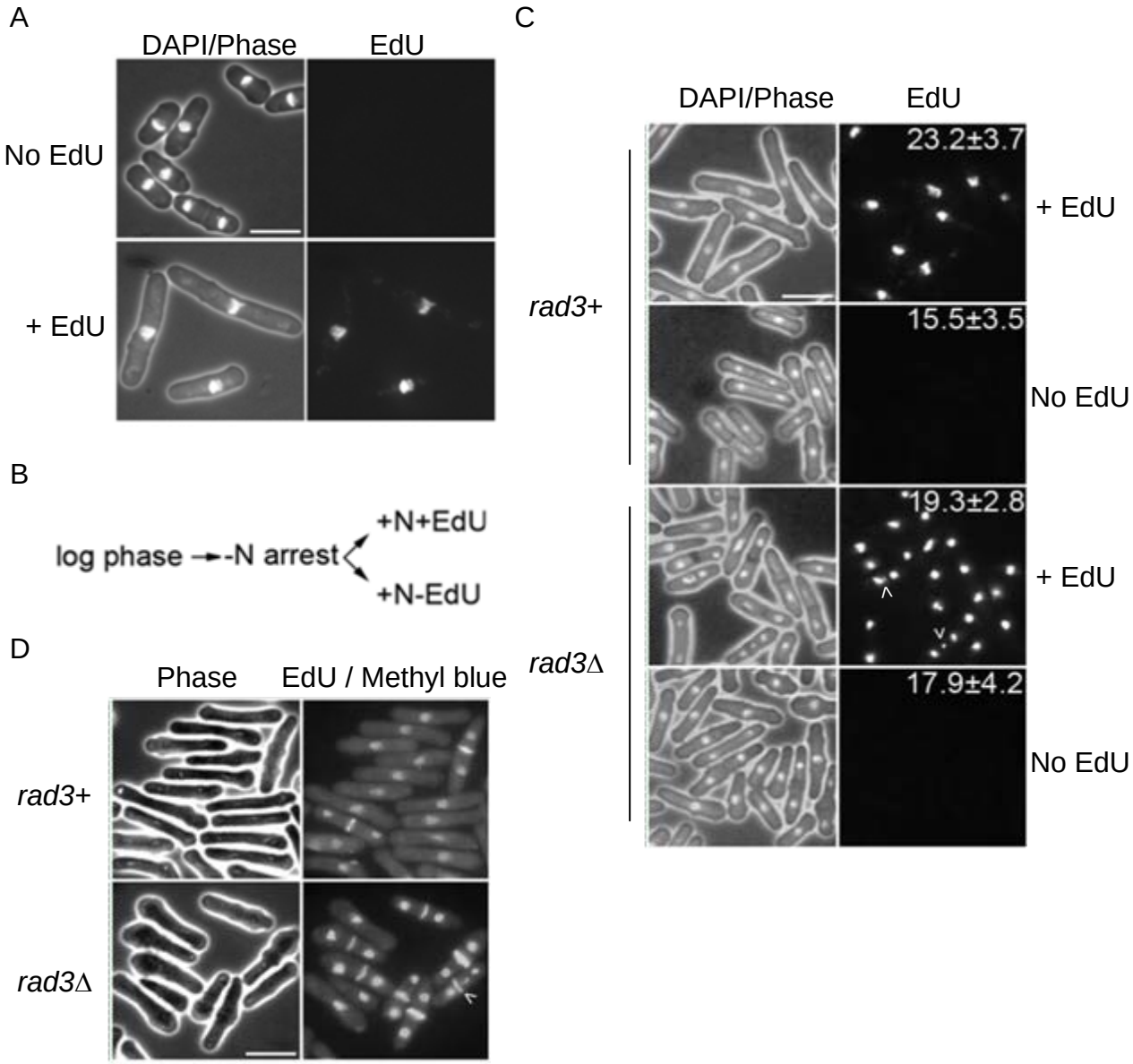


Figure 3.5 Effects of EdU incorporation on cell cycle progression

(A) Cell elongation following growth in EdU. Lower panels show *TK hENT1* [*adh1:tk adh1:hENT1* (P2470)] cells grown in 1 μM EdU (YE3S medium) for 5 h and imaged following DAPI staining and EdU detection; upper panels show control cells from a culture lacking EdU. Bar=10 μm,

(B) Scheme of experiment. *rad3*+ [*adh1:tk adh1:hENT1* (P2470)] and *rad3Δ* [*adh1:tk adh1:hENT1 rad3Δ* (P2472)] cells were arrested in G1 by growing in EMM-N for 16 h at 26ºC, then released from the cell cycle block in EMM+N medium at 32ºC in the presence (1 μM) or absence of EdU for 10 hours and then fixed in 70% EtOH.

(C) Cells following protocol in (B) were imaged following DAPI staining and EdU detection. Bar=10 μm. Mean cell length shown in the EdU panels (μm) was calculated by measuring at least 100 cells and standard deviation is shown,

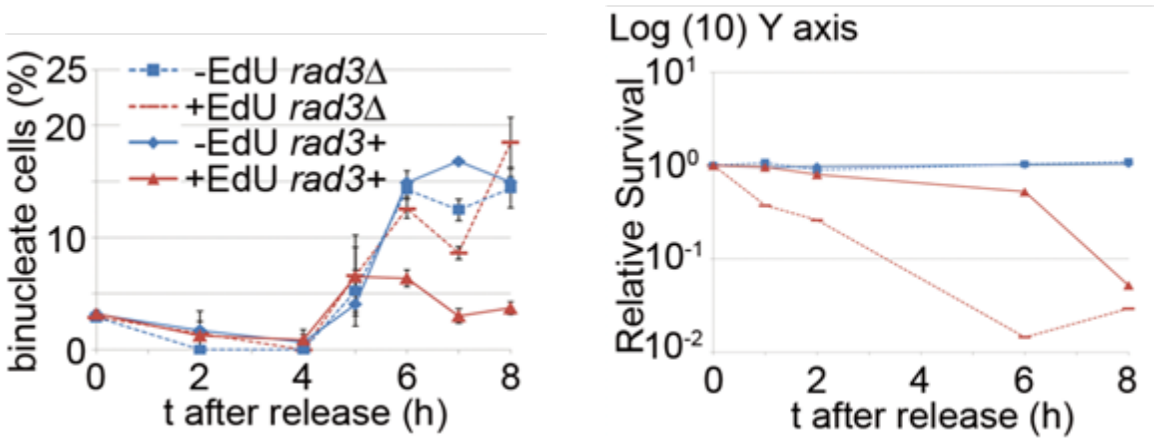
(D) As (C) except septa were also stained with 50 μg/ml methyl blue and only cells with EdU treatment are shown. *rad3Δ* cells grown in EdU execute mitosis (see also Fig. 3.6A) and form septa, but a high proportion of cells fail to carry out later stages of cytokinesis and arrest as septated binuclear cells. Arrows show defective mitoses in *rad3Δ* cells grown in EdU.

Viability started to drop after 2 hours culturing with EdU and it is more obvious if cells were incubated with EdU for longer time, i.e. 6 or 8 hours (Fig. 3.6A right panel). This is consistent with the speculation that problems caused by EdU are related to its incorporation into DNA, as in this nitrogen starvation and release experiment (Fig. 3.7 C), replication starts approximately between 1~2 hours after release, which will be discussed in details in next section. Loss of viability was exacerbated in the *rad3Δ* strain, presumably because there is no checkpoint responding to the EdU-induced problems to arrest cells to permit DNA repair before proceeding into mitosis (Fig 3.6 A right panel). Toxicity effects of EdU on mammalian cells have also been reported (Diermeier-Daucher *et al*. 2009), suggesting that EdU may not be suitable for continuous labelling studies.

3.2.3 Effects of Incorporation of EdU on DNA replication

To determine whether incorporation of EdU affects the kinetics of DNA replication, *TK hENT1* cells were arrested in G1 by nitrogen starvation and released from the block in the presence or absence of the nucleoside (Fig. 3.7 A). Cells from both cultures were stained with SYTOX Green without coupling EdU to fluorescent azide and analyzed by flow cytometry (Fig. 3.7 B). Both cultures started S phase at about the same time (~2 h after release) and bulk replication was largely complete at 4 h, indicating that the duration of S phase

A



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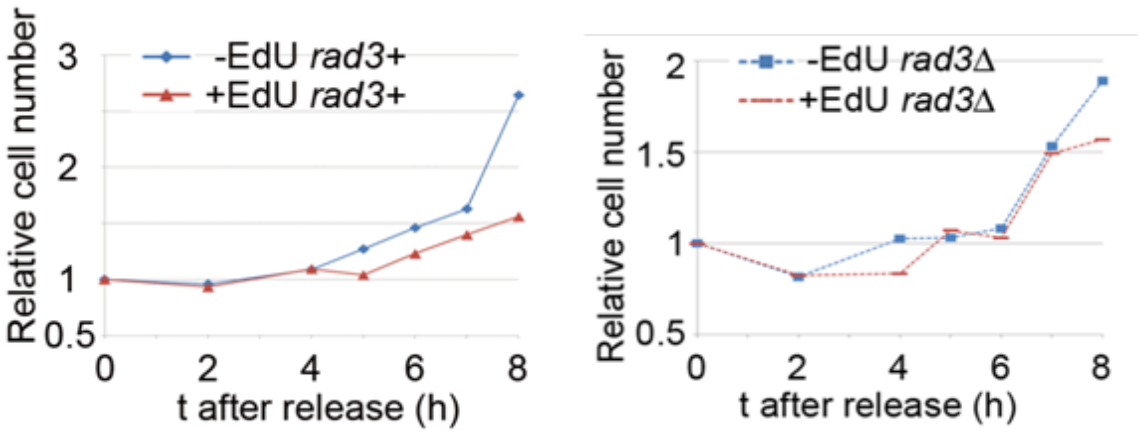


Figure 3.6 Effects of EdU incorporation on cell cycle progression, cell viability and cell division.

(A) Following the protocol in Fig. 2.5 (A), *rad3*+ [*adh1:tk adh1:hENT1* (P2470)] and *rad3Δ* [*adh1:tk adh1:hENT1 rad3Δ* (P2472)] cells were arrested in G1 by nitrogen starvation, then released from the cell cycle block in EMM+N medium at 32ºC in the presence (1 μM) or absence of EdU.

Left panel: cells were fixed in 70% EtOH at the time points shown, imaged following DAPI staining and EdU detection, and then the percentage of binucleate cells were determined.

Right panel: at the indicated time points, cells were gently sonicated and around 1000 cells were plated on YE3S plates, incubated for several days until appearance of colonies. Cell viabilities were determined by counting the numbers of colonies.

(B) Effect of EdU on cell division of *rad3*+ (left panel) and *rad3Δ* cells (right panel). As (A) except cells were fixed in 80% formyl saline and cell concentrations were monitored.

was similar. For cells grown in EdU, the SYTOX Green histogram peaks showing DNA content for cells 4-5 h after release from the G1 block are shifted slightly to the left of the peaks for the minus EdU culture (Fig. 3.7 B). Cells from the EdU-containing culture were also conjugated to fluorescent azide and analysis of these cells by flow cytometry showed commencement of DNA replication at around 2 h in agreement with the SYTOX Green histogram (Fig. 3.7 C). On prolonged incubation, the peak of Alexa Fluor 488 fluorescence associated with EdU incorporation does not shift to the right of the peak shown at 5 h (Fig. 3.7 C), indicating that EdU is only incorporated into a single strand of DNA and that cells are arrested in the first cycle after release.

The leftward shift of the SYTOX Green histograms in Fig. 3.7 B – lower panel could in principle be due to incomplete DNA replication in the presence of EdU. However I think this is unlikely as first, more EdU incorporation can induce a greater leftward shift of the 2C peak without an increase in the S phase population (i.e cells with fluorescence that is intermediate between 1 and 2C peaks), which may not be expected if there were an inhibitory effect on DNA replication (compare Fig. 3.8 A to Fig. 3.7 B). Also, a similar shift was seen when BrdU, rather than EdU, was incorporated (Fig. 3.9 A), and BrdU does not inhibit S phase completion (Sabatinos and Forsburg 2009). Meanwhile, when higher concentration of BrdU (10μM rather than 1μM) was used, reduction of STYOX Green fluorescence, i.e. leftward shift of the 2C peak after BrdU

incorporation, seemed to be more dramatic (compare Fig. 3.9 B to A). In addition, when different concentrations of EdU were used to label DNA synthesis during a synchronized pre-meiotic S phase, and DNA content of samples taken after completion of pre-MS phase (5.5 hours after meiotic induction) was analyzed by flow cytometry after SYTOX Green staining, the relationship between EdU concentration and the leftward shift of the peaks representing replicated DNA content is very clear (Fig. 3.8 C). Finally, analysis of a *rad3*Δ strain shows that this mutation has no effect on the leftward shift, suggesting that it is not due to activation of the DNA replication checkpoint and cell arrest (Fig. 3.8 B). In summary, the leftward shift of SYTOX Green histogram peaks showing DNA content for cells at 4-5 hours after release from the G1 block is intriguing, and a similar effect was seen with BrdU. For the reasons stated above, I think it should be attributed to some effect of EdU on SYTOX Green fluorescence rather than incomplete replication.

Meanwhile, it is worthwhile to note that the 2C double peak at ca 4-5 hours merges into a single peak at later time points (Fig. 3.7 B). This can be explained by referring to a paper from Mochida and Yanagida (Mochida and Yanagida 2006). Basically, they reported that fission yeast cells arrested by nitrogen starvation are mainly in G1 (1C cells) but 15-20% of the population are in G2 (2C). Also, they have shown that when cells are released in to +N medium, the G1 cells (i.e. the 1C population) carry out S phase about 3 h after

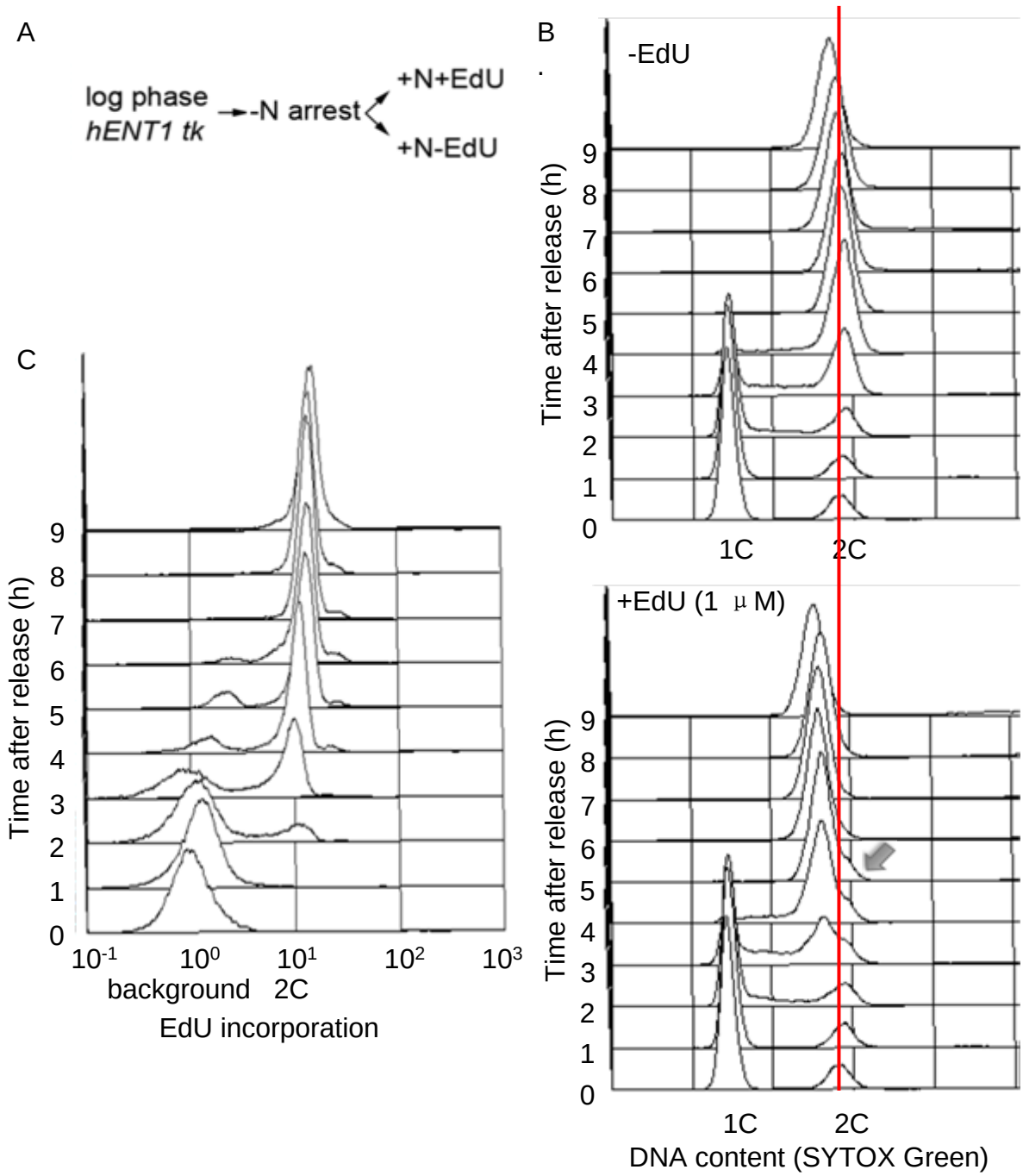


Figure 3.7 Effects of EdU on the kinetics of DNA replication.
(A) Scheme of experiment. *adh1:tk adh1:hENT1* (P2470) cells were arrested in G1 then released into EMM containing nitrogen, in the presence (1 µM) or absence of EdU,
(B) Upper panel: cells from the -EdU culture were processed for analysis of DNA content after SYTOX Green staining by flow cytometry in the absence of conjugation to fluorescent azide;
Lower panel: cells from the +EdU culture were processed as above.
The main 2C peak generated by replication of 1C cells is shifted to the left of the 2C position defined by the 'G2 minus-nitrogen population' (indicated by the arrow), probably due to some effect of EdU incorporation on SYTOX Green fluorescence (see text for further discussion).
(C) Cells from the +EdU culture were conjugated to fluorescent azide (Alexa Fluor 488) and fluorescence was quantitated by flow cytometry.

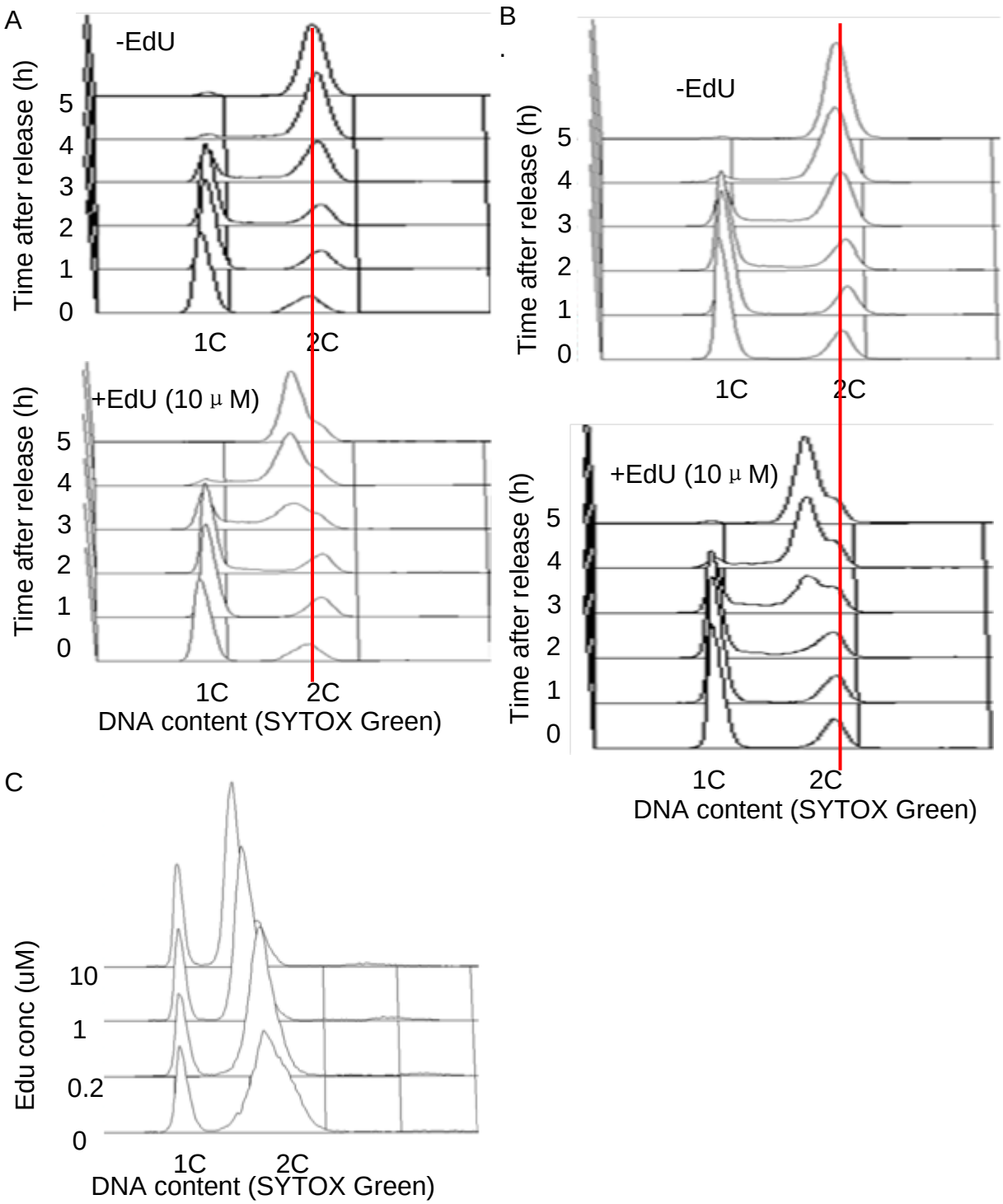


Figure 3.8 Incorporation of EdU induces leftward peak shift of SYTOX Green fluorescence.

(A) As Fig. 3.7 (B) except a higher concentration of EdU was used and a shorter time course was monitored. Upper panel shows cells released in the absence of EdU; lower panel shows cells released in the presence of 10 µ M EdU.

(B) As (A), except a *rad3Δ* [*adh1:tk adh1:hENT1 rad3Δ* (P2472)] strain was used.

(C) *adh1:tk adh1:hENT1 pat1-114* (p3007) cells were released from G1 block into meiosis with the indicated concentrations of EdU for 5.5 hours, following the protocol in Fig. 3.3 (A). Cells were processed for analysis of DNA content after SYTOX Green staining by flow cytometry in the absence of conjugation to fluorescent azide.

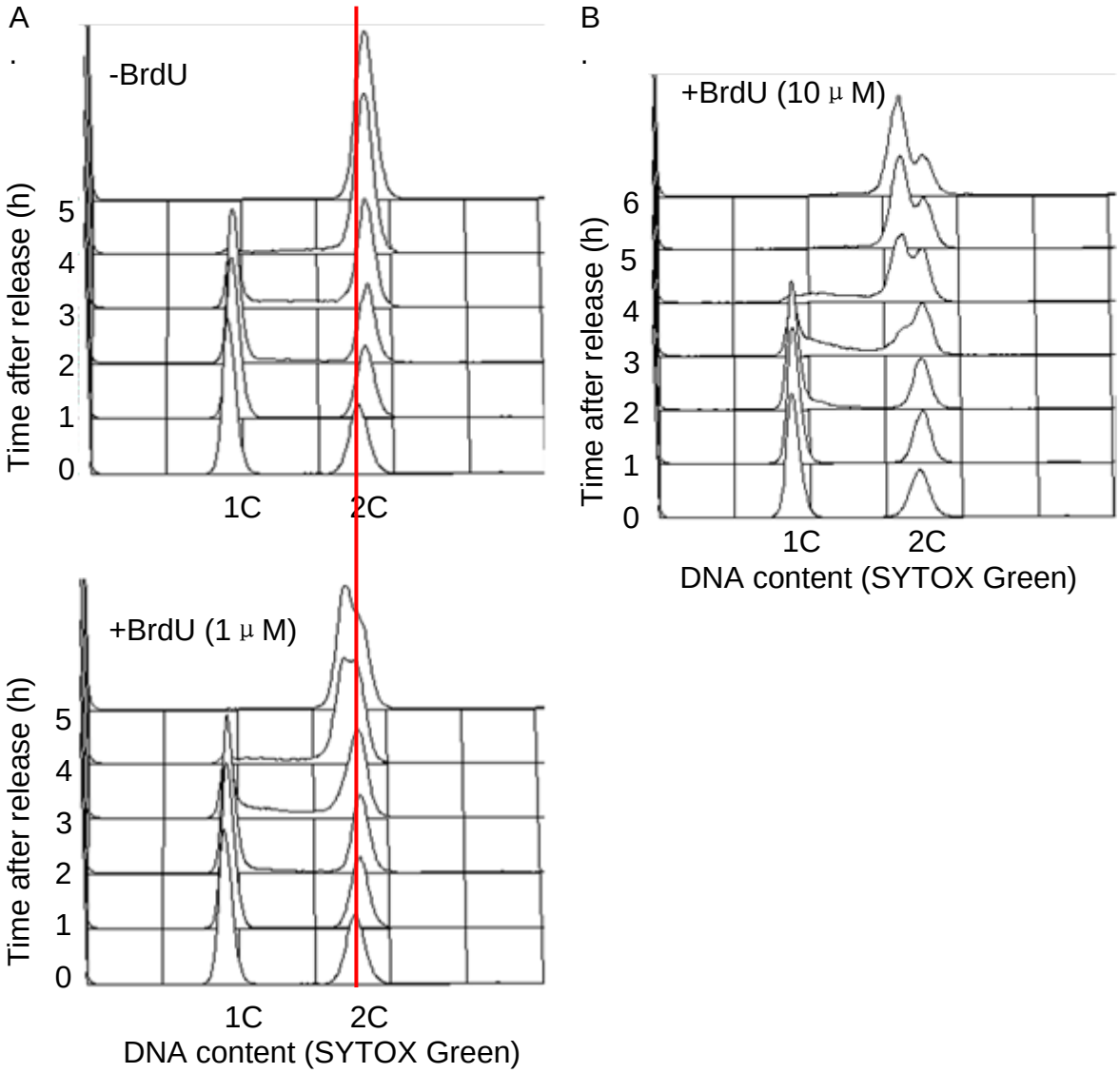


Figure 3.9 Effects on SYTOX Green fluorescence of BrdU incorporation
(A) Flow cytometric analysis of DNA content of cells grown in the presence or absence of BrdU, showing that cells with incorporated BrdU show reduced SYTOX Green fluorescence. *adh1:tk adh1:hENT1* (P2470) cells were nitrogen starved to arrest cells predominantly in G1 and then released following the scheme in Fig. 3.7 (A) except BrdU was used instead of EdU. Cells were processed for SYTOX Green staining in the absence of conjugation to fluorescent azide at the time points shown.
Top panel shows cells released in the absence of BrdU;
Bottom panel shows cells released in the presence of 1 µ M BrdU. The shoulder on the right of the 2C peaks at t=4,5 h in bottom panel represent G2 cells in the nitrogen-starved population that have not carried out S phase at this stage.
(B) Similar but more obvious peak shift was obtained using 10 µ M BrdU to label cells carrying out synchronized S phase after released from G1 block.

release, and the G2 population carries out S phase 6-8 h after release (Mochida and Yanagida 2006). This is consistent with what I see by flow cytometry. At about 3 hours after release, the main G1 population replicates; this generates a peak shifted slightly to the left of the (unreplicated) G2 population which appears as a shoulder on the main 2C peak (arrow in Fig. 3.7 B). At ca 6-7 h after release the G2 population (having carried out mitosis) replicates and the shoulder is lost - cytokinesis is coincident with S phase in *S. pombe* so no 4C peak is formed. These replicated cells now have EdU incorporated and their fluorescence merges with the main peak generated by replication of the 1C cells.

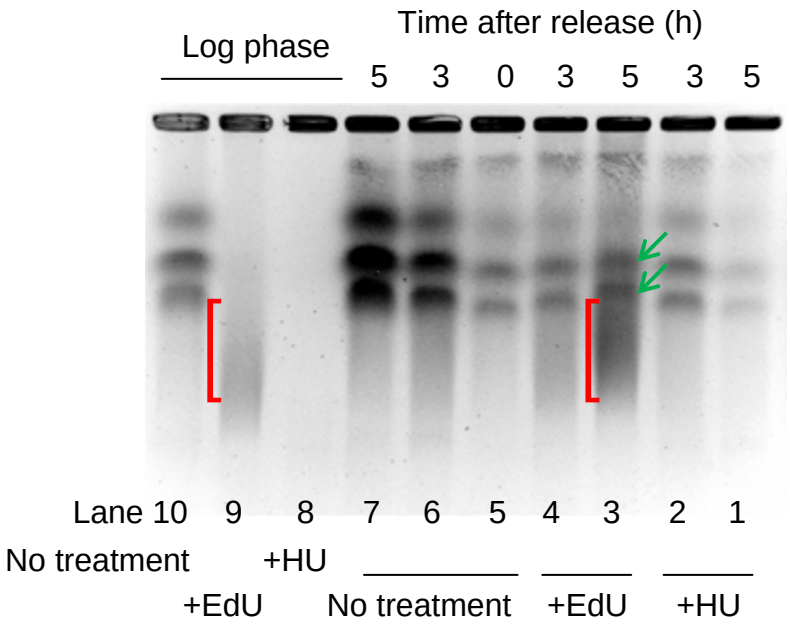
To further investigate whether EdU induces DNA damage after incorporation, I analyzed DNA from cells grown in EdU (10 μM) by pulsed field gel electrophoresis (PFGE), since incompletely replicated chromosomes fail to enter these gels (Santocanale and Diffley 1998). Cells were released from a G1 block in the presence or absence of EdU and samples were taken up to 5 h for analysis by PFGE (Fig. 3.10 A) and flow cytometry (Fig. 3. 10 B). As a control for incomplete replication, chromosomes were analyzed from cells were grown in the presence of hydroxyurea (HU) (Fig. 3.10 A, lanes 1, 2). Chromosomes from cells growing in exponential phase, treated with EdU (Fig. 3.10 A, lane 9) or HU (Fig. 3.10 A, lane 8) for 5 hours are also analyzed by PFGE. Chromosomes are resolved from cells grown in EdU (Fig. 3.10 A,

compare lanes 3 and 7) indicating completion of chromosome replication. However there is a smear of smaller DNA fragments in the +EdU samples, and this smear is more obvious in the samples from log phase cells (Fig. 3.10 A, lane 9), which may reflect the generation of double-strand breaks following EdU incorporation. The faint chromosome band in the +HU 5h sample in the G1 arrest-release experiment (Fig. 3.10 A, lane 1) may be due to the population (15-20%) of G2 cells after nitrogen starvation, which has not entered S phase at this time point (discussed above). The faint chromosome bands in the smear of the +EdU 5h sample (Fig. 3.10 A, lane 3, indicated by the green arrows) may be attributed to both unbroken chromosomes from the G1 population (replicated) and also chromosomes from the unreplicated G2 population (lanes 3, Fig 3.10 A).

Taken together these results suggest that EdU does not inhibit completion of DNA replication, which leaves DNA damage as a likely reason for activation of the checkpoint mechanism, and PFGE analysis confirms that chromosome fragmentation is induced after EdU incorporation. The reduction in SYTOX Green fluorescence seen with DNA containing EdU requires further investigation, but could be due to quenching or reduced binding of the dye.

3.2.4 Using EdU to detect limited DNA synthesis

A



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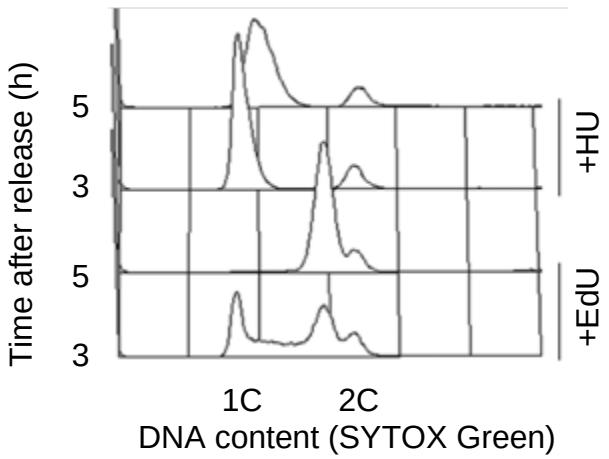


Figure 3.10 Analysis of chromosomes by PFGE.
(A) DNA plugs were prepared from *rad3*+ cells [*adh1:tk adh1:hENT1* (P2470)] released from a G1 arrest as in Figure 3.3 - (A) and grown without (lanes 5–7, 'no treatment') or with 10 μM EdU (lanes 3, 4, '+EdU') for the times shown. As a control for partially replicated chromosomes, cells were grown in the presence of 12mM HU (lanes 6–7 '+HU'). Chromosomes from unsynchronized cells (P2470) grown for 5 hours in YE3S without EdU (lane 10) or YE3S containing 10 μM EdU (lane 9), or 12mM HU (lane 8) were also analyzed. '['indicates the smear in the +EdU sample, suggesting fragmented DNA. Equal quantities of cells (same cell numbers) were processed for each well at lanes 1-7 or lanes 8-10.
(B) Flow cytometric analysis of cells used to prepare samples shown in (A), showing that the HU block to DNA replication can be maintained until 5 h after release

To investigate whether EdU incorporation provides a sensitive method for detecting DNA synthesis where only limited elongation has occurred, G1-arrested cells were released from the block into medium containing both EdU and HU (Fig. 3.11 A). Inhibition of ribonucleotide reductase by HU prevents bulk DNA replication although some fork progression from origins takes place (ca. 4-5 kb in *S. cerevisiae* (Hodson *et al*. 2003). In cells not treated with HU, flow cytometric analysis of SYTOX Green-stained cells showed that S phase occurred at 2-4 h after release (Fig. 3.11 B) while in the presence of HU, no DNA replication was detected as expected (Fig. 3.11 C). Following conjugation to azide, some EdU incorporation could be detected in the +HU+EdU culture by flow cytometry from 2 h (Fig. 3.11 E) but the clearest result was obtained by fluorescence microscopy (Fig. 3.12). Here, EdU incorporation could be detected as early as 2 h after release, around the time that S phase was commencing in the culture lacking HU. The existence of a population of G2 cells after nitrogen starvation can explain why around ~20% of the population did not have nuclear EdU by 5h in the +EdU and +HU experiment (Fig 3.12 B): the time point is too early for the G2 population to have replicated.

EdU can also be incorporated into mitochondrial DNA - at least during meiosis. Normally there is no genomic DNA synthesis between two meiotic nuclear divisions. In agreement with this, if EdU was added after pre-meiotic S phase,

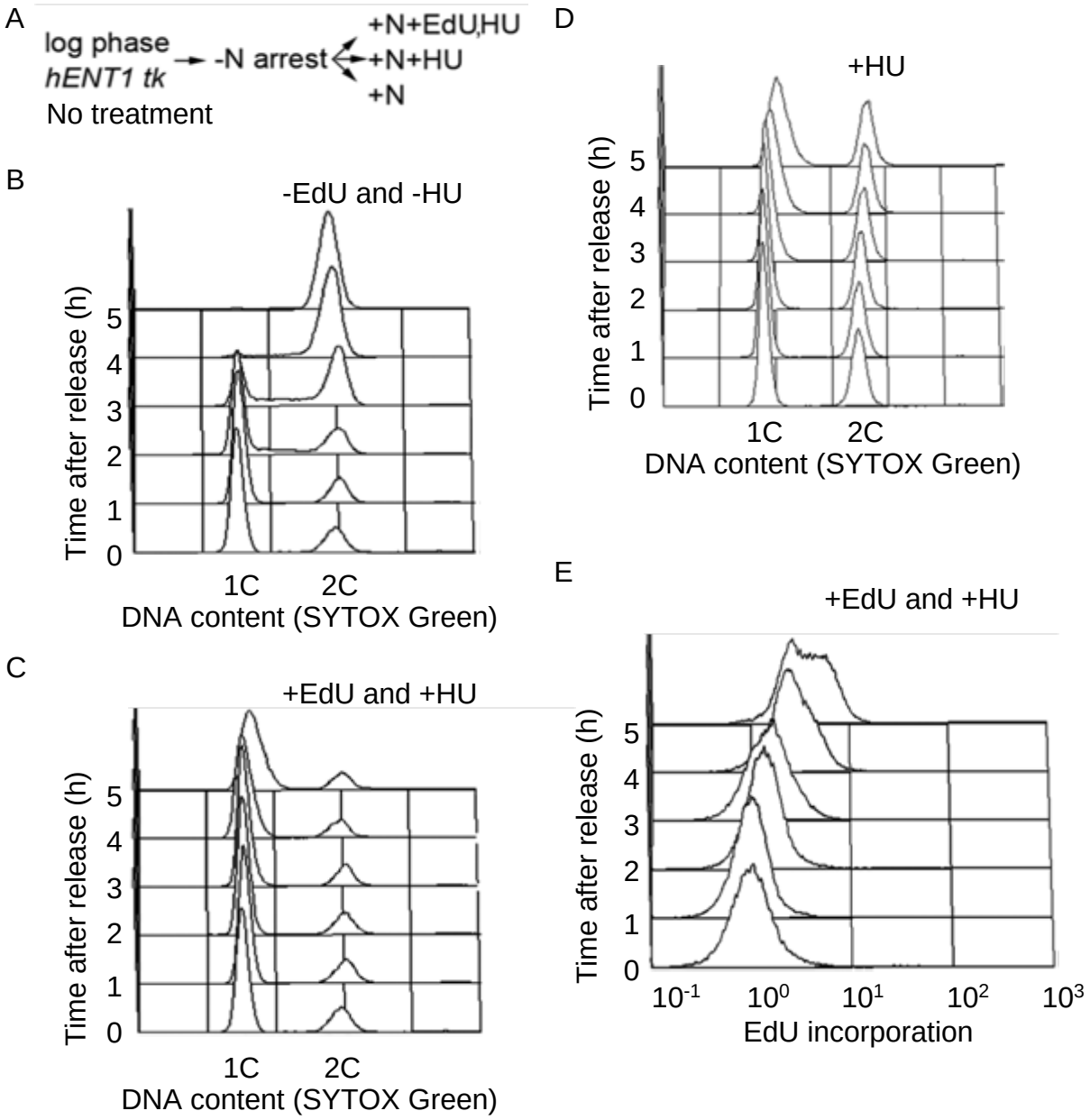


Figure 3.11 Detection of early S phase replication using EdU by flow cytometry.
(A) Scheme of experiment. *adh1:tk adh1:hENT1* (P2470) cells were arrested in G1 by growth in EMM-N for 16 h at 26ºC; cells were released from the cell cycle block by transferring to: (i) EMM only; (ii) EMM containing 12mM HU; (iii) EMM containing 12mM HU and 10 μM EdU (all cultures were incubated at 32ºC)
(B) Analysis of DNA contents of cells from the -EdU, -HU culture showing onset of S phase at around 2 hour.
(C) Analysis of DNA contents of cells from the +EdU, +HU culture confirming early S phase arrest.
(D) Cells from the +HU,-EdU culture as shown in (A), analyzed for DNA content by flow cytometry after SYTOX Green staining, confirming arrest of DNA replication.
(E) Flow cytometric analysis of EdU incorporation in cells from the +EdU +HU culture following conjugation to Alexa Fluor 488 azide.

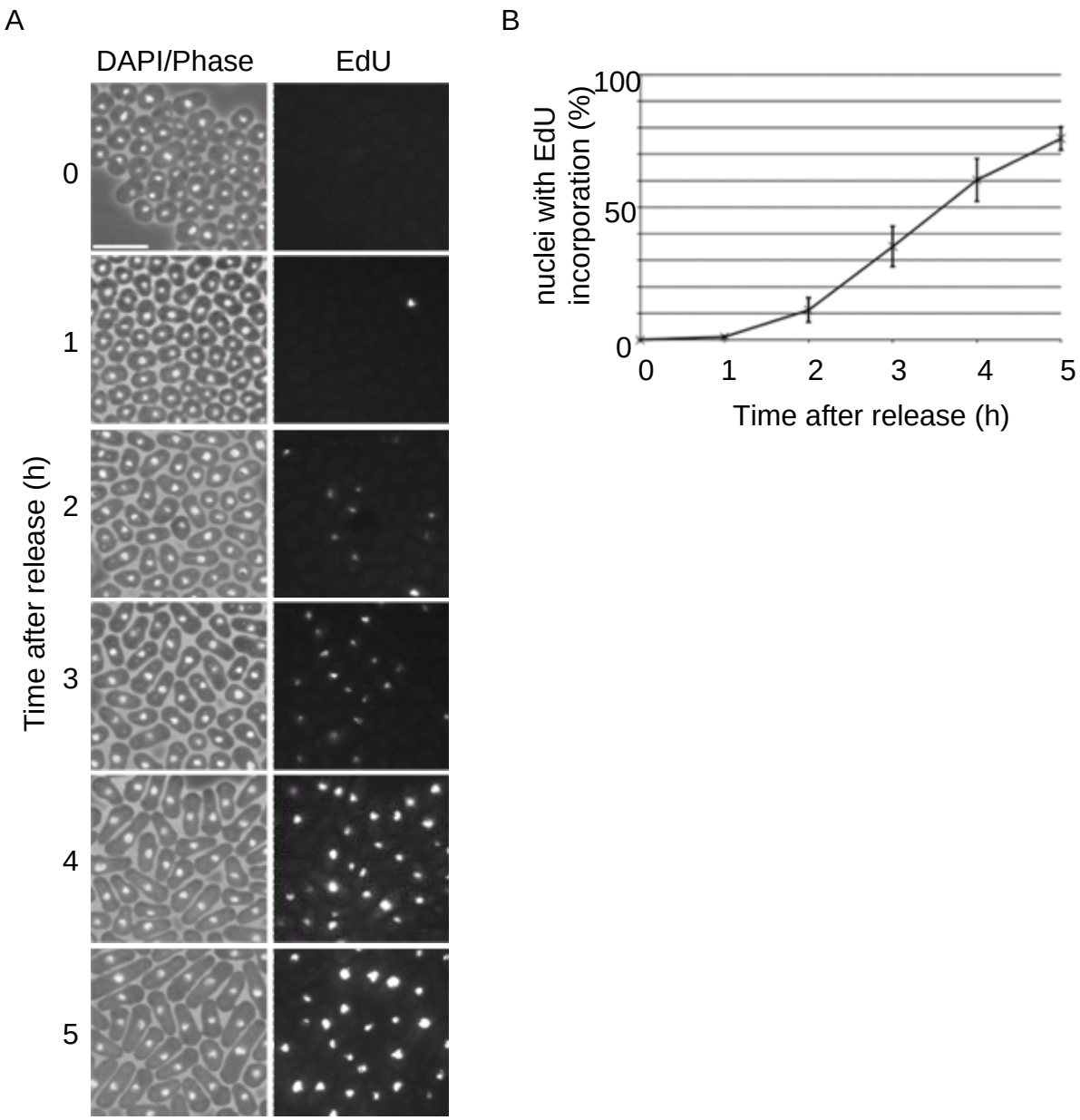


Figure 3.12 Detection of early S phase replication using EdU by fluorescence microscopy.
(A) Cells from Fig. 3.11(C) were analysed by fluorescence microscopy. Bar=10 μm.
(B) Quantitative analysis of cells shown in (A). 100 cells were counted for each sample. Error bars (representing range of two experiments) are shown.

the nuclear EdU signal is negative. But a faint cytoplasmic signal can be detected (Fig. 3.13 A), which suggests that EdU is incorporated into mitochondrial DNA. Compared to EdU incorporation in genomic DNA, the mitochondrial incorporation is much weaker and it is difficult to detect when there is nuclear incorporation of EdU (see Chapter 4). However, it provides another indication of the sensitivity of this method.

3.3 Discussion

Taken together these results indicate that EdU is a useful thymidine analogue for detecting DNA synthesis in experiments involving short labelling periods and single cell cycles. Low concentrations of EdU (1 μM) are sufficient to allow detection of incorporation in cells expressing thymidine kinase and human equilibrative nucleoside transporter 1 (hENT1). Evidence for DNA damage and thus induced cell cycle arrest, caused by EdU in fission yeast is a concern however, potentially limiting EdU's applications for procedures which require labelling over more than one cell cycle. The cell cycle arrest is *rad3*-dependent, and EdU toxicity is more pronounced in a *rad3*- strain. Since *rad3* (*S. pombe* homologue of human DNA damage sensor ATR) is required for both DNA damage and replication checkpoint pathways, EdU incorporation could be causing problems with S phase or its incorporation could be recognized as DNA damage. Data presented in this chapter suggest that the kinetics of S

A

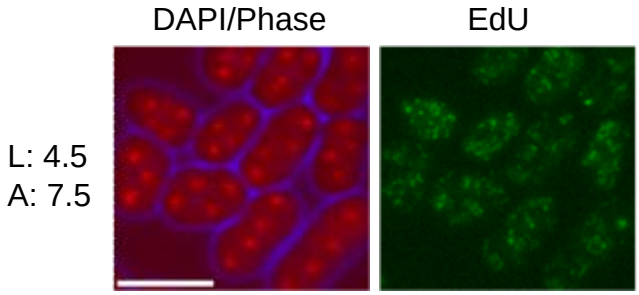


Figure 3.13 Detection of mitochondrial DNA replication during meiosis using EdU by fluorescence microscopy.
(A) *adh1:tk adh1:hENT1 pat1-114 // adh1:tk adh1:hENT1 pat1-114* diploid cells (p3032) were arrested in G1 phase (16 hours in EMM-N medium at 26ºC) and then released into EMM+N medium without EdU. Meiosis was induced by shifting the temperature to 34ºC at the time of release. EdU was added at 4.5 h after meiotic induction, and cells were fixed 3 hours later. The fixed cells were imaged following DAPI staining and EdU detection. Bar=10 μm.

phase are not affected by EdU, suggesting that it does not inhibit DNA synthesis *per se*. PFGE analysis suggests that EdU may be causing DNA damage from the smear of lower molecular weight DNA fragments in the +EdU samples (Fig. 3.10 A), although exactly how the double strand breaks are generated is still unknown. Furthermore, how EdU substituted DNA is recognized as damage, what other proteins are involved in this recognition, and which pathways are used to repair incorporations are still obscure. This can be a future direction in researching the use of EdU in *S. pombe*.

As discussed above, EdU may not be suitable of labelling more than one rounds of replication.

The *rad3*Δ cells can start M phase in the presence of EdU presumably as the DNA damage checkpoint is inactivated. However, *rad3*Δ cells have problems in finishing mitosis, indicated by the accumulation of binucleated cells. Specifically, most of the binucleate cells are arrested with septa, suggesting defective cytokinesis. In fission yeast, an equatorial MTOC (EMTOC) forms mid-way through anaphase and persists until completion of septation (Heitz *et al*. 2001). Maybe the cytokinesis defects of *rad3*Δ cells under EdU treatment is related to this EMTOC, which requires more investigations. In addition, HsOrc6 proteins are enriched at midbody before cytokinesis (Prasanth *et al*. 2002) and depleting DmOrc6 leads to cytokinetic defects (multinucleate cells) (Chesnokov *et al*. 2003), implying that in *S. pombe* ORC may be a candidate

connecting DNA damage to abnormal EMTOC as well.

In combination with DNA combing, newly replicated regions of chromosomes labelled by halogenated nucleotides (BrdU, IdU or CldU) can be directly observed, allowing single molecule analyses of origin firing and rate of fork movement (Patel *et al*. 2006, Lengronne *et al*. 2001). Since detection of EdU is much easier than BrdU, it might be possible to combine EdU labelling and DNA combing, which could reduce the processing time.

The sensitivity and ease of detection of EdU incorporation even when the elongation step of DNA synthesis is largely inhibited should be of value for detecting early replicating parts of the genome, and the limited replication associated with DNA repair and recombination. In the next chapter I use this method to detect inappropriate DNA re-replication between meiosis I and meiosis II, which is too subtle to be caught by conventional flow cytometry.

Chapter 4

Affect on DNA replication by expressing Cdc18 and Cdt1 during late meiosis

4.1 Introduction

As mentioned previously, Cdc18 and Cdt1 are absent during late meiosis (Namdar 2006), which implies that licensing is inhibited and this might constitute the main block to DNA replication between meiosis I and II. Thus a direct thought is to see if forcing expression of these two proteins is enough to induce some re-replication after meiosis I. As both Cdc18 and Cdt1 are essential, there are two ways to achieve this: replace the endogenous *cdc18* and *cdt1* promoter by a constitutive promoter thus to constitutively express Cdc18 and Cdt1 during both cell cycle and meiosis. Alternatively, an additional copy of the *cdc18* and *cdt1* genes can be put under the control of a specific late meiosis promoter that is only activated after meiosis I, i.e. it is off during vegetative growth and other periods of meiosis. I used the second strategy as it allows more accurate control of expression during the desired time and result in fewer disturbances to cell growth.

Detecting total cellular DNA content by staining with certain fluorescent

DNA-dyes such as SYTOX Green and then analyzing by flow cytometry can provide a direct demonstration of DNA re-replication, but this method can give ambiguous results if just limited replication occurs. As I have established in Chapter 3, detecting EdU incorporation is a more sensitive method for monitoring DNA synthesis and it can be detected by both flow cytometry and fluorescence microscopy. Thus I also use this method in this Chapter to detect possible re-replication during MI-MII interval when Cdc18 and Cdt1 are present.

In fission yeast, a common way to study meiosis is by using a temperature sensitive *pat1-114* mutant and in most of my experiments, I also use this method to achieve synchronized meiosis. Basically, by inactivating the meiotic inhibitor Pat1, G1-arrested cells can leave the cell cycle and enter into meiosis with high synchronization, finally forming spores. Fig 4.3 A (upper panel) is a typical flow cytometry profile reflecting this “pat1 meiosis”. After 16 hours incubation in EMM–N medium at permissive temperature (26ºC), meiosis is induced from the nitrogen-starved cells by re-feeding with nitrogen-containing medium and shifting the culture to the non-permissive temperature (34ºC) at the 0 h time point. It takes about 4 hours to finish pre-meiotic S phase. So after 4.5 hours, DNA content is doubled. Meiosis I happens at around 5 or 6 hours whilst meiosis II happens between 6 and 7 hours after meiosis induction, indicated by the frequency of binucleate cells, and tri- and tetra-nucleate cells, respectively, as shown in Fig 4.3 B.

4.2 Results

4.2.1 Is DNA replication control in late meiosis affected by forcing expression of Cdt1 and Cdc18 from integrated constructs?

In order to determine if forcing expression of licensing regulators in the MI-MII interval results in an extra round of DNA replication, a late meiotic promoter, derived from the *mes1* gene, was used to drive *cdt1* and *cdc18* expression. The *mes1* gene encodes an inhibitor of APC/C and thus is important to maintain intermediate CDK levels in the MI-MII interval (Izawa *et al*. 2005). The *mes1* promoter is shut off in vegetative growth and is switched on by Mei4 during late meiosis. i.e. after pre-meiotic S phase (Mata, Lyne *et al*. 2002). *Mes1pr-cdc18* and *mes1pr-cdt1* fragments (Fig. 4.1A) were integrated into chromosomal sites. The *mes1* promoter locus was used for *mes1pr-cdc18-TAP*, and the *ura4* locus was used for *mes1pr-cdt1-myc*, to produce the haploid strain (P2450). The diploid mutant (P2453) was made from the haploid by high-speed centrifugation (see Material and Methods). Western blotting analyses of cell extracts from both strains thus generated confirmed that levels of Cdc18 and Cdt1 expressed from the *mes1* promoter are comparable to their physiological expression levels during pre-meiotic S phase (Fig. 4.1B, C for the haploids and Fig. 4.1D, E for the diploids). However, when DNA content in meiosis is compared with wild type, flow cytometry only detects a slight peak shift when the mutants are allowed to go through synchronized meiosis by inactivating Pat1 (Fig. 4.2 for the haploids and Fig. 4.3 A for diploids). This rightward shift is inhibited by hydroxyurea, indicating

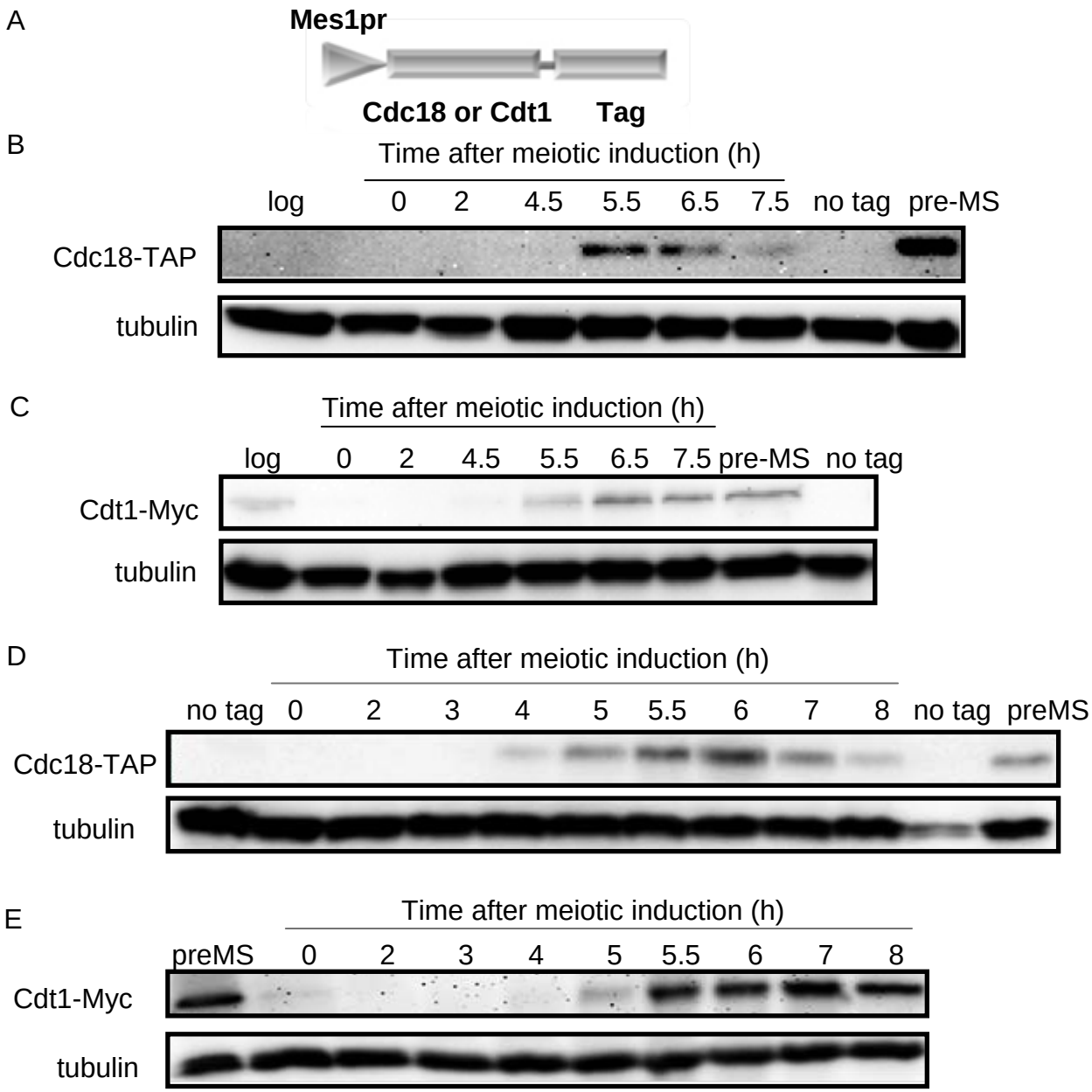


Figure 4.1. Forcing expression of Cdc18 and Cdt1 to physiological levels during late meiosis by using the *mes1* promoter.
(A) Late meiotic expression of Cdc18 and Cdt1 are controlled by *mes1* promoter. The fragment is integrated into appropriate genomic locus thus there is only one copy of each ectopic fragment (*mes1pr-cdc18-TAP* or *mes1pr-cdt1-myc*) per haploid genome.
(B) (C) *mes1pr-cdc18, cdt1* (P2450) cells were arrested in G1 by nitrogen starvation, then released from the block into meiosis. The Cdc18-TAP and Cdt1-myc levels in cell extracts at the time points shown were analyzed by western blotting. Protein samples from pre-meiotic S phase from *pat1-114 cdc18-TAP* cells (P1424) and *pat1-114 cdt1-myc* cells (P1416) are shown as positive controls (preMS level), respectively. No tag = P137.
(D) (E) As (B) (C) except Cdc18-TAP and Cdt1-myc levels during meiosis of a diploid *mes1pr-cdc18, cdt1* strain (P2453) was monitored.

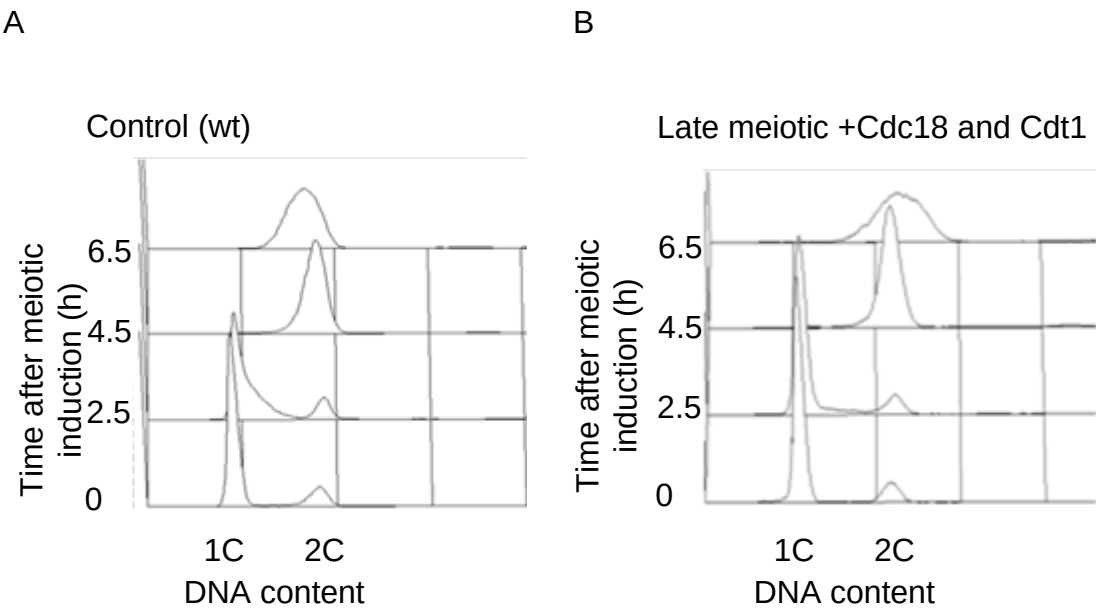


Figure 4.2. In haploids, forcing expression of Cdc18 and Cdt1 to physiological levels during late meiosis can only induce a slight increase in DNA content detectable by conventional flow cytometry.
Control (P937, A) and *mes1pr-cdc18, cdt1* (P2450, B) cells were arrested in G1 and then released into meiosis as in Fig. 2.2, sampled at the time points shown and then processed for analysis of DNA content by flow cytometry after SYTOX Green staining.

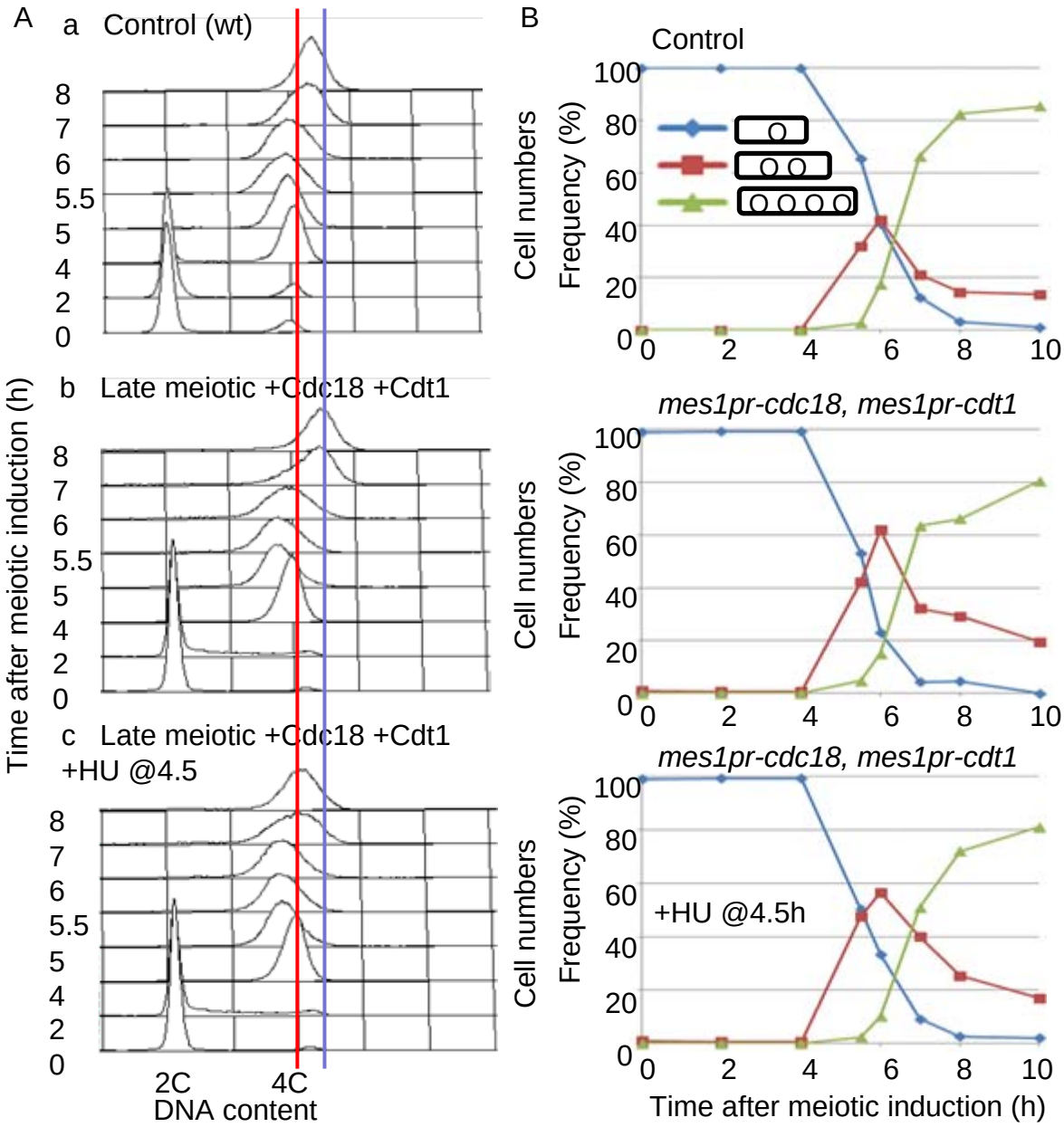


Figure 4.3. Forcing expression of Cdc18 and Cdt1 to physiological levels during late meiosis in diploids gives a similar result to that seen in haploids.
(A) HU prevents the rightward shift of flow cytometry histograms in late meiosis in a diploid strain expressing Cdc18 and Cdt1 during late meiosis. Panel a and b: as Fig. 4.2 (A) except diploid strains ("a": P2454; "b": diploid +Cdc18 & Cdt1, P2453) were used, respectively. Panel-c, as "b" except HU was added after pre-meiotic S phase but prior to MI (4.5 h after meiotic induction).
(B) Cells from (A) were imaged under microscope after DAPI staining and meiotic progress was monitored from the frequency of cells with one nucleus, two nuclei, and three or four nuclei at different time points.

that it represents DNA synthesis (Fig. 4.3 A - c). I also analyzed spore viability in this experiment, which showed dramatically reduced spore viability for the diploid mutant capable of expressing Cdc18 and Cdt1 during late meiosis (reduced to about 6~7% in the *mes1pr-cdc18, cdt1 pat1-114* mutant compared to wt *pat1-114* strain).

The ectopically expressed Cdc18 is localized in the nucleus, as shown by fluorescence microscopy. When a copy of *cdc18-CFP* is under the control of *mes1* promoter, Cdc18-CFP was not detectable during pre-meiotic S phase (2 hours after meiotic induction) but its fluorescence appeared after meiosis I (about 5 hours after meiotic induction), and co-localized with DAPI in binucleate cells (Fig. 4.4).

4.2.2 EdU incorporation indicates DNA re-replication occurs after pre-meiotic S phase if Cdc18 and Cdt1 are present

Detecting the incorporation of EdU is a more sensitive method to detect DNA synthesis compared with standard flow cytometry using DNA binding dyes, as demonstrated in Chapter 3. In order to determine if EdU incorporation can be detected in late meiosis when Cdc18 and Cdt1 are expressed, diploid *pat1-114* cells capable of expressing TK and hENT1 were arrested in G1 by nitrogen starvation and released into EMM+N medium whilst meiosis was induced by inactivating Pat1. EdU was added at different time points during this synchronized meiosis and samples were taken as shown in Fig. 4.5 A. If EdU is added upon the completion of pre-meiotic S phase during normal meiosis,

A

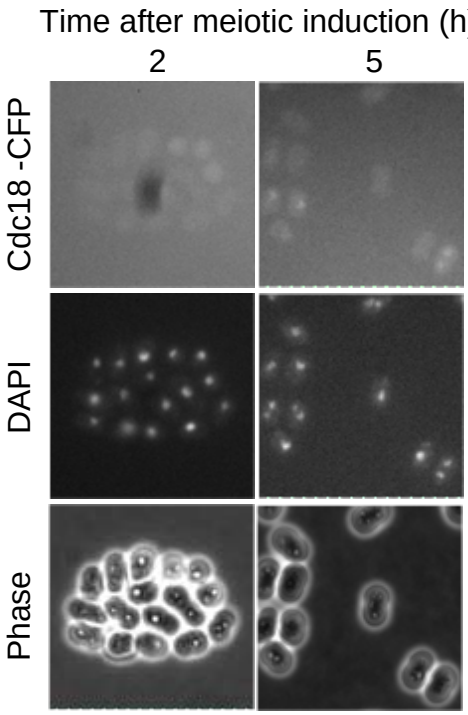


Figure 4.4 Cdc18 is nuclear localized when expressed after meiosis I.
(A) A *pat1-114 mes1pr-cdc18-cfp* strain (P2451) was induced into meiosis. Cells were samples at the indicated time points, and imaged following DAPI staining.

no EdU incorporation could be detected as expected, since there is no ongoing DNA synthesis after pre-meiotic S phase (Fig. 4.5 B right panel and 4.6 A lower panel). But if Cdc18 and Cdt1 are present in late meiosis, EdU incorporation is detected. Co-localization of EdU incorporation with the DAPI-stained DNA indicates that EdU is incorporated into genomic DNA (Fig. 4.5 B – left panel and 4.6 A upper panel).

In order to confirm that EdU incorporation was not occurring towards the end of pre-meiotic S phase or in G2, an experiment when EdU and HU (hydroxyurea) were added at the same time was carried out (Fig. 4.7 A). In this situation, EdU could still be incorporated if there is DNA synthesis, but the signal will be weaker, as HU slows the elongation step of DNA replication by inhibiting RNR (Salic and Mitchison 2008; Hua and Kearsey 2011). Meanwhile, HU treatment triggers the DNA replication checkpoint response due to eventual arrest of replication forks, and entry to meiosis I is prevented (Murakami and Nurse, 1999). Thus if EdU is incorporated during pre-meiotic S phase, in the presence of HU, cells will be arrested before meiosis I (i.e. cells will have a single nucleus) with EdU incorporation (Fig. 4.7 B). When EdU and HU are added to cells at 5.5 hours or 6.5 hours after meiotic induction (i.e. after pre-meiotic S phase) for 1 hour's labelling, the appearance of binucleate and tetranucleate cells with EdU incorporation indicates that the cells had completed meiosis I before EdU incorporation (Fig. 4.7 B and C). In any case, EdU incorporation, like HU, activates the DNA damage checkpoint in fission yeast (Hua and Kearsey, 2011), and cells incorporating this nucleoside during pre-meiotic S phase arrest before MI even in the absence of HU (Fig. 4.5 B).

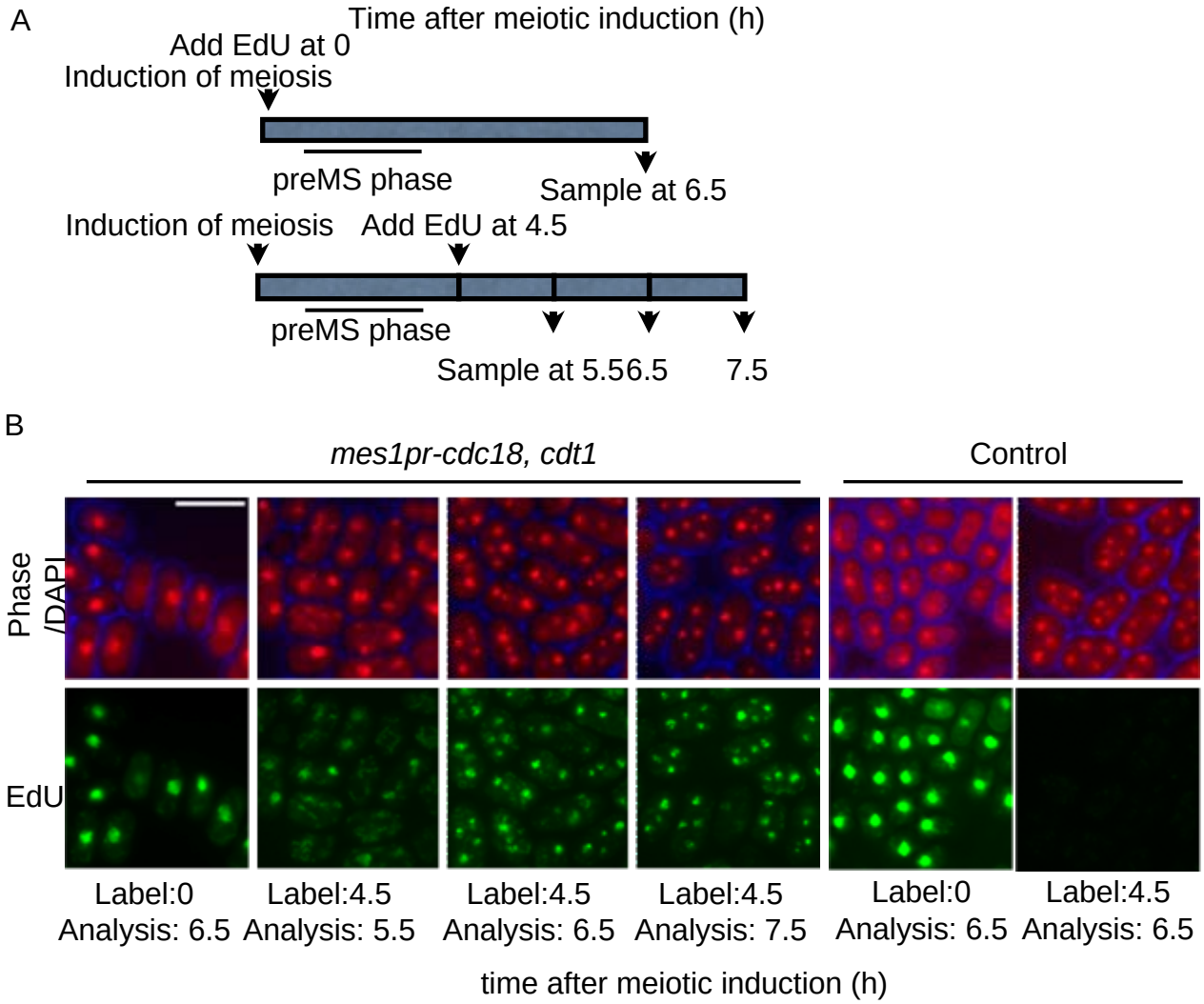


Figure 4.5. Expression of Cdc18 and Cdt1 during the MI-MII interval leads to DNA replication, which can be readily detected by EdU incorporation.
(A) Scheme of experiment.
(B) As in (A), mutant (*mes1pr-cdc18, cdt1,* P3031, left panel) and control (P3032, right panel) strains were induced to undergo meiosis, (i) in the presence of EdU at the time of meiosis induction ("Label: 0"), or (ii) in the presence of EdU after pre-meiotic S phase (at 4.5 h after meiotic induction - "Label: 4.5"). Cells were sampled at the indicated time points ("Analysis"), fixed and imaged following conjugation to fluorescent azide and DAPI staining. Bar=10 μm.

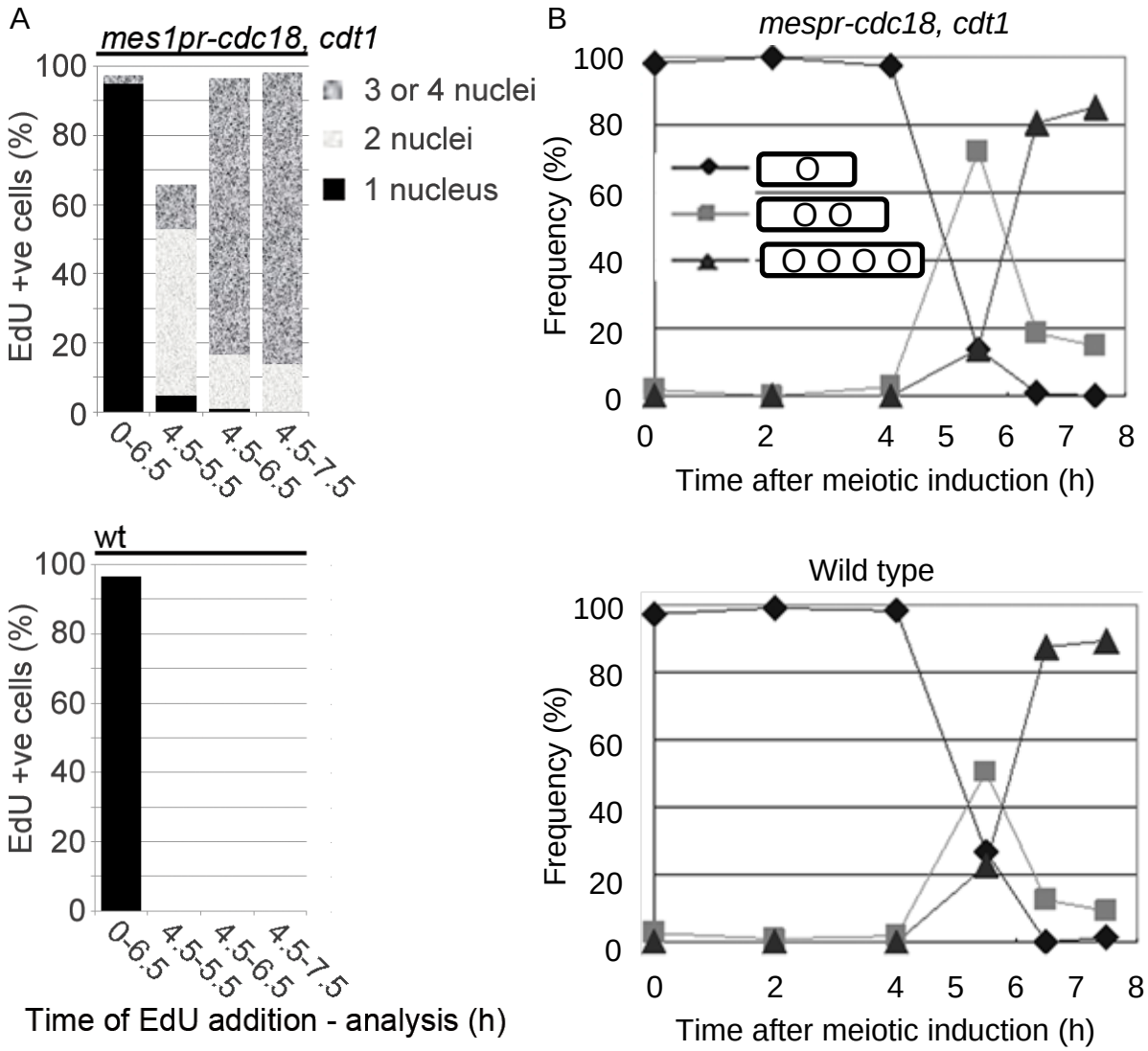


Figure 4.6. Quantitative analysis of cells from Fig. 4.5.
(A) Quantitative analysis of cells from Fig. 4.5-(B): Upper panel, cells expressing Cdc18 and Cdt1 in late meiosis P3031; lower panel, control, P3032. It shows percentage of different types of cells positive for EdU incorporation. At least 100 cells were counted for each point.
(B) Meiotic progress of cells from Fig. 4.5-(B). Meiosis was monitored from the frequency of cells with one nucleus, two nuclei and three or four nuclei at different time points. Upper panel, *mespr-cdc18, cdt1,* P3031; lower panel, control, P3032.

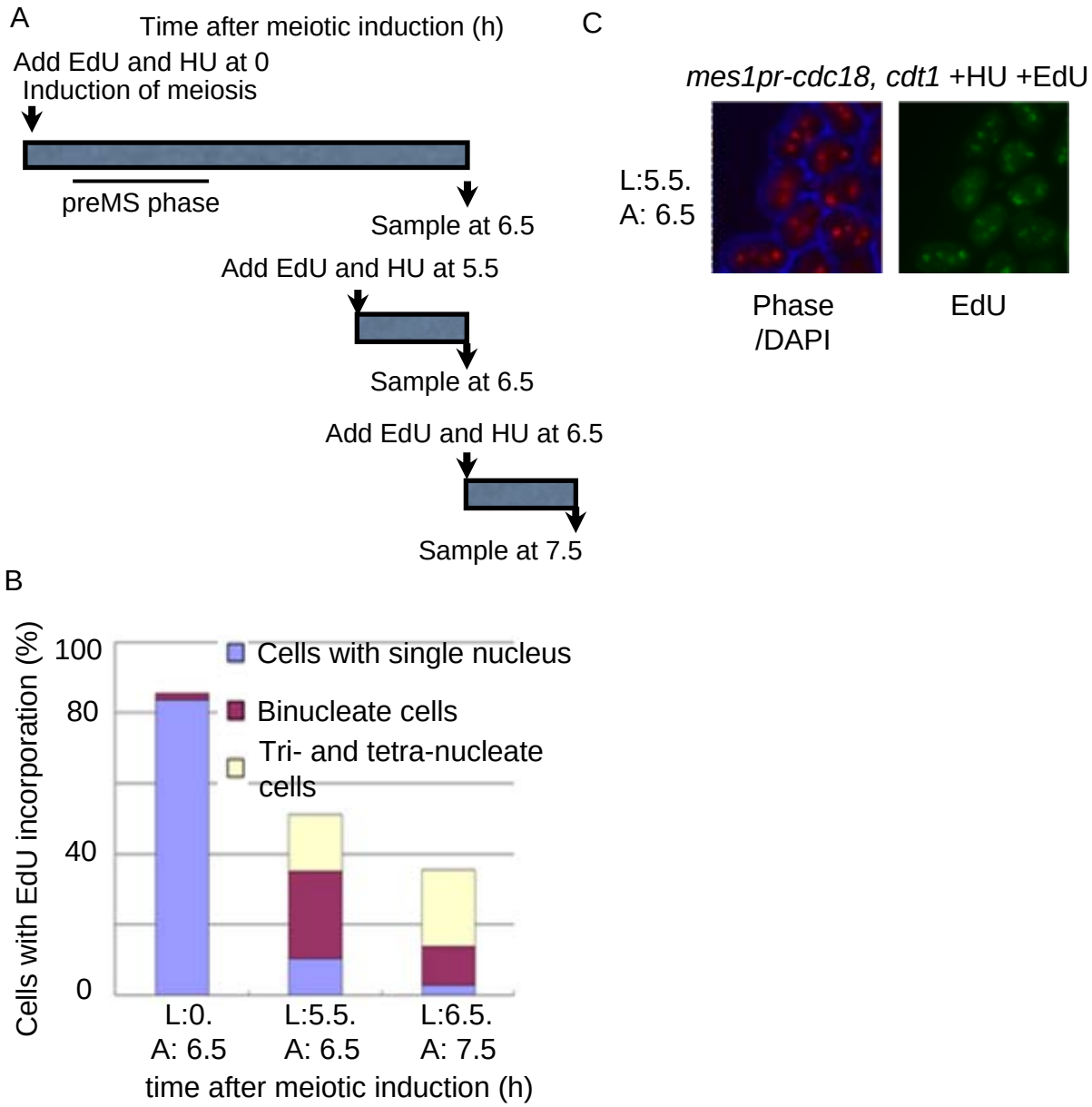


Figure 4.7 EdU incorporation after meiosis I induced by expression of Cdc18 and Cdt1 can still occur with addition of HU.
(A) Scheme of the "+EdU +HU" experiment. Meiosis was induced as Fig 2.2 and all cultures were incubated at 34ºC.
(B) Quantitative analysis showing percentage of different types of cells positive for EdU incorporation in the "+EdU +HU" experiment. Cells expressing Cdc18 and Cdt1 in late meiosis (P3031) were induced to enter meiosis, and both EdU and HU were added at various time points (L: label) as (A). Cells were sampled at the indicated time points (A: analysis), fixed and imaged following conjugation to fluorescent azide and DAPI staining. At least 100 cells were counted for each sample.
(C) One representative image of cells from (B), i.e. when both EdU and HU were added.

However it is noteworthy that *mes1pr-cdc18, cdt1* cells with incorporated EdU can still enter meiosis II with similar kinetics as the wild-type cells that do not incorporate EdU after meiosis I (Fig. 4.6 B). This suggests that there is no DNA replication checkpoint during the MI-MII interval. Furthermore, as suggested in Chapter 3, incorporation of EdU causes DNA damage, so it may also indicate that there is no DNA damage checkpoint between MI-MII.

To determine if the detected replication is dependent on meiotic recombination, a *rec12Δ* strain was analyzed (Fig. 4.8 A). Together with other proteins, Rec12 is responsible for the production of double strand breaks required for meiotic recombination. Previous publications indicated that if the *rec12* gene is deleted in *S. pombe*, double strand breaks in DNA do not appear during meiosis, and there is no DNA synthesis associated with recombination (Young *et al*. 2004, Young *et al*. 2002). When EdU was added after pre-meiotic S phase in a *rec12Δ* strain, EdU incorporation was still detected at a comparable level to the wt strain (Fig. 4.8 A and B), which suggests that the EdU incorporation observed is independent of meiotic recombination, i.e. not associated with DNA recombination.

Re-replication represented by EdU incorporation can also be detected by flow cytometry (Fig. 4.8 B and C). The histogram representing EdU incorporation by DNA synthesis in late meiosis is about 20% of the fluorescence level generated when EdU was added before pre-meiotic S phase in a diploid strain (final DNA content 4C). Since complete re-replication after meiosis I would

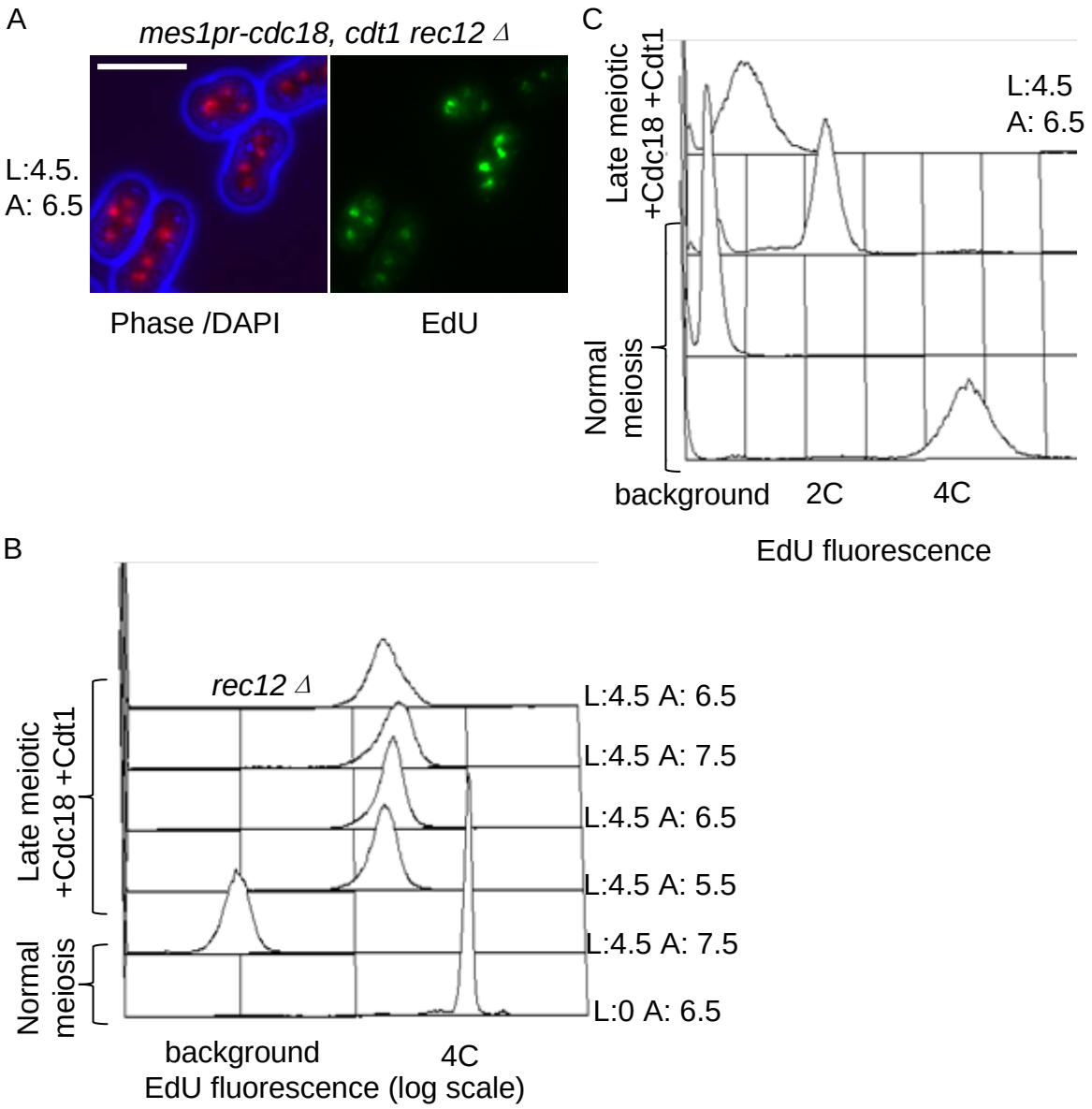


Figure 4.8 EdU incorporation after meiosis I induced by expression of Cdc18 and Cdt1 can be detected by flow cytometry and is independent of meiotic recombination.

(A) As Fig. 4.5 (B) except a *rec12* Δ (diploids, +Cdc18 & Cdt1 in late meiosis, P3021) strain was used: cells were labelled at 4.5h and sampled at 6.5 h after meiotic induction. There are no differences between *rec12* Δ mutant (P3021) and the *rec12*$^{+}$ strain (P3031, Fig. 4.5 B – left panel) when EdU incorporation is visualized by click reaction and observed under fluorescent microscope. Bar=10 μm.

(B) Flow cytometric analysis of cells from Fig. 4.5 (B) and Fig. 4.8 (A). Note that EdU incorporation is a little bit less in the *rec12* Δ mutant compared to the *rec12*$^{+}$ strain, which may reflect the minor influence from meiotic recombination dependent DNA synthesis. L: label; A: analysis.

(C) Flow cytometry analysis of cells from Fig. 4.5 (B) : linear x-axis is used to show estimation of percentage of genome being re-replicated (10%).

result in an 8C DNA content, this implies that only about 10% of the genome has re-replicated (Fig. 4.8 C).

All the experiments described above using diploid strains were also performed with relevant haploid strains, which gave the same results (Fig. 4.9 A and B). This confirms that ploidy is not influencing our results obtained.

The effect of deleting the *mes1* gene when Cdc18 and Cdt1 are expressed during late meiosis was also examined in a haploid mutant. Mes1 competes with Cdc13 (Cyclin B) for binding to Slp1 – an activator of APC/C (Izawa, Goto *et al*. 2005). Therefore presence of Mes1 suppresses efficient proteolysis of Cdc13 mediated by APC/C when cells exit from meiosis I, and deletion of *mes1* allows complete destruction of Cdc13 and a consequent block to meiosis II entry. Presumably, under this circumstance, CDK activity is decreased to a low level after meiosis I, which is not enough to stimulate the second round of nuclear division so that cells are arrested with two nuclei (Izawa *et al*. 2005). Because CDK activity suppresses licensing but is required for initiation of DNA replication, decreasing CDK activity may create an environment facilitating pre-RC formation but may not promote the later steps of replication. Re-replication could still be induced in the *mes1pr-cdc18, cdt1 mes1Δ* strain, but at a lower level compared with the *mes1pr-cdc18, cdt1 mes1*$^{+}$ strain (Fig. 4.9 C).

4.2.3 Over expression of Cdc18 and Cdt1 causes Mcm2 to rebind to chromatin after meiosis I

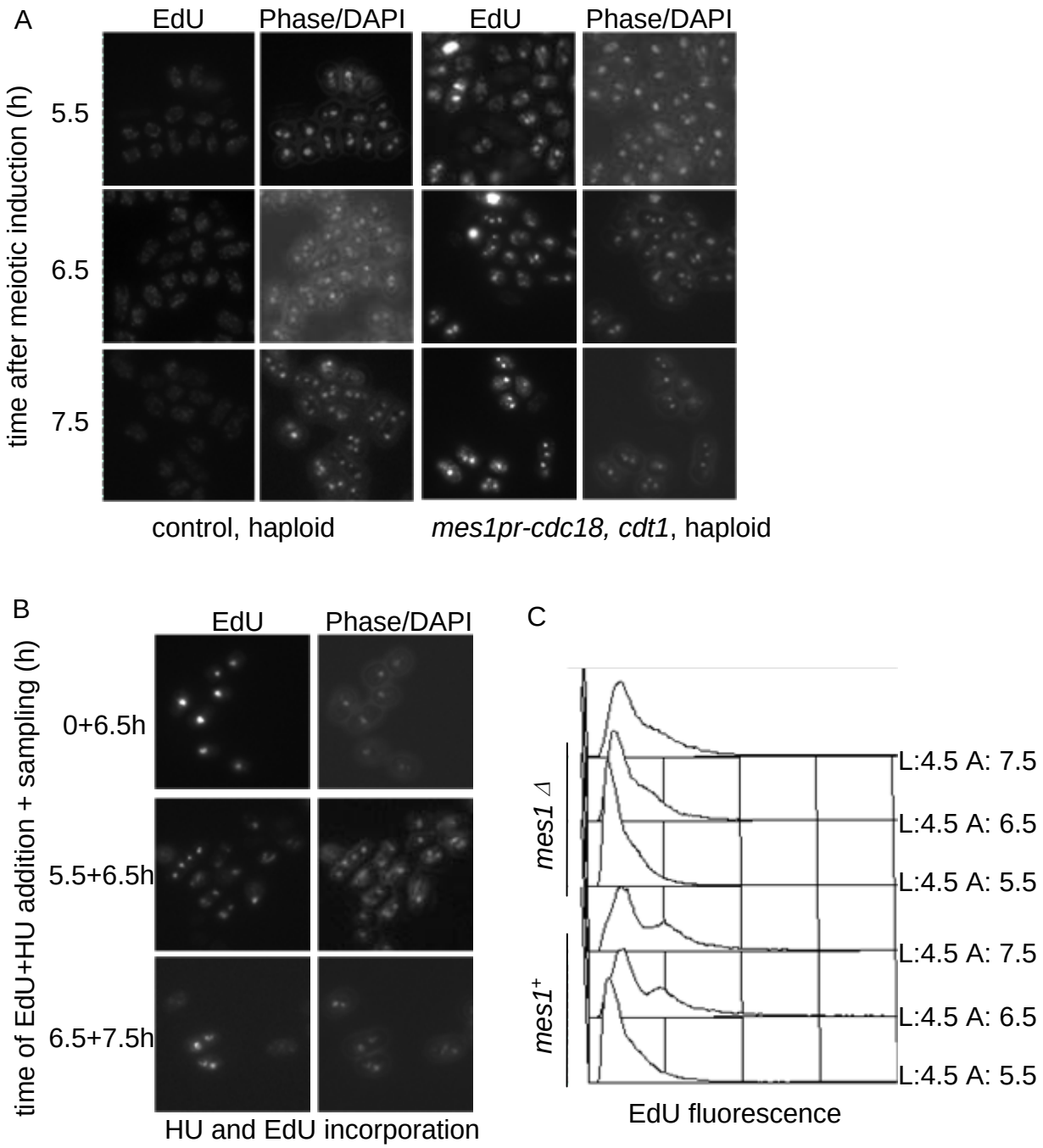


Figure 4.9 EdU incorporation in haploid strains.
(A) As Fig 4.5 – (B) except haploid strains (right panel: *mes1pr-cdc18, cdt1,* P2457; left panel: control, P2465) were used.
(B) As Fig 4.7 – (C) except haploid strains (*mes1pr-cdc18, cdt1*: p2457) were used.
(C) Flow cytometric analysis of cells after click reaction to visualize EdU incorporation: cells expressing Cdc18 and Cdt1 during late meiosis in a *mes1*$^{+}$ (P2465) or *mes1* Δ background (P2460) were induced to undergo meiosis as Fig 2.2.

It is possible that failure to observe dramatic re-replication in the experiment described in section 4.2.1 was due to factors that actively inhibit DNA synthesis in the MI-MII interval. Previous reports suggested that in vegetative cell cycle, over expression of Cdc18 and Cdt1 seems to be able to overcome whatever mechanisms are blocking re-replication (Yanow *et al*. 2001). Therefore, a similar experiment was attempted in meiosis to see if further elevation of Cdc18 and Cdt1 levels during the MI-MII transition can override whatever blocks re-replication after pre-meiotic S phase. Over expression was achieved by taking advantage of plasmids to control *cdc18* and *cdt1*'s expression instead of the previously used integration strategy (Fig. 4.10 A). When cells with plasmid borne *mes1pr-cdc18*, *cdt1* were induced into meiosis by inactivating Pat1 after G1 arrest, western blotting analysis shows a higher level of Cdc18 and Cdt1 expression during the MI-MII interval compared with the pre-meiotic S phase level and that in the experiments with integrated constructs (Fig. 4.10 B, compared with Fig. 4.1).

Use of a chromatin binding assay for Mcm protein can give information about licensing efficiency (see Methods and Materials). Following the experimental scheme shown in Fig. 4.11 A, Mcm2-CFP cells were released into a synchronised meiosis, and at the time points shown, cells were permeabilized, detergent extracted, and fixed before imaging to detect chromatin-bound Mcm2-CFP. In a wild-type meiosis, Mcm2 chromosome binding was detected around the time of pre-meiotic S phase (2.5 h after meiotic induction), but not later (Lindner *et al*. 2002). When Cdc18 and Cdt1 are expressed to levels

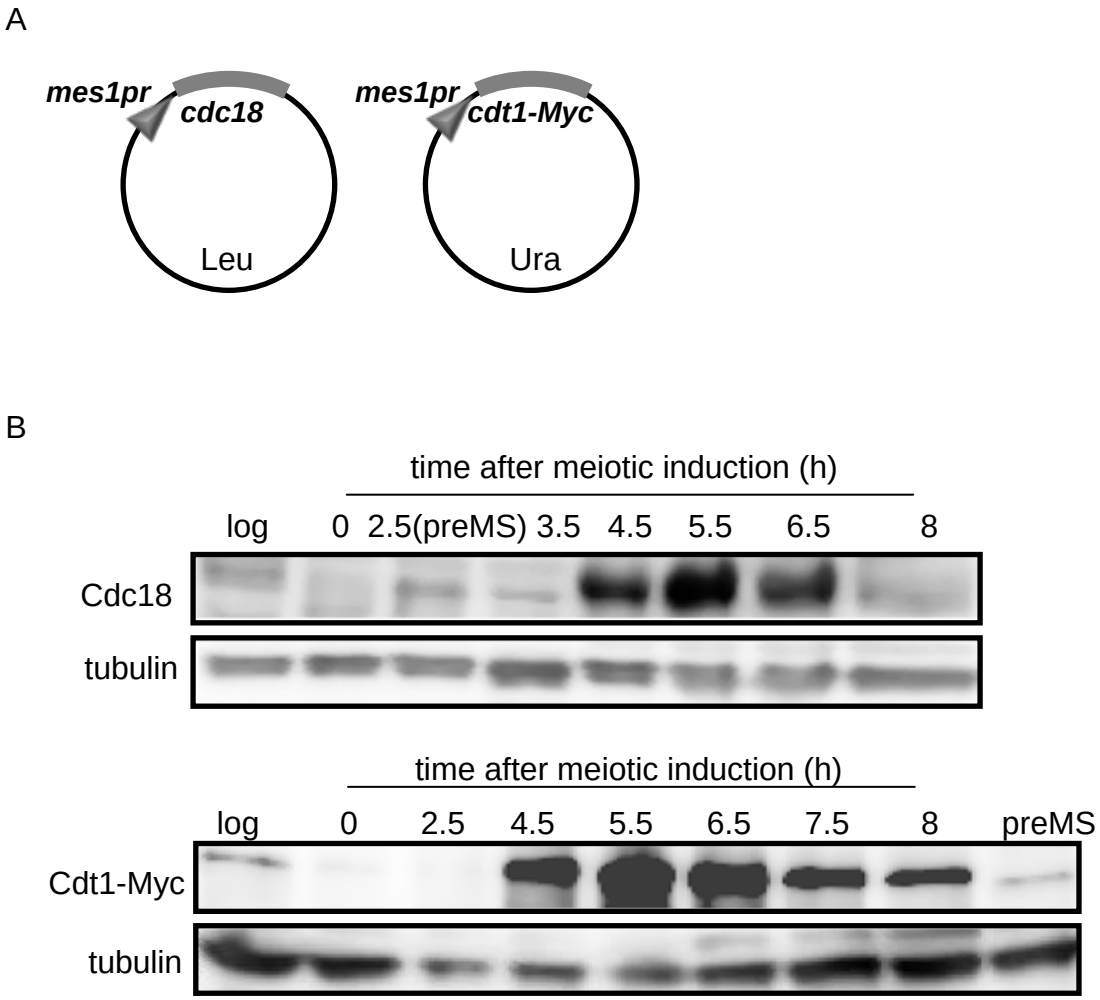


Figure 4.10 Over-expressing Cdc18 and Cdt1 in late meiosis by using plasmids.
(A) Plasmids used to over-express *cdc18* and *cdt1* after meiosis I. Selectable marker of the [*mes1pr-cdc18*] plasmid is *LEU2* (from budding yeast), and for the [*mes1pr-cdt1-myc*] plasmid is *ura4*$^{+}$.
(B) Western blot analysis of Cdc18 and Cdt1 levels in meiosis when both *mes1* promoter constructs are expressed from plasmids (P2466). Cells were arrested in G1 and meiosis was induced by inactivating Pat1 as in Fig. 2.2. Plasmid borne *mes1pr-cdc18* is not tagged thus an anti-Cdc18 antibody was used (see section 2. for antibodies).

comparable to those in the pre-meiotic S phase from the integrated constructs (see section 4.2.1), Mcm2 chromatin rebinding after preMS phase cannot be detected, even when *mes1* is deleted, suggesting that inefficient licensing is occurring in this situation (Fig. 4.11 B – left panel). If Cdc18 and Cdt1 are over-expressed from plasmids, Mcm2 chromatin binding can be detected in both *mes1*$^{+}$ and *mes1Δ* cells after meiosis I (Fig. 4.11 B and 4.12 A). The intensity of chromatin bound Mcm2-CFP fluorescence is comparable to that seen in pre-meiotic S phase (Fig. 4.12 B), indicating that licensing is efficient after meiosis I when Cdc18 and Cdt1 are over-expressed during the same period.

Nonetheless, improving the efficiency of licensing by over-expression of Cdc18 and Cdt1 levels during the MI/MII transition (i.e. where the genes were plasmid based) does not lead to a full round of replication (Fig. 4.13 A). EdU incorporation was also examined in the strain over expressing Cdc18 and Cdt1, the result of which is consistent with the flow cytometric analysis of DNA content after SYTOX Green staining: re-replication is still partial (Fig. 4.13 B). It implies that there are still other mechanisms blocking replication in the MI-MII interval even though licensing appears to be efficient. Probably initiation or elongation becomes rate limiting for DNA replication in this situation.

4.2.4 Comparing effects of over-expressing Cdc18 and Cdt1 during meiosis and the mitotic cell cycle

It seems that over expressing Cdc18 and Cdt1 after the first round of meiotic

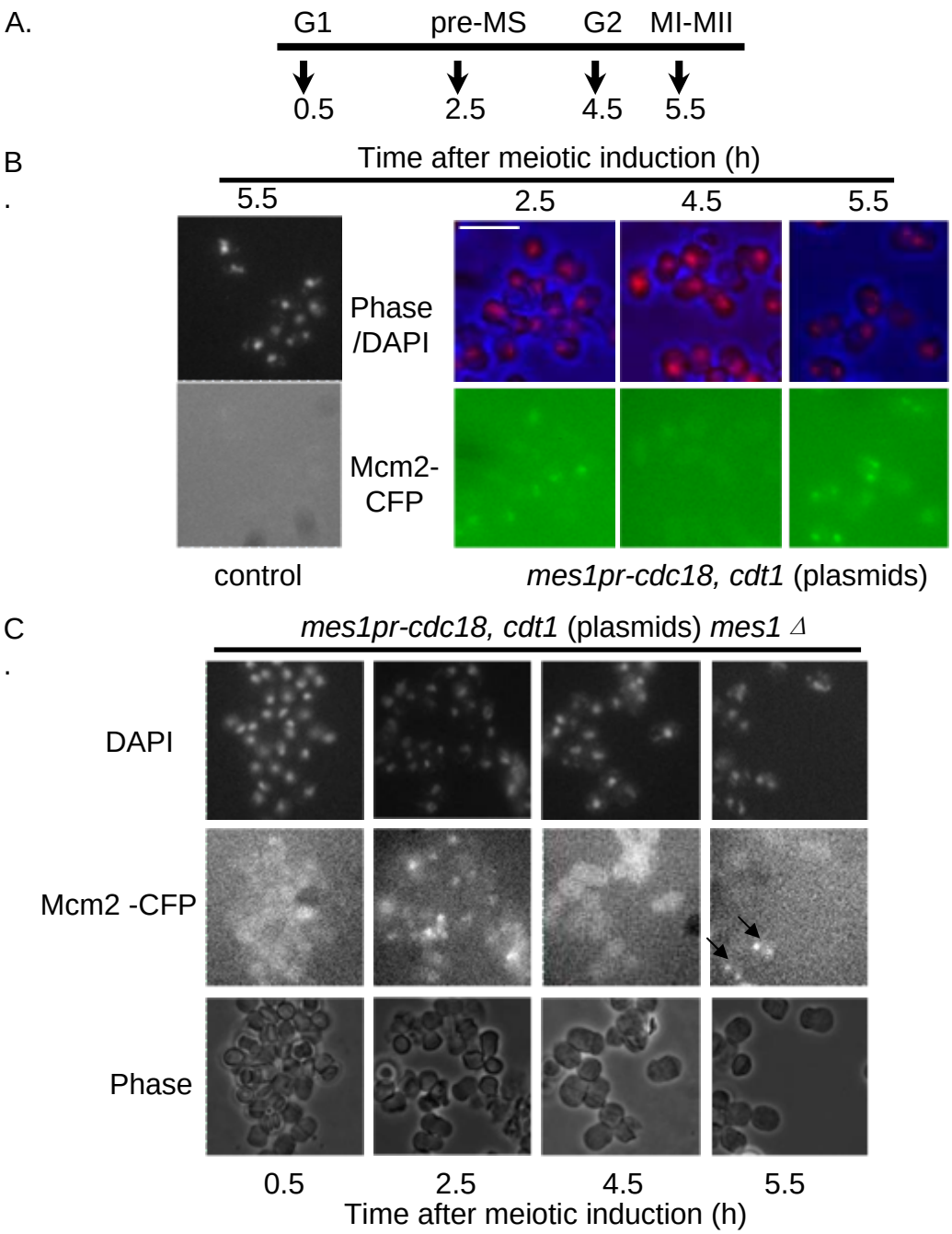


Figure 4.11. Effects on Mcm2 chromatin binding of over-expressing Cdc18 and Cdt1 from plasmids in late meiosis.

(A) Scheme of experiment for Mcm2-CFP chromatin binding assay. Mcm2-CFP (P2466) cells were released into a synchronised meiosis by inactivating Pat1, and at the time points shown, cells were zymolyase digested, detergent extracted, fixed and examined by fluorescence microscopy.

(B) Mcm2 rebinds to chromatin after meiosis I if Cdc18 and Cdt1 are over expressed (right panel, P2466). Cells after detergent extraction were imaged by fluorescence microscopy. Bar=10 μm. Mcm2 rebinding is not seen if Cdc18 and Cdt1 are expressed at physiological level even in a *mes1 Δ* background (left panel, P1756 – samples shown corresponds to 5.5 h after meiotic induction).

(C) As (B) except that *mes1 Δ* mutant cells over expressing Cdc18 and Cdt1 in late meiosis (P2467) were subjected to chromatin binding assays.

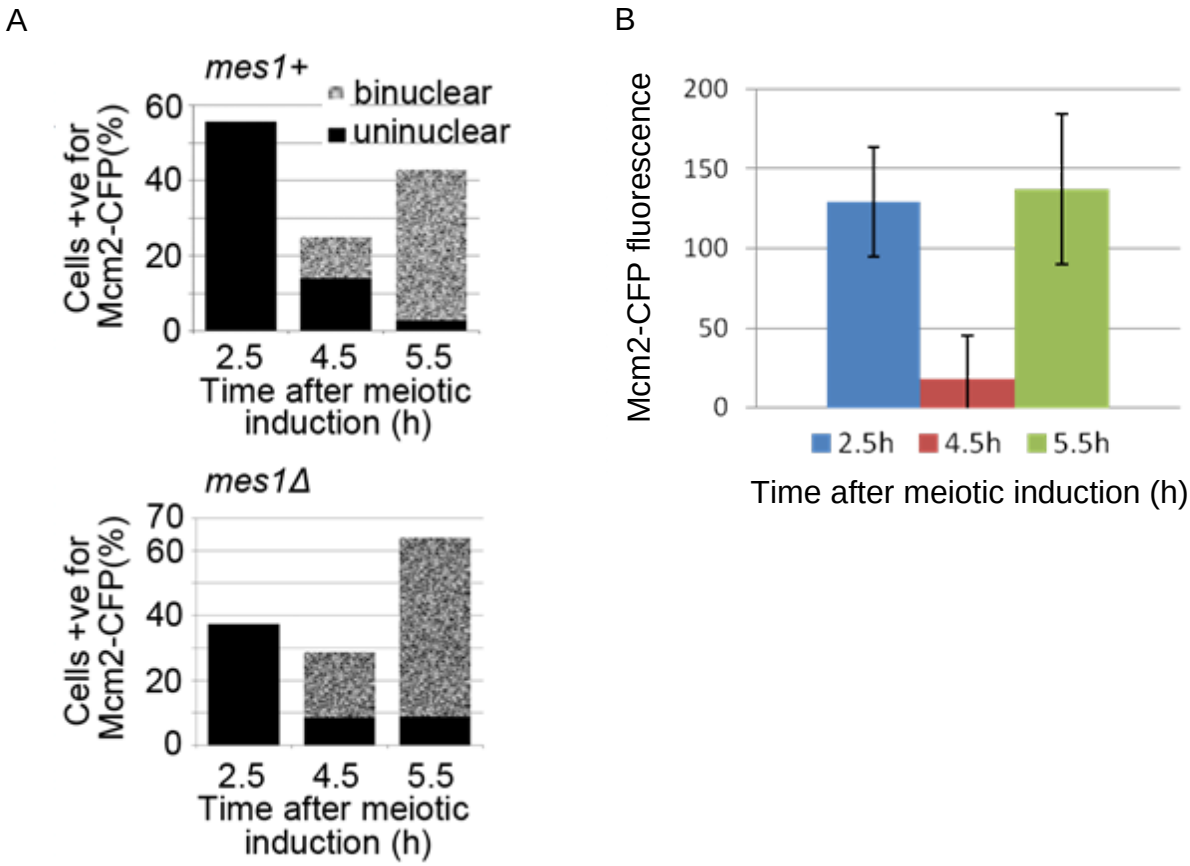
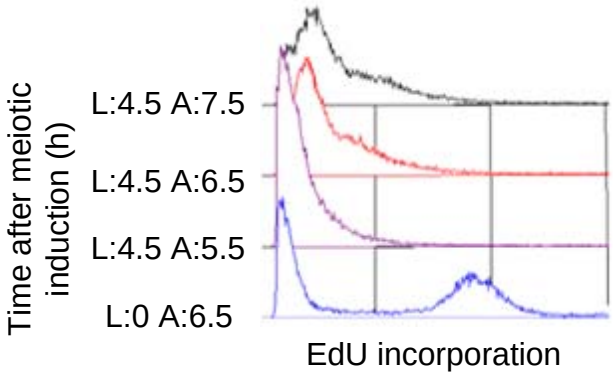


Figure 4.12. Quantitative analysis of cells shown in Fig. 4.11.
(A) Quantitation of percentage of cells from Fig. 4.11 B - right panel (P2466) and Fig. 4.11 C (P2467) showing Mcm2-CFP chromatin binding. 100 cells were counted for each sample and standard deviation is shown. Upper panel: *mes1*$^{+}$ strain P2466; lower panel: *mes1* Δ strain P2476.
(B) Quantitation of fluorescence intensity of nuclear Mcm2-CFP for *mes1*$^{+}$ (P2466); experiment shown in (A). 100 cells were measured for each sample and standard deviation is shown.

A



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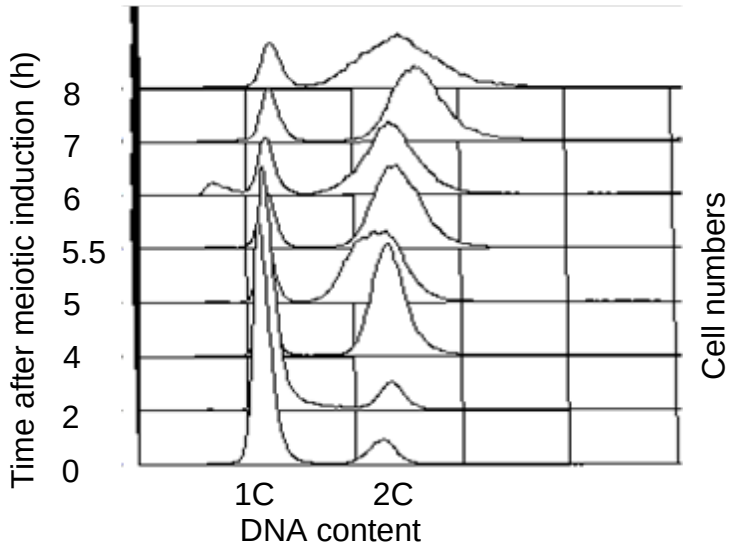


Figure 4.13. Effects on DNA replication of over-expressing Cdc18 and Cdt1 from plasmids in late meiosis.
(A) *pat1-114 TK hENT1* cells capable of over-expressing Cdc18 and Cdt1 during late meiosis (P2461) were induced to undergo meiosis, (i) with EdU at the time of meiosis induction or (ii) in the presence of EdU after pre-meiotic S phase. Cells were samples at the indicated time points, fixed and analyzed by flow cytometry after click reaction to detect EdU incorporation. L: label; A: analysis.
(B) +Cdc18 & Cdt1 over-expression (P2474) cells were arrested in G1 and then released into meiosis, sampled at the time points shown and then processed for analysis of DNA content by flow cytometry after SYTOX Green staining.

nuclear division only leads to limited re-replication in meiosis, which is very different, compared to the previous reports that over expressing Cdc18 and Cdt1 during G2 phase of the vegetative cell cycle can evoke efficient DNA re-replication (DNA contents accumulate to 64C or more), which is easily detected by conventional flow cytometry (Yanow *et al*. 2001). Therefore, I compared expression levels in meiosis with the G2 phase in mitosis in order to see whether there are meiotic-specific mechanism(s) repressing DNA synthesis after pre-meiotic S phase, which makes it more difficult to induce re-replication during MI/MII interval compared with G2 phase.

In this experiment, mitotic Cdt1 levels were stabilized by deletion of the gene *cdt2*. Over-expression of Cdc18 was regulated by the *nmt81* promoter and expressed from plasmids. G2 arrest was achieved by using *cdc25-22* mutant. Following the experiment scheme described by Yanow, Lygerou *et al*. (Yanow *et al*. 2001) (Fig. 4.14 A), cells were cultured without thiamine for 12 hours at the permissive temperature ($25^{O}C$) before shifting to the non-permissive temperature ($37^{O}C$) to arrest cells in G2 phase. Western blotting analysis suggested that at 21 hours after thiamine removal, expression of Cdc18 by the *nmt* promoter is less than the over expression levels in late meiosis controlled by *mes1* promoter (Fig. 4.14 C). However, flow cytometric analysis indicated that more re-replication was induced in the G2 cells than meiotic cells (Fig. 4.14 B). This means that when Cdc18 are expressed to similar levels in G2 and late meiosis, it is more difficult to induce re-replication after meiosis I than in G2 phase, implying meiosis specific inhibition of DNA replication after MI.

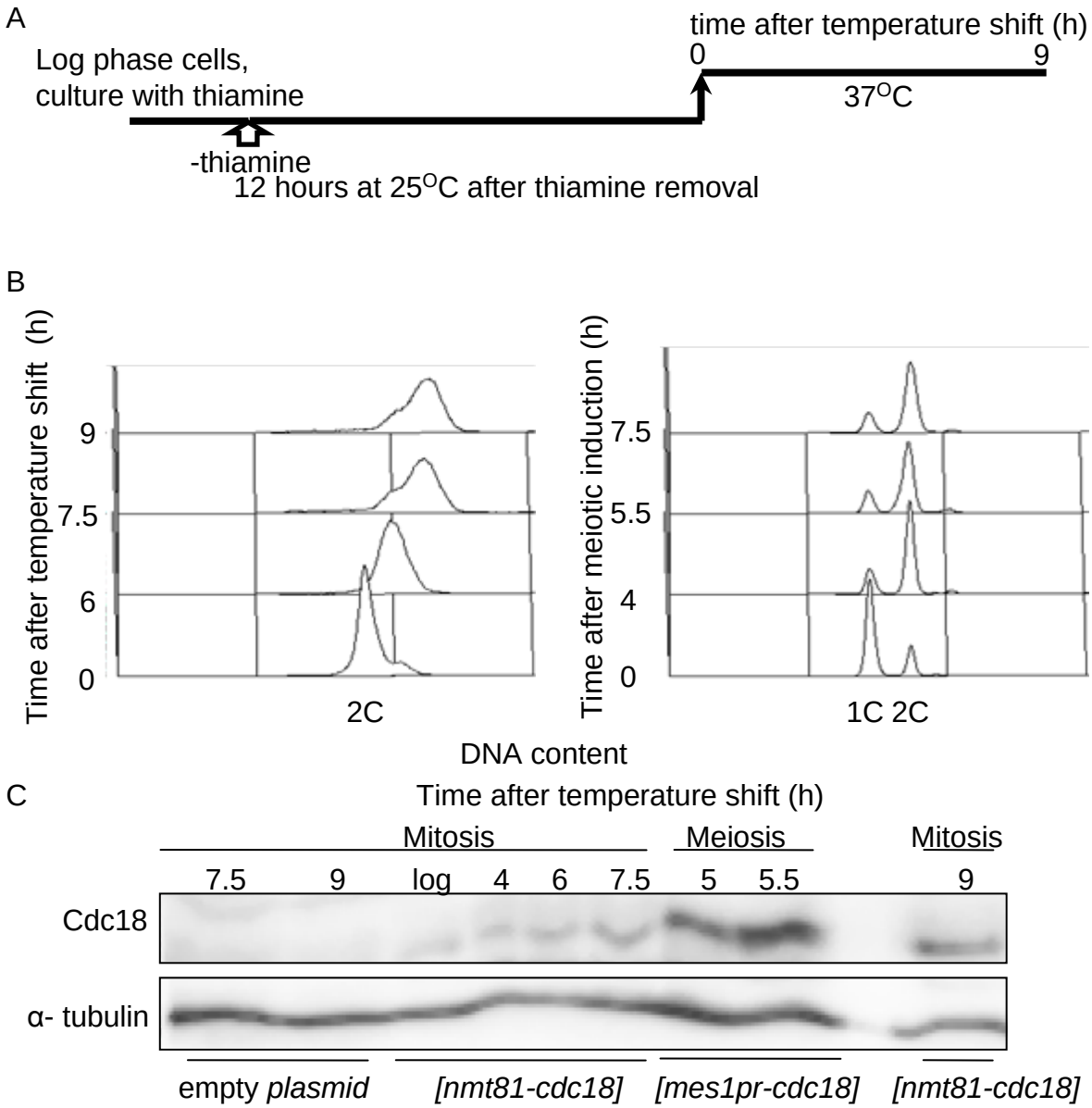


Figure 4.14 Effects of over-expressing Cdc18 and Cdt1 on G2 phase arrested cells.

(A) Scheme of experiment. *cdc25-22* cells were firstly cultured in EMM + thiamine medium to log phase, and then moved to EMM medium without thiamine at permissive temperature (25ºC) for 12 hours to switch on the *nmt* promoter. Then temperature was increased to the non-permissive temperature (37ºC) to induce G2 arrest. Normally after 3 hours at 37ºC all cells should be in G2 phase.

(B) Left panel: flow cytometric analysis of *cdc25-22 cdt2⊿ [nmt81X-cdc18]* cells (2950) that were induced to G2 arrest following protocol in (A). Right panel: flow cytometric analysis of *pat1-114 [mes1pr-cdc18] [mes1pr-cdt1]* cells (2951) that were induced into meiosis by inactivating Pat1.

(C) Western blotting analysis confirms that by using the strategy in (A), *nmt81-cdc18* could be expressed in G2 arrested cells (mitosis, *[nmt81-cdc18]*), and the expression level is less than that of the over expression during late meiosis regulated by *mes1* promoter (meiosis, *[mes1pr-cdc18]*). The numbers above: time after temperature shift (h) for those labelled “mitosis”, or time after meiotic induction (h) for those labelled “meiosis”.

4.2.5 DNA replication/damage checkpoint during MI/MII interval

Previous reports indicated that in budding yeast there is no chromosome segregation checkpoint after meiosis I (Shonn *et al*. 2000). In my project, I found that cells can still proceed into meiosis II when an incomplete round of DNA replication is initiated in the MI-MII interval (section 4.2.1 and 4.2.2), implying that there is no DNA replication checkpoint regulating entry into MII in fission yeast.

No previous reports have discussed if there is any DNA damage checkpoint after meiosis I in *S. pombe*. When diploid *pat1-114* cells (*rec12Δ* or *rec12*$^{+}$) were treated with bleomycin (to DSB-causing agent) after meiosis I, the kinetics of meiosis were similar to the mock-treated cells (Fig. 4.15 A and C). Pulsed-field gel electrophoresis confirms that under the experimental conditions bleomycin can produce broken chromosomes during either the vegetative cell cycle or in late meiosis (Fig. 4.15 B and D), as expected. In summary, these results suggest there is neither a DNA replication nor a damage checkpoint during late meiosis.

4.3 Discussion

In this Chapter I demonstrate that expression of Cdc18 and Cdt1 during the MI-MII interval leads to an incomplete round of DNA replication. The re-replication is inefficient, irrespective of whether Cdc18 and Cdt1 are

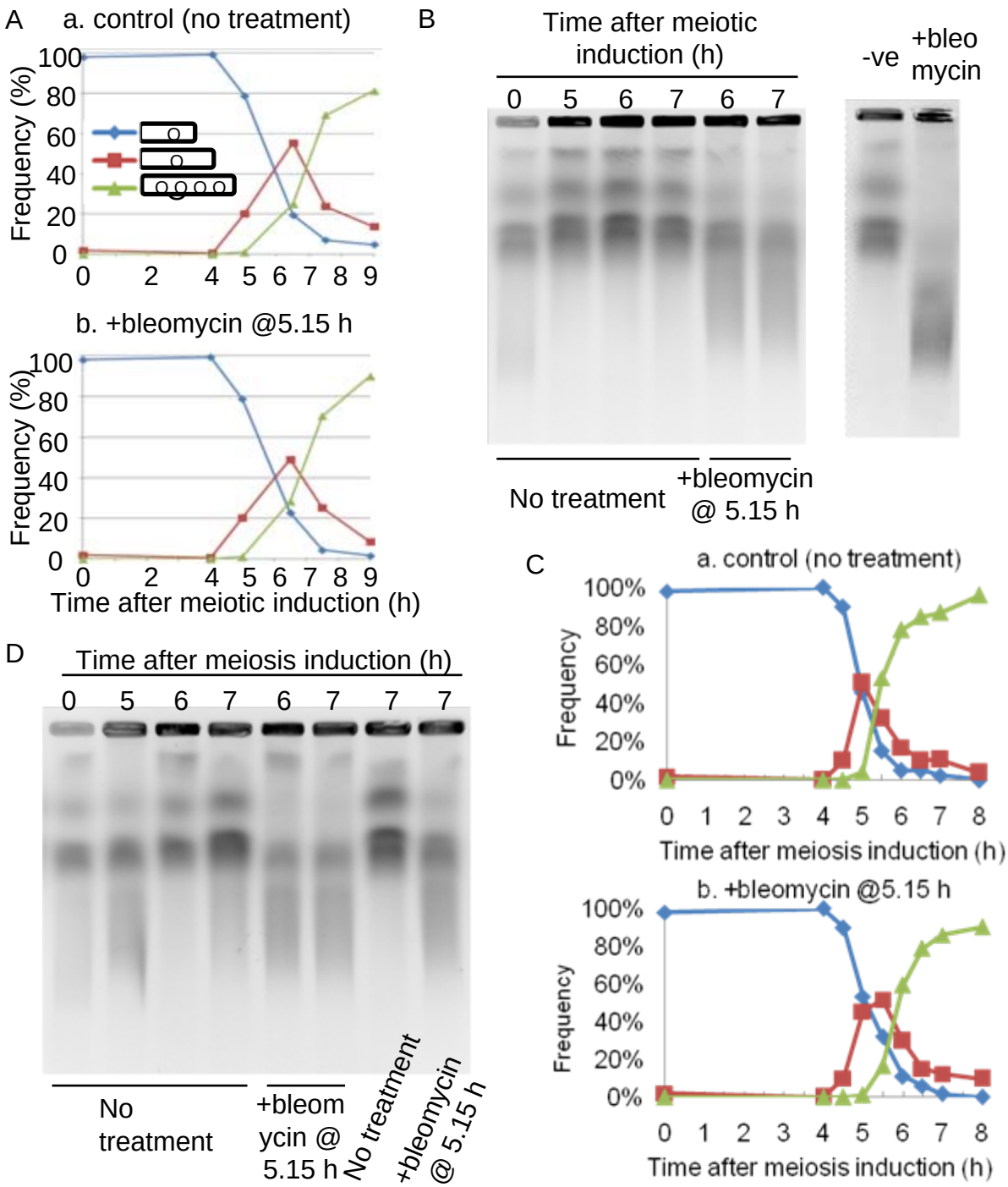


Figure 4.15 Effect of DNA damage on meiosis II execution.
(A) Diploid *pat1-114 rec12Δ* cells (p2456) were induced to undergo meiosis without bleomycin (a = control) or with addition of bleomycin (30 μg/ml) at 5.15 hours after meiosis induction (b). Meiotic progress was monitored by calculating the frequency of cells with one nucleus, two nuclei, and three or four nuclei at time points shown.
(B) PFGE analysis confirms that bleomycin (30 μg/ml) can lead to fragmented chromosomes in either synchronized meiosis (a) or unsynchronized log phase culture (b. 26°C, 1.5 hours bleomycin treatment,). The same strain as in (A) was used.
(C) As (A) except *rec12*$^{+}$ (diploid *pat1-114*) cells (strain p3008) were used.
(D) As (B)-a except strain p3008 was used.

expressed to levels comparable to their pre-meiotic S phase levels or over-expressed. There might be several reasons for the limited re-replication. Pre-RC complexes might only form on a subset of the origins, or initiation may be inefficient, or most origins perhaps fire but the elongation step could be rate limiting. To first clarify if licensing is still restricted, I carried out a chromosome-binding assay for Mcm2-CFP. This assay is crude but global compared to the ChIP (chromatin immuno-precipitation) assay and can give some indications as to the efficiency of pre-RC formation. I found that when Cdc18 and Cdt1 are over-expressed, re-licensing during MI-MII interval can be as efficient as that in pre-meiotic S phase. In addition, when Cdc18 and Cdt1 were expressed at levels found in pre-meiotic S phase, MCM chromatin binding was not detected, even if the *mes1* gene was deleted to allow Cdc13 proteolysis and thus depression of CDK activity, indicating inefficient licensing. This suggests that licensing is repressed not only by absence of Cdt1 and Cdc18, but also by Cdc13-independent mechanisms. Even in the mitotic cell cycle, when high levels of re-replication occur with Cdc18 and Cdt1 over-expression, Mcm chromatin binding is modest compared to the levels seen in a normal S phase (Yanow *et al*, 2001), suggesting that there may be additional constraints on MCM chromatin loading.

According to the MCM chromatin binding results, a direct question is that whether there are more restrictions for DNA synthesis during late meiosis in addition to regulation of pre-RC components. Another question is that if it is more difficult to induce re-replication during MI-MII interval than in mitotic cell cycle, suggesting meiotic-specific regulation of DNA replication.

In section 4.2.4, I show that In *S. pombe*, over-expressing Cdc18 and stabilizing Cdt1 can result in more dramatic re-replication during the mitotic G2 phase. So it is more difficult to induce re-replication in the MI-MII interval than in G2 phase, suggesting meiosis specific regulations of replication. It may be worth investigating if the fission yeast situation appears similar to other meioses, i.e. determining whether it is more difficult to induce re-replication in meiosis than in mitosis.

Previous reports suggest that in mammalian cells disruption of licensing control can lead to re-replication because it permits origins to be repeatedly licensed and fired during one cell cycle. Basically, if re-licensing of previously fired origins occurs during S phase via depletion of geminin, and the ATR-mediated intra-S phase checkpoint pathway was defective, such as in certain tumour cell lines, substantial re-replication occurs, resulting in an accumulation of DNA content more than 4C, and this is detectable by conventional flow cytometry (Zhu and Dutta 2006, Liu *et al*. 2007, Dorn *et al*. 2009). In this case, arresting cells in G2 phase is critical for increasing DNA content, as a deficient G2/M checkpoint prevents accumulation of over-replicated cells, and G2 arrest mediated by silencing cyclin A can restore the re-replication phenotype when the G2/M checkpoint is inactivated (Zhu and Dutta 2006). Therefore, the short time span between MI and MII may be one restriction limiting re-replication in *S. pombe*. To address this question, I will use a *spo4Δ* mutant to arrest cells in the MI-MII interval, and this will be described in Chapter 6.

There appears to be neither a DNA damage nor a replication checkpoint in the MI/MII transition in *S. pombe*. Once the first meiotic nuclear division occurs, this also seems to commit cells to meiosis II. It will be intriguing to see if this is a specific characteristic for unicellular organisms or whether it is a common feature for all eukaryotes. In higher eukaryotes, meiosis is always the only way to produce the next generation, so maintaining genome stability in meiosis may be more important. Also, meiosis does not occur under conditions of poor nutrition in higher eukaryotes. Thus multi-cellular organisms may restore the surveillance mechanism during meiosis if it is worthwhile to ensure genome stability in gametes. Meanwhile, considering that meiosis can be considered to have evolved from two consecutive mitotic cell cycles and DNA synthesis of the second cell cycle is “omitted” or “lost” during evolution (Furuno *et al*. 1994, John, 1990), it will be interesting to think about that how the checkpoint regulation is lost as well, and if there is any other biological significance to this. Maybe one reason is that re-replication is already strictly blocked by several “safeguard” mechanisms, i.e. repressing not only licensing but also other steps such as initiation and elongation, so there is no need for a checkpoint mechanism.

Chapter 5

Analysis of some other replication factors during late meiosis

5.1 Introduction

In Chapter 4 I showed that subverting the block to licensing in the MI-MII interval does not lead to an efficient round of DNA replication, suggesting initiation and/or elongation are also restricted. In addition, it is easier to induce re-replication in mitotic G2 phase than in the MI-MII interval when block to licensing is overcome, indicating meiotic-specific mechanism preventing re-replication between MI and MII. The dual blocks to licensing and initiation/elongation are not surprising. In principle, cells are not prepared for DNA synthesis during late meiosis, thus there may be multiple restrictions limiting DNA replication, such that when replication origins are efficient licensed, only partial re-replication is induced. This fact directly leads to the question: are there any other relevant replication factor(s) besides Cdc18 and Cdt1 that are influencing DNA replication in this interval? In this chapter, I have examined whether the initiation factor Dfp1, Sld2 and Sld3 might be relevant to inefficient replication in late meiosis.

The roles of these proteins (Dfp1, Sld2 and Sld3) in DNA synthesis have been reviewed in section 1.1.3. Sld2 and Sld3 are initiation factors that form an 11-3-2 (Sld2-Sld3-Dpb11) complex with Dpb11. It mediates association of Cdc45 and GINS, both of which are components of the CMG (Cdc45-MCM-GINS) complex functioning as a helicase (Tanaka *et al*. 2007, Zegerman and Diffley 2007). Dfp1 is the regulatory subunit of kinase DDK, which is required for activation of the MCM complex by phosphorylating the MCM proteins (Sheu and Stillman 2006, Chuang *et al*. 2009). In *S. pombe*, DDK also triggers chromatin association of Sld3 and then Cdc45 to form the Cdc45/MCM/GINS (CMG) complex that activates Mcm2–7 replicative helicase (Yabuuchi *et al*. 2006). In addition, Hsk1-Dfp1 has been suggested to have an additional role in meiotic recombination and first meiotic division (segregation of homologous chromosomes). In budding yeast, phosphorylation of Mer2 (an ancillary protein of Spo11) by Cdc7-Dbf4 (*S. cerevisiae* orthologues of Hsk1-Dfp1) is required for chromatin binding of Spo11 (*S. cerevisiae* homologue of *S. pombe* Rec12, which is required for generation of DSBs before meiosis I, see section 1.2.3) (Sasanuma *et al*. 2008; Wan *et al*. 2008). Meanwhile, chromatin associations of Rec114 and Mei4 that may facilitate Spo11 chromatin binding, are also blocked in a *cdc7Δ* mutant (Sasanuma *et al*. 2008). So mutants with deficient Hsk1 or Dfp1 have a reduced meiotic recombination frequency and are arrested in prophase I (Ogino *et al*. 2006,

Wan, Niu *et al*. 2008). DDK is also required in *S. cerevisiae* for expression of *NDT80*, a meiotic transcriptional factor promoting expression of genes involved in multiple late meiotic events (Lo *et al*. 2008, Matos *et al*. 2008). Meanwhile, DDK helps homologous chromosomes to bi-orient during prophase I, by regulating monopolar attachment of sister-chromatids to spindle via promoting recruitment of the monopolin complex to kinetochores (Marston 2009, Matos *et al*. 2008). Monopolin complex in budding yeast is composed of Hrr25, Lrs4, Csm1 and Mam1, among which phosphorylation of Lrs4, which is a critical step for monopolin localization rather than assembly, is regulated by DDK, and the polo-kinase Cdc5 in complex with Spo13 (Marston 2009, Matos *et al*. 2008).

Additionally, DNA synthesis also requires a supply of dNTPs as they are the building blocks. Therefore I also examined subunits and an inhibiting subunit of ribonucleotide reductase. Levels of these proteins were checked during a synchronized meiosis, mainly using western blotting and fluorescence microscopy techniques.

5.2 Results

5.2.1 Sld2 and Sld3 are up-regulated during late meiosis

In *S. pombe*, microarray analysis regarding the transcriptional program suggests that Sld2 is expressed to a constant level throughout cell cycle, whilst during meiosis, Sld2 mRNA slightly peaks during pre-meiotic S phase and before MI. Sld3 mRNA level goes up in meiotic G2 phase and then the level goes down after MII (Mata *et al*. 2002). However, how these two proteins are regulated during meiosis is still obscure.

Thus protein levels of Sld3 and Sld2 (Drc1) were examined during a synchronized meiosis to see if they are detectable during the MI-MII interval. Western blotting indicated that after nitrogen starvation, Sld3 levels are detectable but lower than in a log phase culture (Fig 5.1 A). After meiotic induction Sld3 levels rise around the time of pre-meiotic S phase (2h to 3h) and then remain at a similar level for the rest of the time course (Fig 5.1 A). Progress of meiosis of this Sld3 FLAG tagged strain was monitored by flow cytometry and fluorescence microscopy (Fig. 5.1 B and C).

Sld2 is at a similar level in G1 arrested cells and log phase cells. Interestingly two Sld2 bands are seen, which may correspond to different phosphorylation states of the protein (Fig 5.2 A). At t=0 h the faster migrating band predominates but 2-4 h after meiotic induction, Sld2 migrates as a single slower migrating band (Fig 5.2 A). At late time points the overall level of Sld2 appears to slightly increase and the proportion of Sld2 in the faster migrating

form increases. Sld2 is known to be phosphorylated by CDK at around the time of S phase initiation (Zegerman and Diffley 2007) and it is possible that the slower migrating band corresponds to the phosphorylated version of the protein.

In summary, Sld2 and Sld3 are abundant during late meiotic stages, suggesting that they are not the limiting factors for DNA replication during this period, although this analysis does not show whether they are in their active CDK phosphorylated states.

5.2.2 Dfp1 is down-regulated after meiosis I

Previously, Hsk1 was found to be up-regulated during late meiosis (Namdar 2006). I found that Dfp1, the regulatory subunit of replication kinase DDK, was not detected in nitrogen-starved G1 arrested cells but became detectable 3 h after release into meiosis (Fig. 5.3 A, B). Levels peaked at around 5h, i.e. Dfp1 was abundant in a period before meiosis I including late pre-meiotic S phase and the subsequent G2 phase (Fig. 5.3 A, B), consistent with previous publications that Hsk1-Dfp1 is involved in meiotic specific double strand breaks formation and meiotic chromosome orientation, as reviewed in section 5.1 (reviewed in Marston 2009).

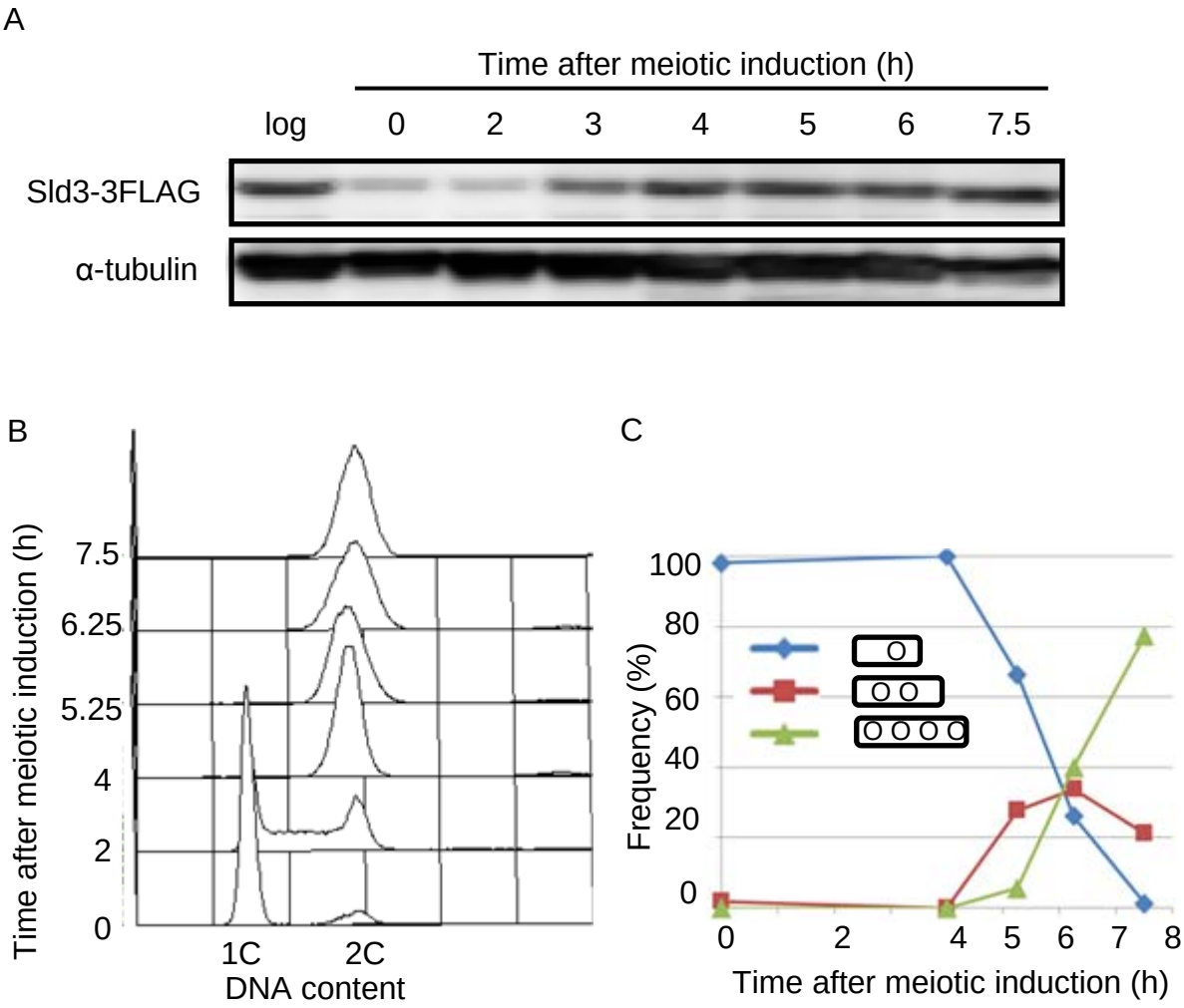


Figure 5.1 Levels of Sld3 during meiosis.
(A) Cells of *sld3-3FLAG pat1-114* strain (P3019) were arrested in G1 by nitrogen starvation and released into meiosis at time 0. Western blotting analysis using anti-FLAG antibody showing that Sld3 is expressed during meiosis. Protein level of α-tubulin is shown as a loading control.
(B) Flow cytometric analysis of DNA content of cells from (A).
(C) Meiosis progress was monitored from the frequency of cells with one nucleus, two nuclei and three or four nuclei at time points shown.

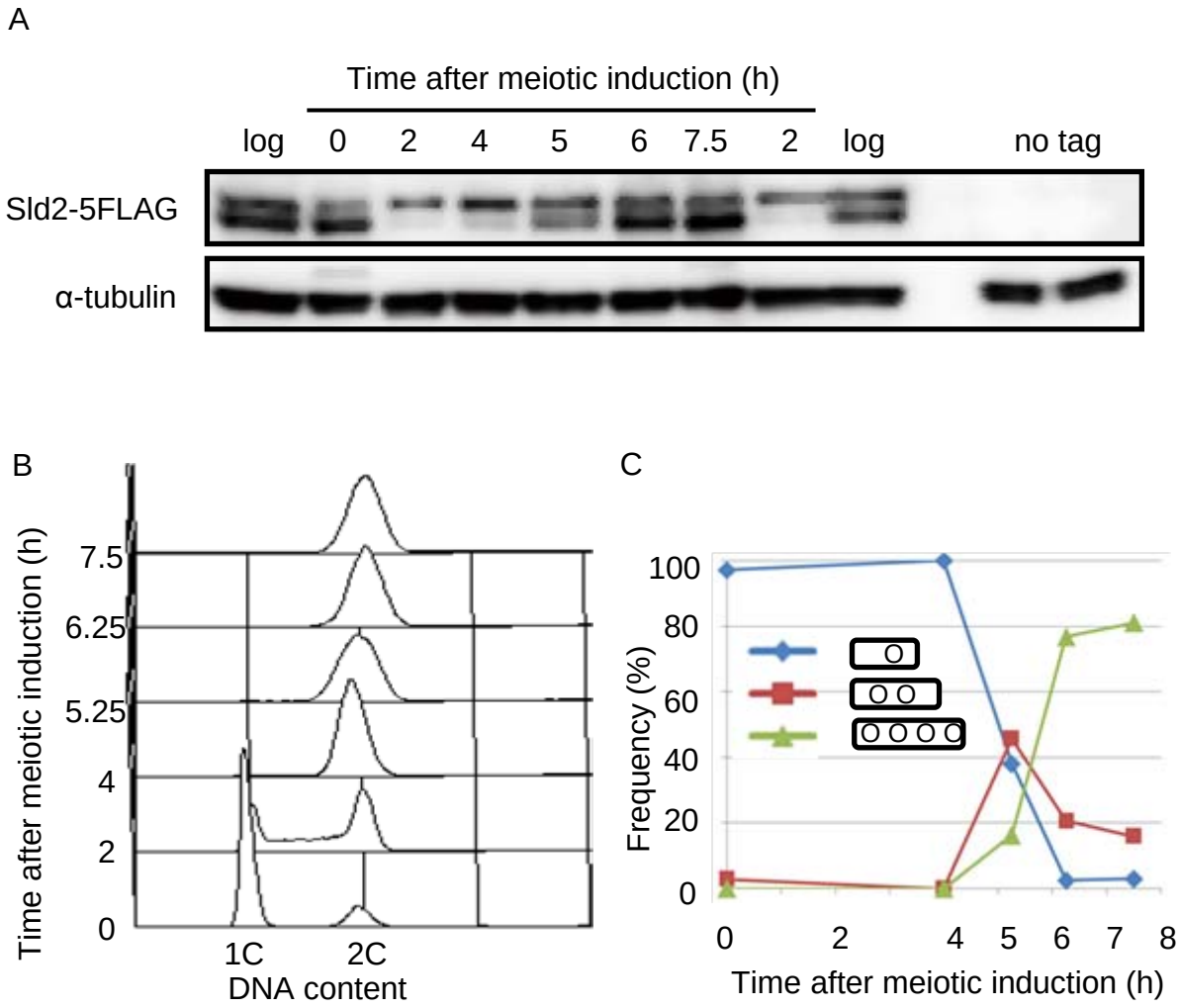


Figure 5.2 Levels of Sld2 during meiosis.
(A) Cells of *sld2-5FLAG pat1-114* strain (P3022) were arrested in G1 by nitrogen starvation and released into meiosis at time 0. Western blotting analysis of cells sampled at time points shown using anti-FLAG antibody Protein level of α-tubulin is shown as a loading control.
(B) Flow cytometric analysis of cells from (A).
(C) Meiosis progress was monitored from the frequency of cells with one nucleus, two nuclei and three or four nuclei at time points shown.

Dfp1 disappears in late meiosis (Fig. 5.3 A, 7~8 h). Further fluorescence microscopy analysis indicates that Dfp1 is absent in binucleate cells that have completed meiosis I and tetranucleate cells (Fig 5.3 B, 5~8 h), which implies its down-regulation after metaphase I. Previous reports also suggests that in budding yeast during meiosis the highest level of Dbf4 (orthologous to *S. pombe* Dfp1) is in metaphase I (Lo *et al*. 2008, Matos *et al*. 2008). Presumably this down-regulation in meiosis I anaphase is also related to Anaphase-Promoting Complex (APC) mediated proteolysis as happens in mitotic anaphase. These findings suggest that Dfp1 may be one crucial factor restricting DNA replication during late meiosis besides Cdc18 and Cdt1.

5.2.3 Regulation of RNR components during late meiosis

Ribonucleotide reductase (RNR) catalyses the direct reduction of ribonucleotides to the corresponding deoxyribonucleotides, which are building blocks of DNA synthesis (Elledge *et al*. 1992). RNR is an iron-dependent enzyme, composed of four subunits: two large RNR1 subunits (Cdc22 in *S. pombe*) and two small ones RNR2 (Suc22). RNR2 contains a stable tyrosyl radical, critical for initiation of the radical-dependent nucleotide reduction (Stubbe and Riggs-Gelasco 1998). During replication, RNR plays an important role in regulating the rate of DNA synthesis by modulating availability of dNTPs (Herrick and Sclavi 2007). Disturbance of RNR function, such as using

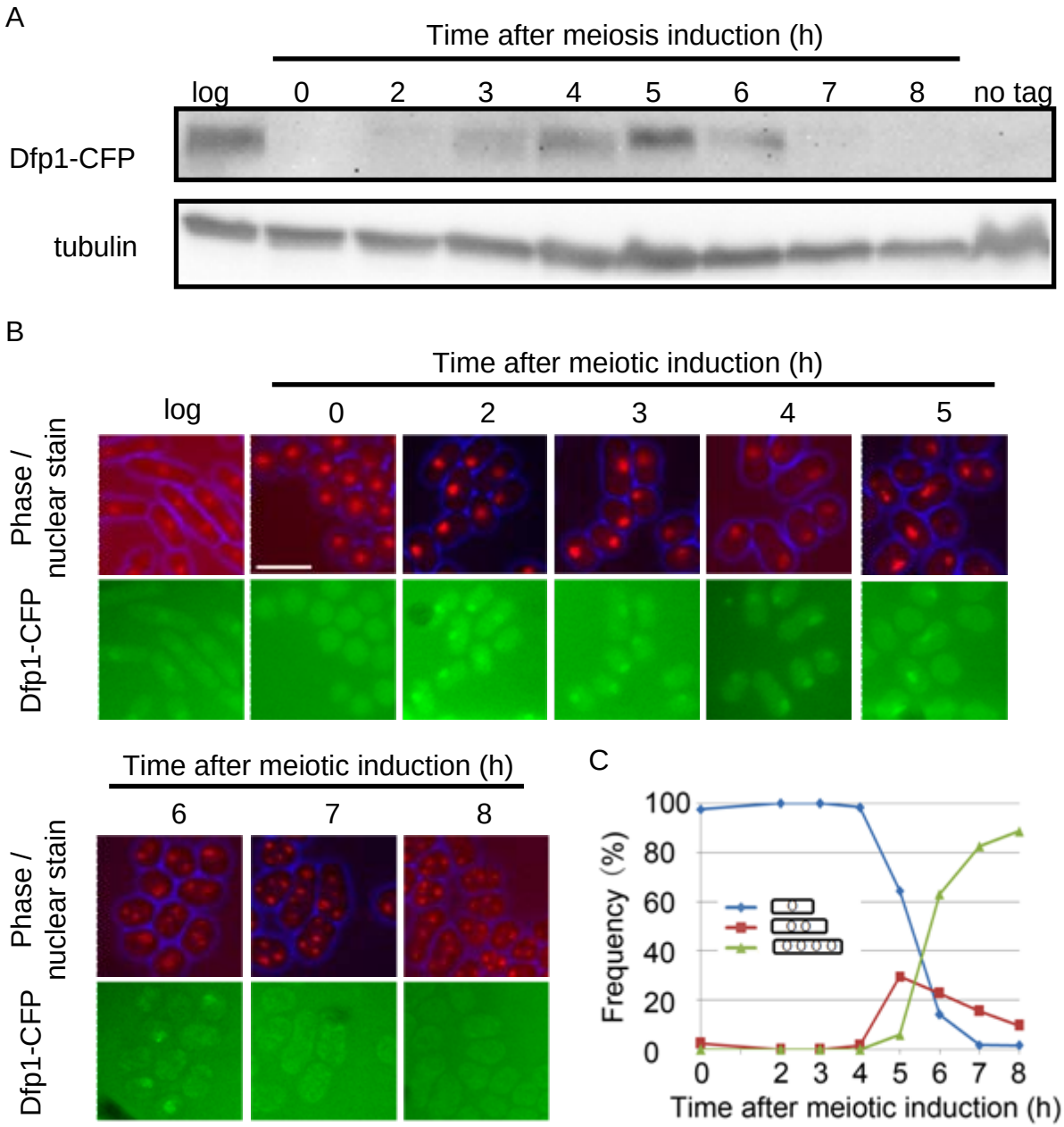


Figure 5.3 Protein levels of Dfp1 during meiosis
(A) Cells of *dfp1-CFP pat1-114* strain (P3026) were arrested in G1 by nitrogen starvation and released into meiosis at time 0. Western blotting analysis using anti-GFP antibody showing that Dfp1 is down-regulated during late meiosis. Protein level of α-tubulin is shown as a loading control. No tag = p138.
(B) Microscopy analysis of cells from (A) indicates that Dfp1 is only detectable in cells with a single nucleus. Bar = 10μm
(C) Meiosis progress was monitored from the frequency of cells with one nucleus, two nuclei and three or four nuclei at time points shown.

hydroxyurea to inhibit RNR by inactivating its tyrosyl radical (Yarbro 1992), perturbs dNTP pools and causes cell cycle arrest in S phase due to paused replication forks. So during replication the dNTP concentration via RNR should be just right: too high and replication is mutagenic due to high dNTP levels, whilst if too low, the replication checkpoint is activated due to arrested replication forks (Mathews 2006).

RNR protein levels are subject to transcriptional regulation. In addition, post-translational regulation of RNR activity includes sub-cellular compartmentation. In budding yeast, RNR1 is always cytoplasmically localized whilst RNR2 is sequestered in the nucleus for most of the cell cycle, only entering the cytoplasm in S phase and in response to DNA damage, thus forming the active complex with RNR1 (Lee and Elledge 2006). In fission yeast, localizations of RNR subunits are similar. In addition, a small *S. pombe* protein Spd1 inhibits RNR activity by (i) promoting Suc22 nuclear import; and (ii) inhibiting RNR by binding to Cdc22 (Nestoras *et al*. 2010, Hakansson *et al*. 2006). Proteolysis of Spd1 in S phase or after DNA damage is mediated by the Pcu4–Ddb1–CSN ubiquitin ligase, where Cdt2 is a regulatory subunit. It results in nuclear export of Suc22 and release of inhibition of Cdc22, which correlates with a requirement for dNTP during DNA replication or damage repair thus activation of RNR (Liu *et al*. 2005, Liu *et al*. 2003).

Therefore, to see if cells are competent to provide enough dNTPs at all stages of meiosis, the expression patterns of Cdc22, Suc22 and Spd1 during meiosis were examined by western blotting and fluorescence microscopy. Suc22 levels were constant through meiosis; in spite of that its level seemed to be slightly low after nitrogen starvation and in late meiosis, i.e. the highest level of Suc22 is in the period of pre-meiotic S phase (Fig 5.4 A). The nuclear signal of Suc22 is stronger than its cytoplasmic signal in pre-meiotic S phase cells or cells in vegetative growth; however, nuclear Suc22 is less obvious in the tetra-nucleate cells in late meiosis (Fig. 5.4 B). Cdc22 was up-regulated during late meiosis (Fig. 5.5 A), and it was always pancellular (both in nucleus and cytoplasm) in meiosis (Fig. 5.5 B).

Interestingly, despite the fact that both Cdc22 and Suc22 are present in late meiosis, Spd1 was down-regulated during pre-meiotic S phase but re-appeared afterwards (Fig. 5.6 A), suggesting that the dNTP pool might be disturbed during late meiosis. Fluorescence microscopy analysis indicated a similar result and in late meiosis, both cytoplasmic and nuclear Spd1-YFP fluorescence was much higher than that in pre-meiotic S phase (Fig. 5.6 B). After meiosis II the Suc22-GFP signal seemed to be increased in the cytoplasm, which may suggest that even if Spd1 inhibits RNR activity during late meiosis, probably it is not through anchoring Suc22 inside the nucleus.

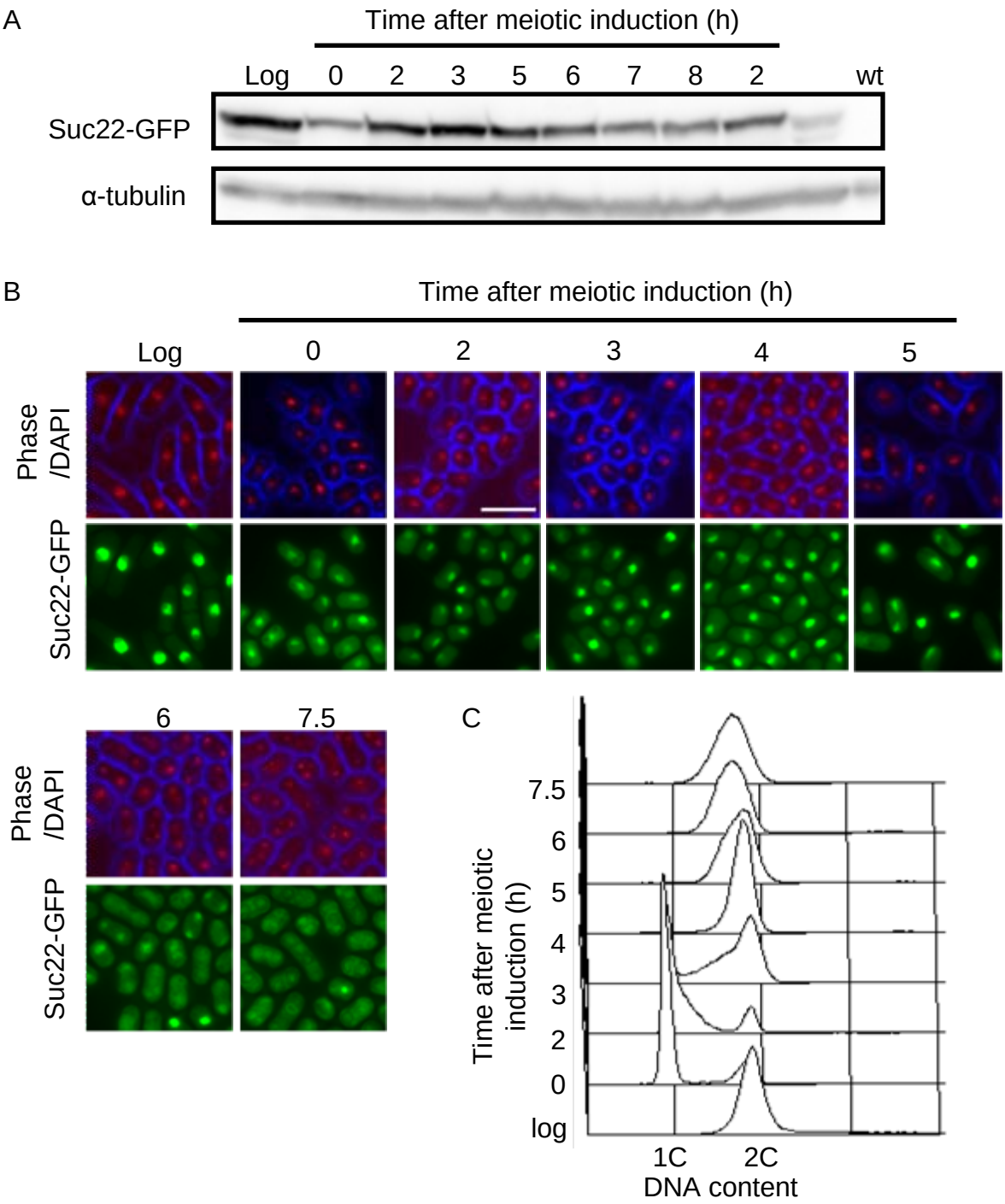


Figure 5.4 Protein levels of RNR subunit Suc22 during meiosis.
(A) Cells of *suc22-GFP pat1-114* strain (P3017) were arrested in G1 by nitrogen starvation and released into meiosis at time 0. Western blotting analysis using anti-GFP antibody showing that Suc22 is expressed throughout meiosis.
(B) Cells from (A) were fixed in methanol/acetone and observed by fluorescence microscopy following DAPI staining. Note that though the Suc22 protein level does not change too much during meiosis according to western blotting analysis, it seems to diffuse into cytoplasm in the tetra-nucleate cells. Bar = 10μm.
(C) Flow cytometric analysis of DNA content of cells from (A).

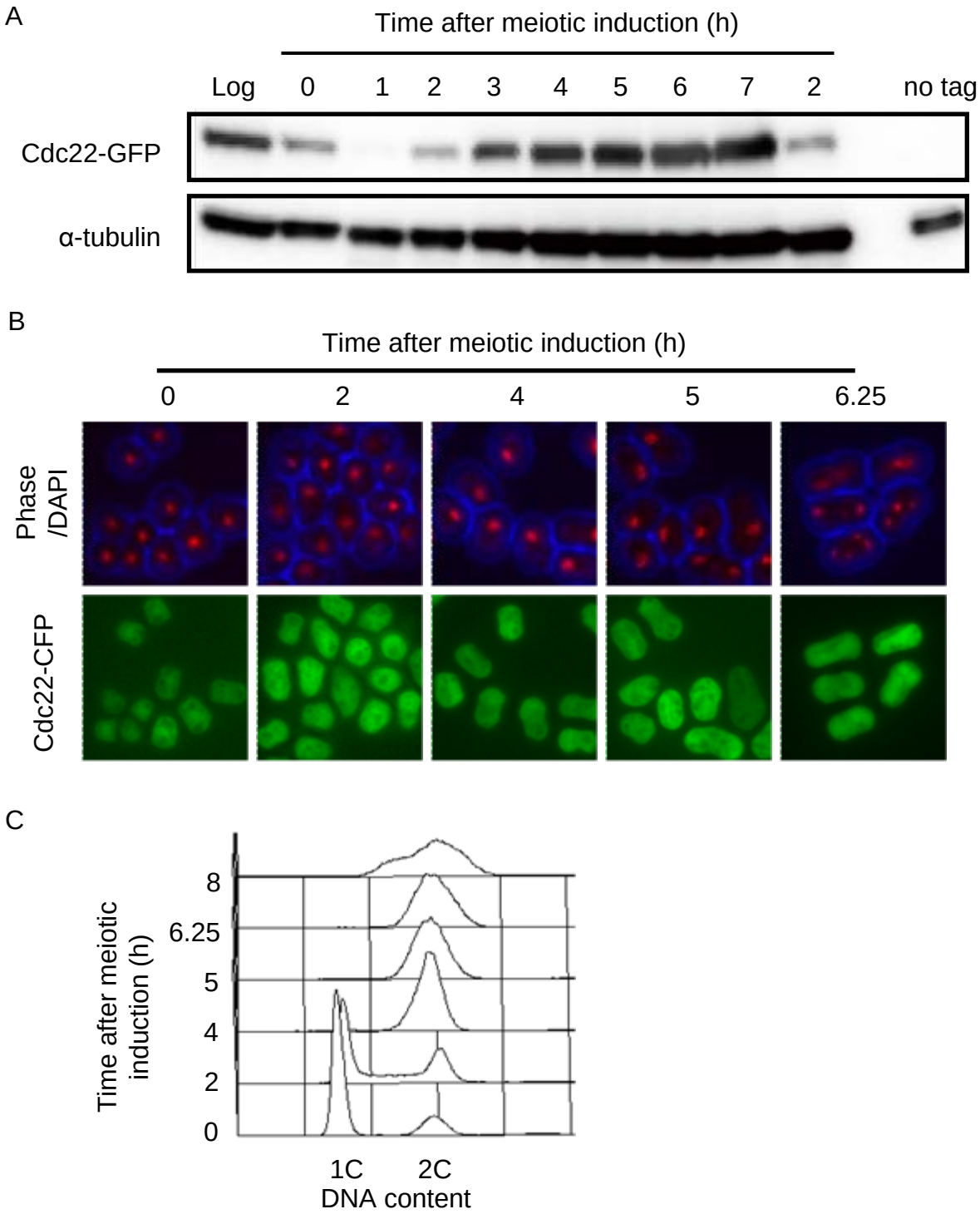


Figure 5.5 Protein levels of RNR subunit Cdc22 during meiosis.
(A) Western blotting analysis shows that Cdc22 is present from pre-meiotic S phase to late meiosis (strain P3018).
(B) Cells from (A) were fixed in methanol/acetone and observed by fluorescence microscopy following DAPI staining. Bar = 10μm.
(C) Flow cytometric analysis of DNA content of cells from (A).

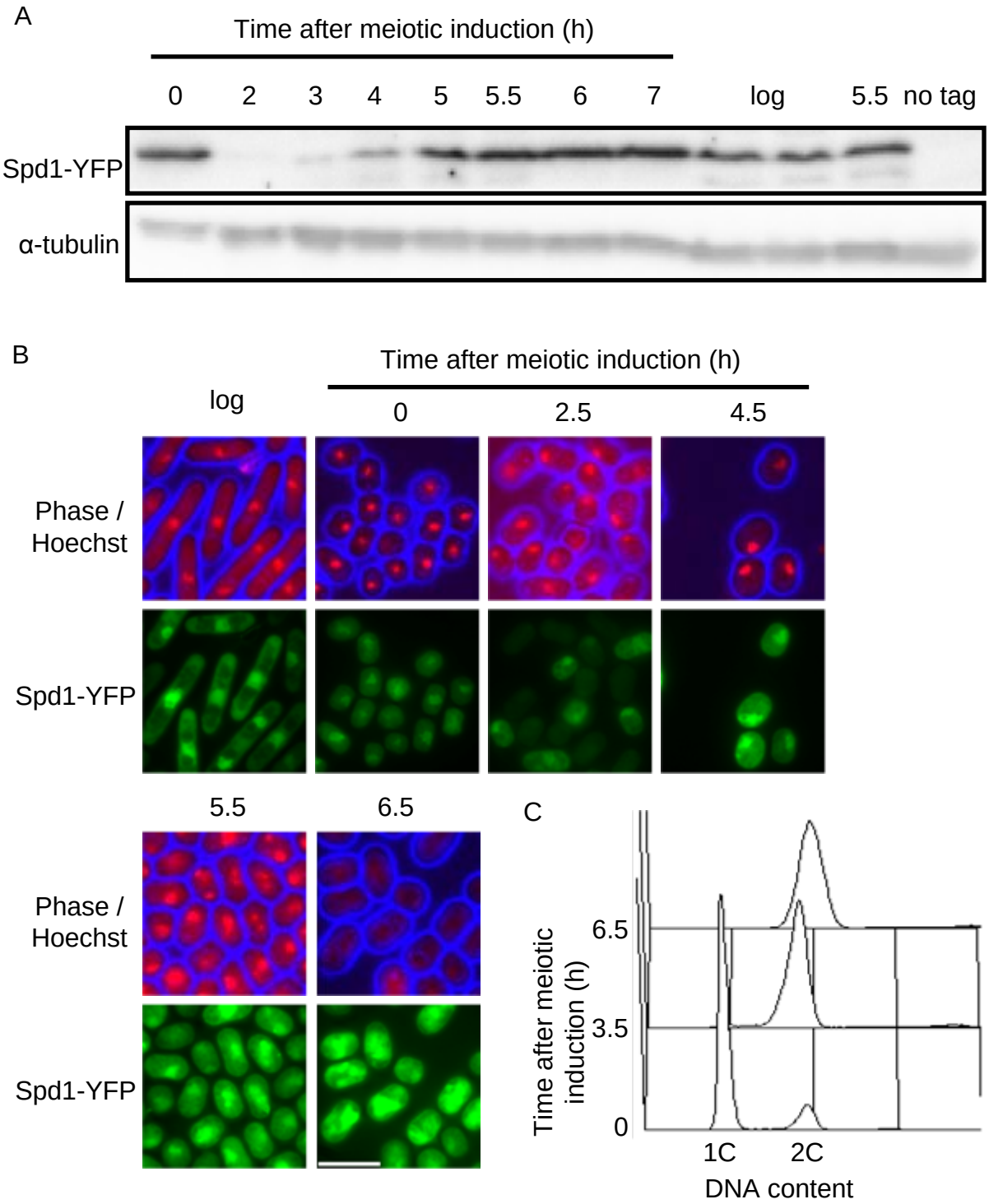


Figure 5.6 Levels of RNR inhibitor Spd1 during meiosis.
(A) Western blotting analysis shows that Spd1 is down-regulated during pre-meiotic S phase but up-regulated during late meiosis (strain P2383).
(B) Cells from (A) were stained by Hoechst and analyzed by fluorescence microscopy. Note that Hoechst cannot get into spores when they formed in late meiosis. Bar = 10μm.
(C) Flow cytometric analysis of DNA content of cells from (A).

5.3 Discussion

In this chapter, I have examined the expression levels of some other replication factors in meiosis. One kinase, DDK (Hsk1-Dfp1), seems to be inactivated after meiosis I by proteolysis of its regulatory subunit Dfp1, possibly mediated by APC/C complex as in mitosis. DDK is required for promoting initiation, together with CDK. In addition DDK regulates origin efficiency during replication; in *S. pombe* increasing or decreasing DDK levels correspondingly increases or decreases initiation efficiency (Patel *et al*. 2008). Also, concentrating DDK near an origin can promote firing efficiency of this origin (Patel *et al*. 2008). So less availability of Dfp1 during late meiosis may prevent efficient initiation.

At the same time, elevated protein levels of Spd1 may inhibit RNR activity after pre-meiotic S phase, though RNR subunits themselves seem to be stable during late meiosis. So elongation may be restricted due to limited dNTPs, which could constitute another mechanism for a replication block in late meiosis.

It is not surprising that several pathways may be coordinated to repress replication between the MI-MII interval. Similar basic components in processes such as replication or chromosome segregation are used in both mitosis and

meiosis, so cooperation between different regulators may provide a reliable and efficient way to fine-tune these processes to satisfy those specific requirements in meiosis. At the same time, because meiotic progenies form the basis for a new individual, it makes sense to ensure that error rates in DNA replication are decreased to the minimum during meiosis, thus multiple parallel safeguard mechanisms may be used to prevent mistakes. Given that genomes may contain hundreds (yeast) or many thousands (higher eukaryotes) of replication origins, and just one misfiring origin could promote genome instability, it makes sense not only to block origin firing but, if one origin does misfire, to stop the forks from moving too far. In addition, this paradigm should not only be relevant to replication control, but also may apply to other physiological pathways such as regulation of recombination and sister chromatid mono-polar attachments in MI. Thus in these processes, if one regulator is untimely activated, the target protein may still be kept inactivated as other regulator(s) are not functional. For example, sequential phosphorylation of Mer2 by CDK and DDK are necessary to promote DSBs, and dual phosphorylations of Lrs4 by DDK and Cdc5-Spo13 to ensure sister-chromatid monopolar attachment, may provide a failsafe mechanism to minimize frequency of error occurrence in meiosis (Marston 2009).

Chapter 6

Further investigations into DNA replication induced in the MI-MII interval

6.1 Introduction

Following the previous Chapter, I will focus on proteins that show interesting expression patterns during late meiosis, especially Spd1 and Dfp1, in an attempt to identify additional regulatory mechanisms responsible for the incomplete replication when licensing is induced after meiosis I. As explained in the previous chapter, Spd1 is an inhibitor of RNR. So it influences replication by regulating dNTP levels via repressing RNR (Liu *et al*. 2003, Nielsen 2003). Dfp1 is the regulatory subunit of an S phase protein kinase DDK (Dpf1-dependent kinase) that is required for origin firing by stimulating assemblies of multiple proteins at origins that activate the helicase activity of Mcm2–7 complex (Francis *et al*. 2009) (see Chapter 1 for details).

In addition, I have tried to fine-tune CDK activity after meiosis I to see if it can lead to more re-replication when Cdc18 and Cdt1 are present. CDK has opposing effects in DNA replication depending on which stage of replication is

being considered, and it is already well established that intermediate CDK activity is maintained during the MI-MII interval as an evolutionarily conserved feature. Some of these fission yeast experiments will be similar to those have done in *Xenopus* eggs previously, for example, examining role of Wee1 and phosphorylation state of Cdc2 in late meiosis, and inhibition of protein synthesis after meiosis I, to see how different these two systems are. Finally, by using DNA combing analysis, I have examined if initiation and/or elongation are restricted when block to licensing is subverted.

6.2 Results

6.2.1 Stabilizing Dfp1 during MI-MII interval by deleting its N-terminal APC box

In budding yeast, levels of Cdc7 (orthologue of *S. pombe* Hsk1) are constant throughout the cell cycle but Dbf4 (orthologue of *S. pombe* Dfp1) is subject to APC/C mediated proteolysis in mitotic anaphase and remains very unstable during pre-Start G1 phase (Ferreira *et al*. 2000). In the previous chapter (section 5.2.2), I have also demonstrated that in fission yeast, Dfp1 is unstable during anaphase of meiosis I and thereafter, as the CFP tagged Dfp1 was present in cells with single nucleus in early meiosis but disappeared in the binucleate and tetra-nucleate cells (Fig. 5.3 B).

To stabilize this protein in late meiosis, I constructed a mutant Dfp1 with a deletion from its N-terminal second to the thirteenth amino acids, which removes an APC destruction box (a conserved signal sequence “RxxL” that is recognized by APC/C) (Fig. 6.1 A, see section 2.1.4 for details). A strain expressing this N-terminal mutated *dfp1* (named *dfp1M*) instead of the wild-type *dfp1* is viable, indicating that it is functional.

To determine whether this mutation affected the stability of Dfp1M, the strain was analysed by fluorescence microscopy and western blotting. During vegetative growth, wild type Dfp1-CFP is only present in cells with a single nucleus and the Dfp1-CFP signal is very weak (Fig. 5.3 B) but Dfp1M-CFP is highly stable and localized in the nucleus throughout the cell cycle (Fig. 6.2 A), indicating its stabilization during mitotic anaphase. In meiosis, Dfp1 wt disappeared after meiosis I (Fig. 5.3 B) whilst Dfp1M is present in binucleate cells in late meiosis (Fig. 6.2 A), indicating that the Dfp1M protein is not degraded during meiosis I. Western blotting also indicates its stabilization, if comparing the Dfp1 protein levels at the same time point. For example at 5.5 hours, the level of Dfp1M was much higher than the wild-type protein (Fig. 6.2 B).

Therefore, a strain was constructed expressing Dfp1M and also expressing

A

MNLGRCPLAPRSANIVLPKHDAVSKQKEYRIEEKTNEAQREEIITWKDNRED
EGEVKTDFEVVNNENIITTTPKHQTVITPKSYRKSVKRIKHDAPQNEDIPVMKG
LAPINADTESKAESMAAGKVLGSKNSSQKARLQEWKRQYKKAFPHFRFYLD
GCDPSVAHRVKKQIQQLGGHVETFFSGNVTHVATVRAIQDVSVKYAKQDVIT
KARQLNMKIWSMEKLCNRVLKTLMENDQCTTNAPTKQGNDLSYLLYVEKVQ
GTNERDLSVPRQDFVHFRGPYLYVHDIANIYKPILLREWQKPLPDRDVPWPT
FRATSIGRCPFVPETKYRLSTSKSLVAKNDQQLLQRQSQEPSLILRANSMKAS
LPDISNTGISGMNTNTTYNTNINNTPQTAISGITQDTSPSIRTNCHHCLDDGMQ
ASGIVQSNLTSAAMSNNSAIRSGSAASVPVVTINGRDIAELKKRIIQQKSGMIG
KDYSYKAMLHNTSQRKIRVDAKPGYCENCREKFDNFESHIRSSRHRRFAENN
DNFKDLDELFALVQRPLRPD

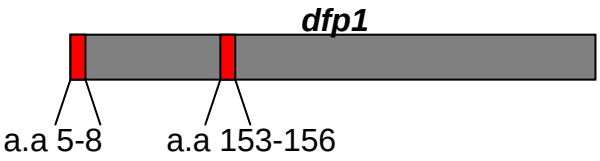
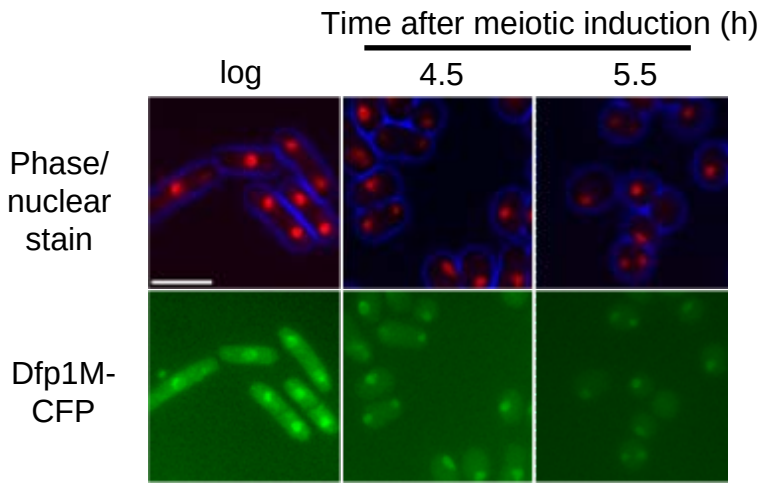


Figure 6.1 Protein sequence of Dfp1 and stick diagram showing two APC destruction boxes in red.

A.



B

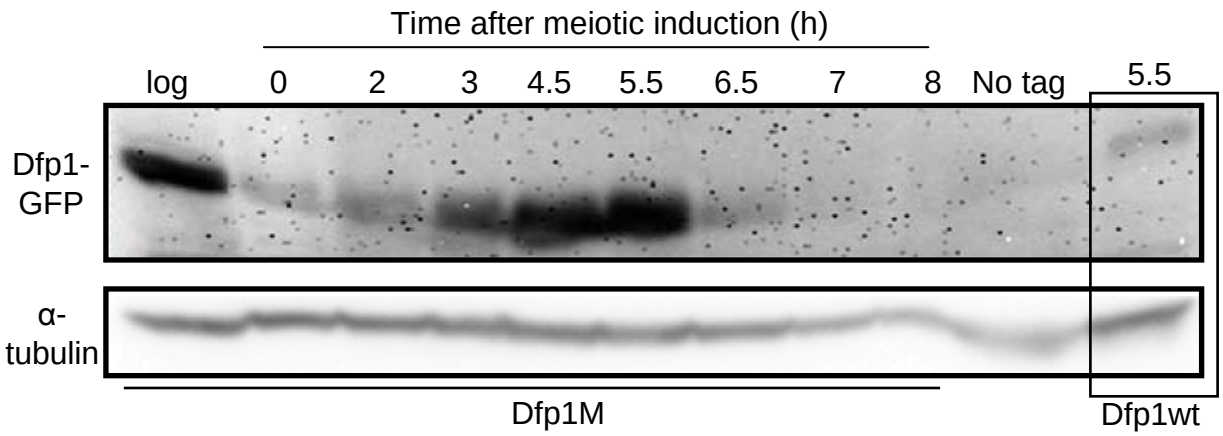


Figure 6.2 Dfp1 can be stabilized after meiosis I by deleting its N-terminal APC destruction box.
(A) Microscopy analysis of cells with *dfp1M* mutant (*dfp1M-CFP-hyg::dfp1 pat1-114*. P3025) indicates Dfp1's stabilization during mitotic anaphase and anaphase of meiosis I.
(B) Western blotting analysis of Dfp1M (P3025) during a synchronized meiosis. Protein level for wt protein (from P3026) during late meiosis (5.5 h time point) is also shown (right lane) for comparison. Protein levels of α-tubulin are shown as a loading control. No tag = P138.

Cdc18 and Cdt1 in late meiosis from the integrated constructs. Meiotic DNA replication was analyzed in this strain (Fig. 6.3 A). The re-replication observed is not more obvious compared to a strain expressing wild type Dfp1 (compare Fig. 6.3 A to Fig. 4.3 A). When Cdc18 and Cdt1 are over-expressed, i.e. from plasmids (so licensing is efficient as demonstrated in section 4.2.3), stabilizing Dfp1 after meiosis I also does not enhance the re-replication between meiosis I and II (Fig. 6.3 B, compared to Fig. 4.13 A).

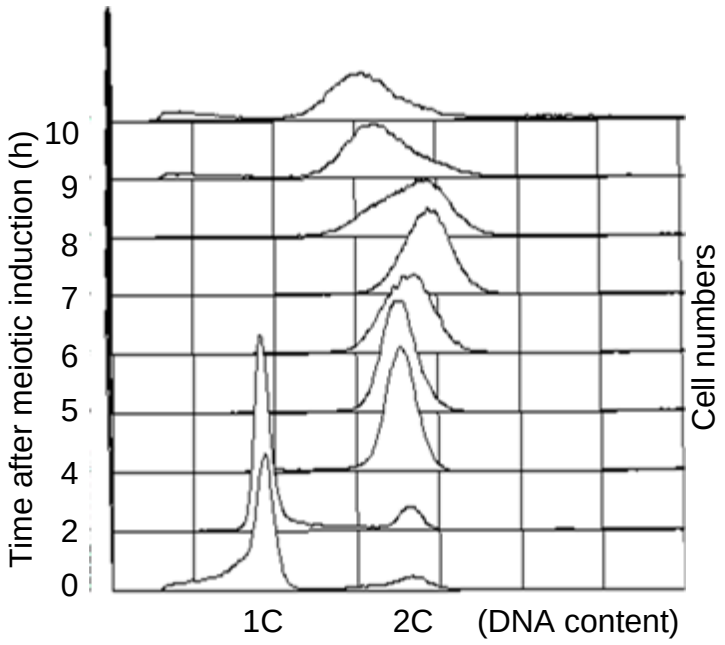
It is noteworthy that mutant protein Dfp1M still disappeared during late meiosis (Fig 6.2 B), suggesting that it is degraded during meiosis II. Thus there may be an additional and meiotic-specific pathway for the proteolysis of Dfp1 during meiosis II and the process of spore formation. It is also possible that its second potential APC box (RFYL) located at amino acids 153 to 156 (Fig 6.1 A) functions only in anaphase of meiosis II.

6.2.2 Stabilizing Cdt1 and maintaining dNTP levels by *ddb1* and *spd1* deletions

A previous publication suggests that if Spd1 is present in pre-meiotic S phase, meiosis cannot be completed, presumably due to the reduced dNTP level and inhibition of pre-meiotic S phase (Holmberg *et al*. 2005). Therefore, it is interesting to investigate whether the presence of Spd1 during late meiosis

A

integrated *mes1pr-dfp1M, mes1pr-cdc18, mes1pr-cdt1*



B

dfp1M and plasmid expressed *[mes1pr-cdc18] [mes1pr-cdt1]*

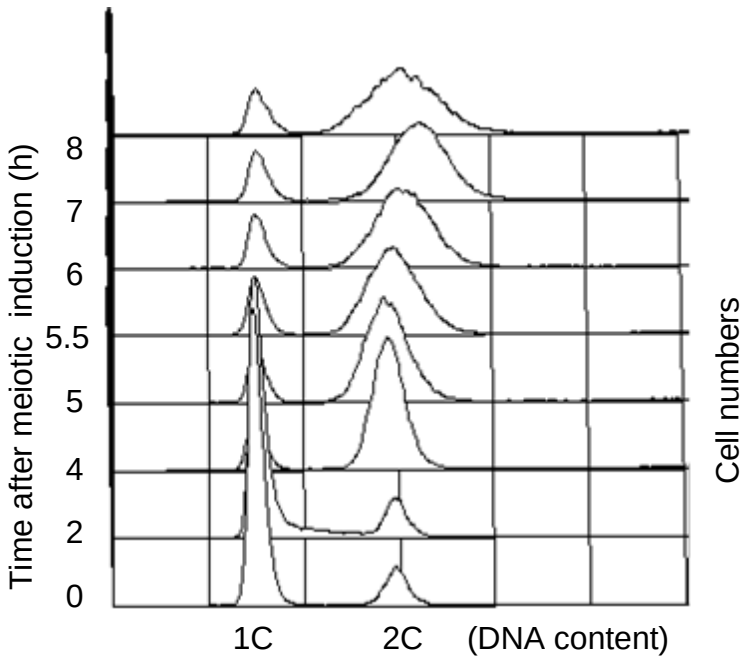


Figure 6.3 Stabilization of Dfp1 after meiosis does not enhance the re-replication induced by expressing *cdc18* and *cdt1* after MI.
(A) Flow cytometric analysis of cells of strain P3020 (*pat1-114 mes1pr-dfp1M-kanMX6::mes1pr mes1pr-cdc18-hyg::mes1pr mes1pr-cdt1-ura4::ura4-294*).
(B) As (A) except strain P3014 (pat1-114 *dfp1M-hyg::dfp1 [mes1pr-cdc18-leu+] [mes1pr-cdt1-ura4+]*) was used.

(Fig. 5.6 A) is another reason for incomplete re-replication. In order to examine this, the *spd1* gene was deleted. To ensure that Cdt1 was present in late meiosis, the *ddb1* gene was deleted to inhibit the normal programme of Cdt1 proteolysis (Ralph *et al*. 2006). To ensure that Cdc18 was present in late meiosis, a *mes1* promoter controlled *cdc18* gene construct was integrated into the *ddb1 spd1* double deletion strain.

Examination of DNA replication when meiosis is induced in this strain indicates that deletion of the *spd1* gene does not lead to increased replication (Fig. 6.4 B, compare to Figs. 4.2 A and 4.3 A) Western blotting confirms Cdt1's stabilization and presence of Cdc18 after meiosis I, although the Cdt1 level appears to fall 5.5 hours after meiosis induction (Fig. 6.4 C). Meiosis was monitored from the frequency of cells with one nucleus, two nuclei and three or four nuclei at different time points shown (Fig. 6.4 A). When Cdt1 is stabilized by *spd1Δ ddb1Δ* during meiosis, it is localized in the nucleus (Fig. 6.4 D). Further elevation of the Cdc18 level by using a plasmid expressing *mes1pr-cdc18* gives a similar result (Fig. 6.4 E, compare to Fig. 4.13 A).

These results suggest that deleting the *spd1* gene does not alleviate the block to DNA replication between MI and MII even when Cdc18 is expressed during this interval and Cdt1 is stabilized by deletion of the *ddb1* gene. However, it is possible that the Cul4-Ddb1 ubiquitin ligase has other targets whose

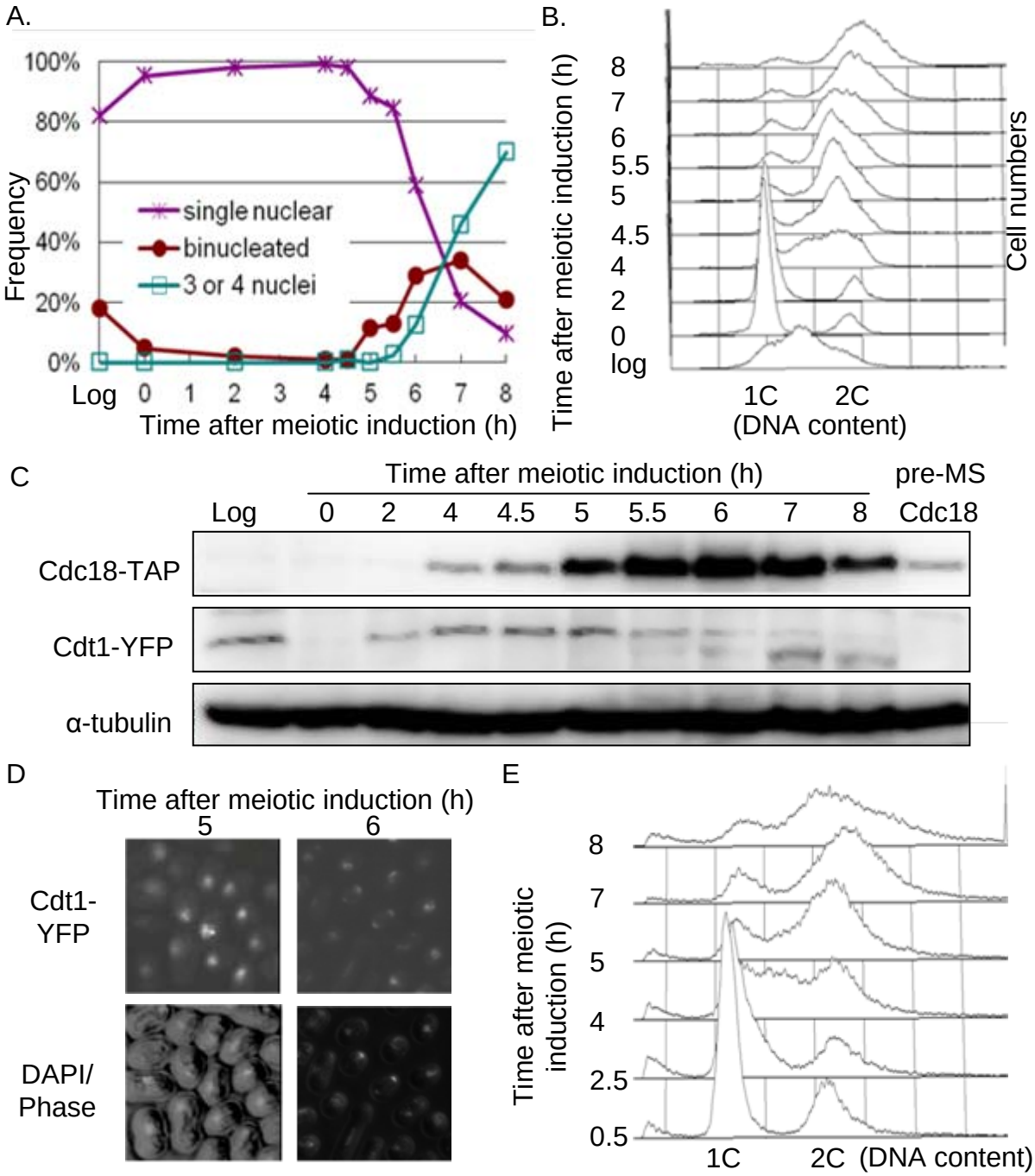


Figure 6.4 Deleting *spd1* and *ddb1* when Cdc18 is expressed after meiosis I does not lead to dramatic re-replication in late meiosis.

(A) *pat1-114 spd1Δ ddb1Δ mes1pr-cdc18* cells (P2027) were induced to undergo meiosis. Meiotic progress was monitored from the frequency of cells with one nucleus, two nuclei and three or four nuclei at time points shown.

(B) Flow cytometric analysis of cells from (A).

(C) Western blotting analysis of cells from (A) showing Cdc18 and Cdt1 levels. Protein level of α-tubulin is shown as a loading control.

(D) Images of cells from the indicated time points from (A), showing that Cdt1 is localized in the nucleus when it is stabilized during late meiosis by the *ddb1Δ* mutation.

(E) As (B) except that a strain over expressing Cdc18 was used (P2474, *pat1-114 spd1Δ ddb1Δ [mes1pr-cdc18-leu+]*)

stabilization has a negative effect on DNA replication. To control for this, I examined the effect of deletion of the *ddb1* gene in the vegetative cell cycle when Cdc18 is over-expressed from the *nmt1* promoter. During vegetative growth, if over expression of Cdc18 is controlled by different types of *nmt* promoter, re-replication happens in a Cdc18-dose dependent way (Nishitani *et al*. 2000). When Cdc18 is over-expressed, deletion of the *ddb1* and *spd1* genes enhances re-replication (Fig. 6.5 A) and increases lethality (Fig. 6.5 B), compared to the wild-type strain, presumably via stabilization of Cdt1. Taken together, these results suggest that deletion of *ddb1* and *spd1* has a stimulatory effect on re-replication on Cdc18 over-expression in the vegetative cell cycle, but the same phenotype is not seen in meiosis, possibly reflecting the effect of other regulatory controls.

In a final experiment, a more simple strategy was used. The *spd1*Δ mutation was introduced into a *mes1pr-cdc18, cdt1* strain. However, in this strain, lacking Spd1, does not show a stimulation of re-replication (Fig. 6.6 A, compared to Fig. 4.2 A). Similarly, over-expressing *cdc18* and *cdt1* from plasmids when the *spd1* gene is deleted still does not induce dramatic re-replication (Fig. 6.6 B, compared to Fig. 4.13 A). In summary, presence of Spd1 during late meiosis is not the crucial reason for the incomplete re-replication.

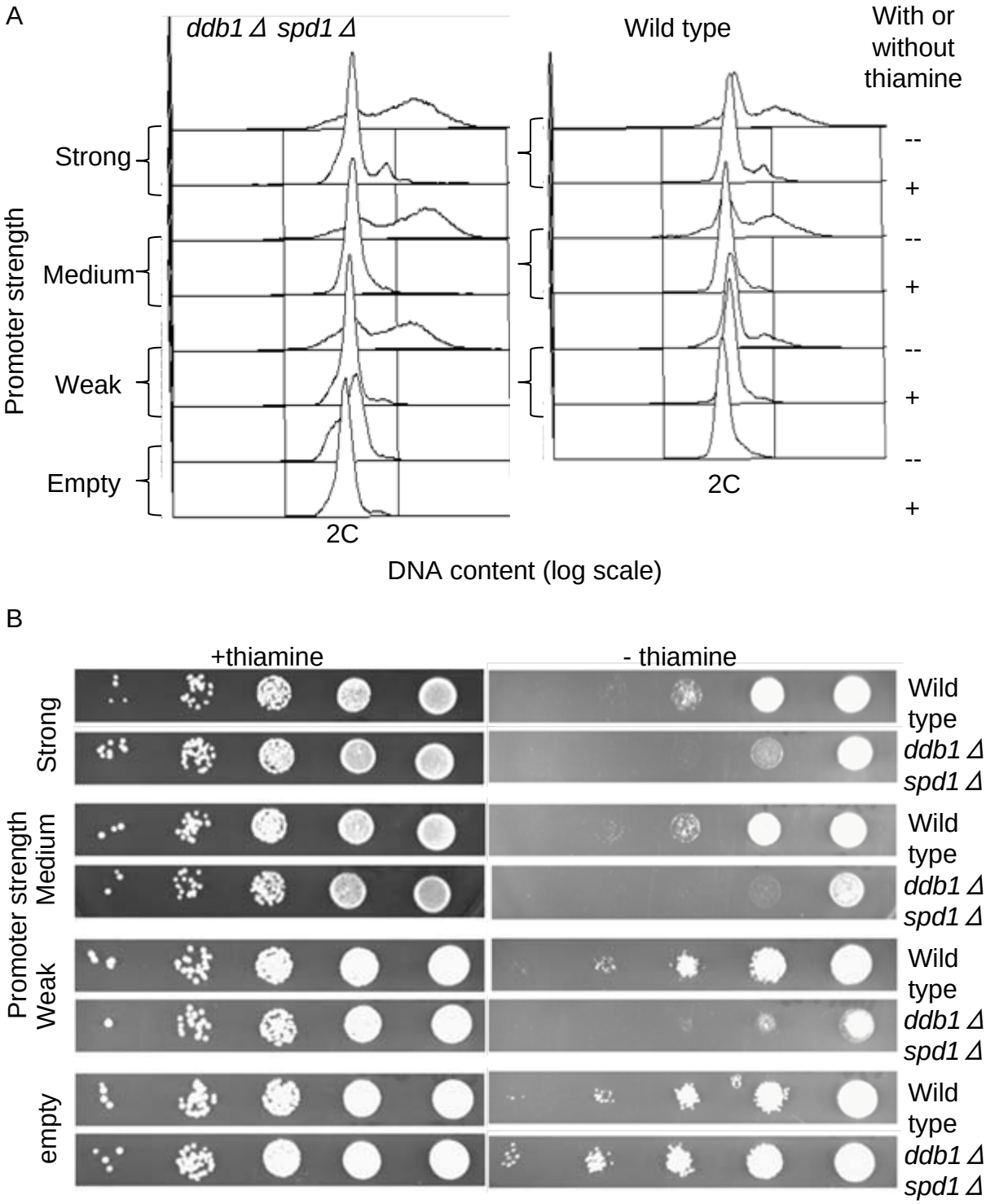
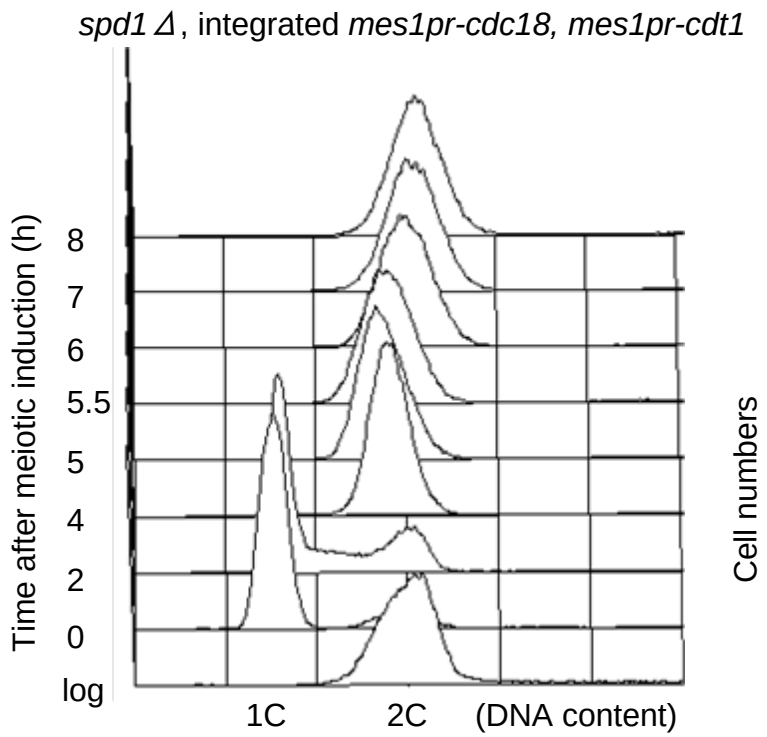


Figure 6.5 Cdt1 stabilization can enhance the phenotype of Cdc18 over-expression.
(A) *Ddb1* and *spd1* deletions enhance the re-replication induced by Cdc18 over-expression during the cell cycle. Strains: Wild type+Nmt3X-Cdc18 (strong): P2086; Wild type+Nmt41X-Cdc18 (medium): P2087; Wild type+Nmt81X-Cdc18 (weak): P2088; Wild type+Nmt3X (empty): P2089; *ddb1Δ spd1Δ*+Nmt3X-Cdc18 (full strength): P2090; *ddb1Δ spd1Δ*+Nmt41X-Cdc18 (medium): P2091; *ddb1Δ spd1Δ*+Nmt81X-Cdc18: P2092; *ddb1Δ spd1Δ*+ Nmt3X (empty): P2093.
(B) Spot test of mutants in (A), showing reduced viability by *ddb1Δ spd1Δ* when grown on – thiamine plates.

A



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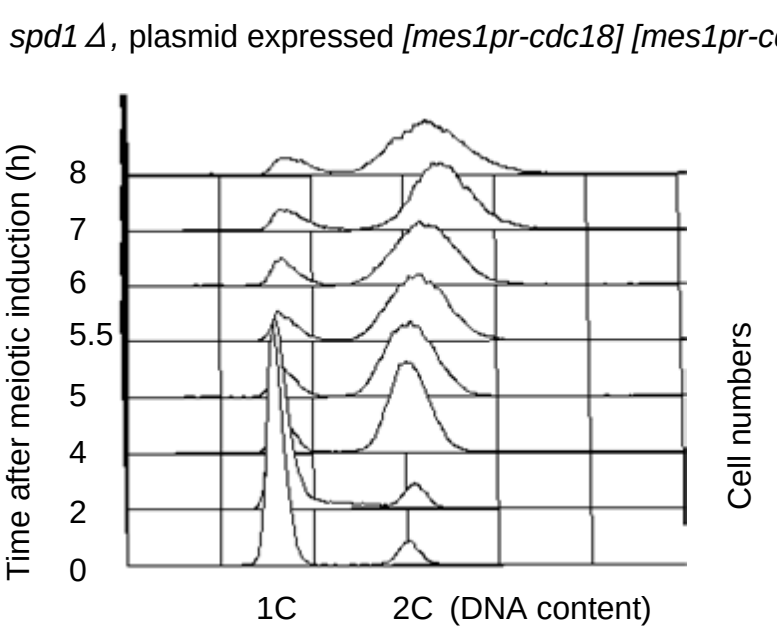

Figure 6.6 Deleting *spd1* when Cdc18 and Cdt1 are present after meiosis I does not lead to full round of re-replication in late meiosis.
(A) Flow cytometric analysis of the *spd1* deletion and *mes1pr-cdc18, cdt1* mutant (P3024, *pat1-114 mes1pr-cdc18-hyg::mes1pr mes1pr-cdt1-ura4::ura4-294 spd1Δ::natMX*)
(B) As (A) except the over-expression strain with *spd1* deletion (P3021, *pat1-114 spd1Δ::natMX [mes1pr-cdc18-leu+] [mes1pr-cdt1-ura4+]*) was used.

6.2.3 Ime2 orthologue in *S. pombe*

In *S cerevisiae* if Ime2 (a meiosis-specific protein kinase) is ectopically expressed in the mitotic cell cycle when cells are released from G1 arrest, DNA replication could be repressed (Holt *et al*. 2007). Also licensing is restricted during the MI/MII interval in budding yeast, partially due to phosphorylation of pre-RC components (Cdc18, Mcm6 and Orc6) by Ime2 (Holt *et al*. 2007; Ofir *et al*. 2004) . Therefore I examined the role of Ime2 orthologues in fission yeast meiosis.

There are two potential Ime2 orthologues in *S. pombe*, Mde3 and Pit1. The *mde3* gene was initially identified as a target of Mei4 (a meiosis-specific transcription factor required for late meiosis progress and sporulation), i.e. expression of *mde3* is stimulated by Mei4 ("mde" = Mei4 dependent expression) (Abe 2000). Both Mde3 and Pit1 (*S. pombe* Ime-two) have large sequence similarities to the Ime2 protein (Abe 2000). Meanwhile, deleting *mde3* or *pit1* resulted in defective spore formation. Although deleting both genes exacerbated this deficiency, occurrence of pre-meiotic S phase and meiosis I seemed not to be affected by absence of Mde3 and Pit1 (Abe 2000).

Because the mRNA of Pit1 is present during both the mitotic cell cycle and meiosis at constant levels but Mde3 is only expressed in late meiosis (Abe

2000), I focused on Mde3. The *nmt1* promoter was used to over express Mde3 during the mitotic cell cycle to see if it can inhibit or delay S phase. Although *mde3* gene has 2 introns, western blotting confirms that when the unspliced DNA sequence is under the control of an *nmt1* promoter, a protein of the expected size can be detected when the promoter is on by removing thiamine (Mde3 is tagged with TAP) (Fig. 6.7 B). This indicates that the Mde3 mRNA can be correctly spliced during the vegetative cell cycle, i.e. expression of Mde3 does not require meiosis-specific splicing.

However, I found that cells released from a G1 arrest to synchronized S phase in the presence of Mde3 (Fig. 6.7 A for experimental scheme) still finish DNA replication (Fig. 6.7 C – upper panel) with similar kinetics to the +thiamine control (Fig. 6.7 C – middle panel) or wild type (Fig. 6.7 C – lower panel). Interestingly, during the synchronized S phase (about 2 hours after G1 release), the protein level of Mde3 seems to decrease, and after S phase it increases again (Fig. 6.7 B). Because the gene is under the control of the *nmt* promoter, it should be constitutively expressed once the promoter is switched on. Thus the disappearance of Mde3 after activation of *nmt* promoter may indicate active degradation of Mde3 during S phase.

Whether Mde3 restricts replication when licensing is induced in the MI-MII interval was also examined. Deleting the *mde3* gene in addition to expressing

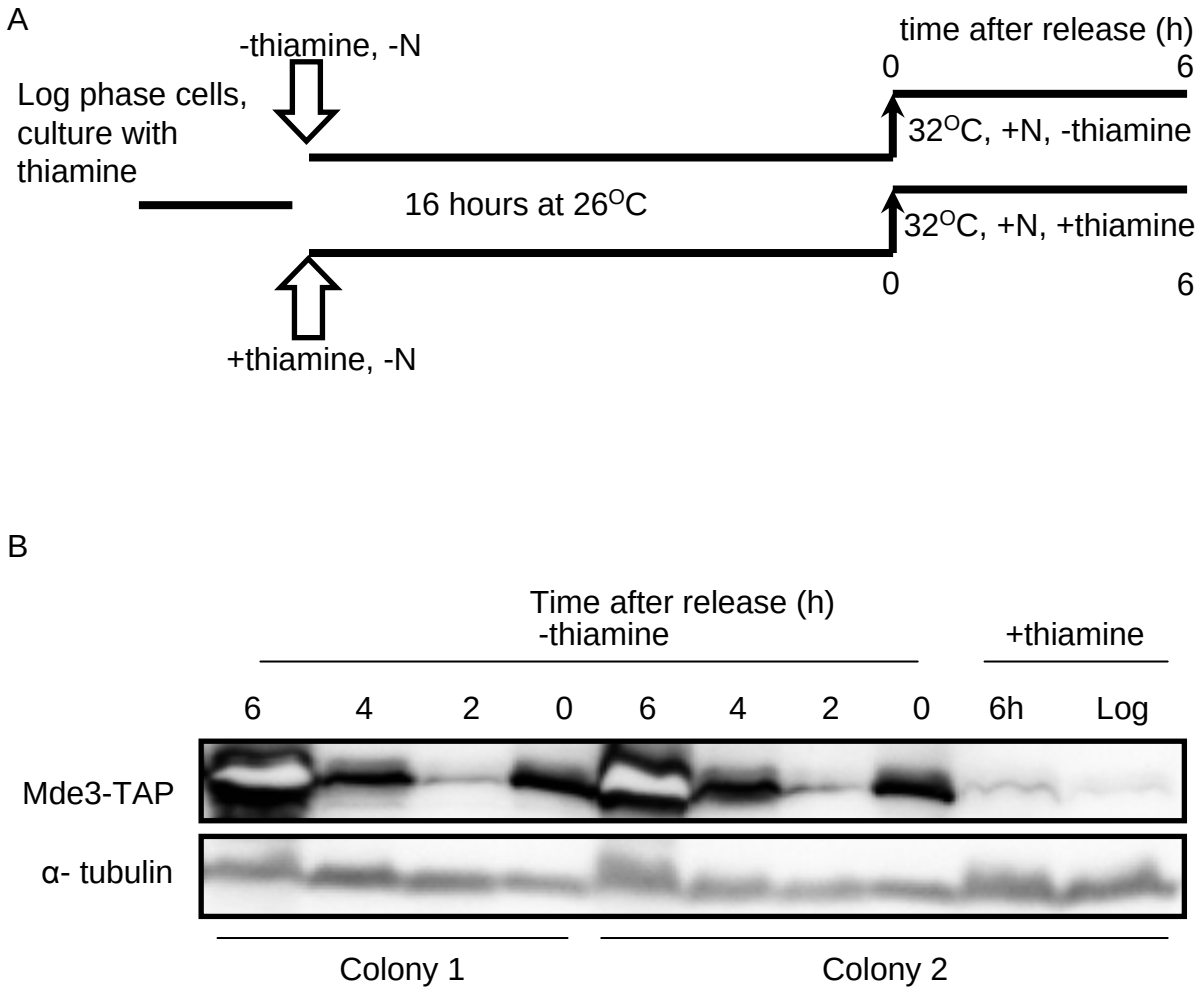

Figure 6.7 Effect of Mde3 expression in DNA replication.
(A) Scheme of experiment. Log phase cell culture was split into two, one was cultured in EMM-N medium with thiamine, the other was cultured in EMM-N-thiamine; after 16 hours, both cultures were released into EMM+N medium, with or without thiamine, respectively.
(B) Western blotting confirms that *mde3* can be expressed during vegetative growth under the regulation of the *nmt1* promoter. Protein extracts from two transformants (colony 1 and 2, P3013 and P3010) are analyzed.

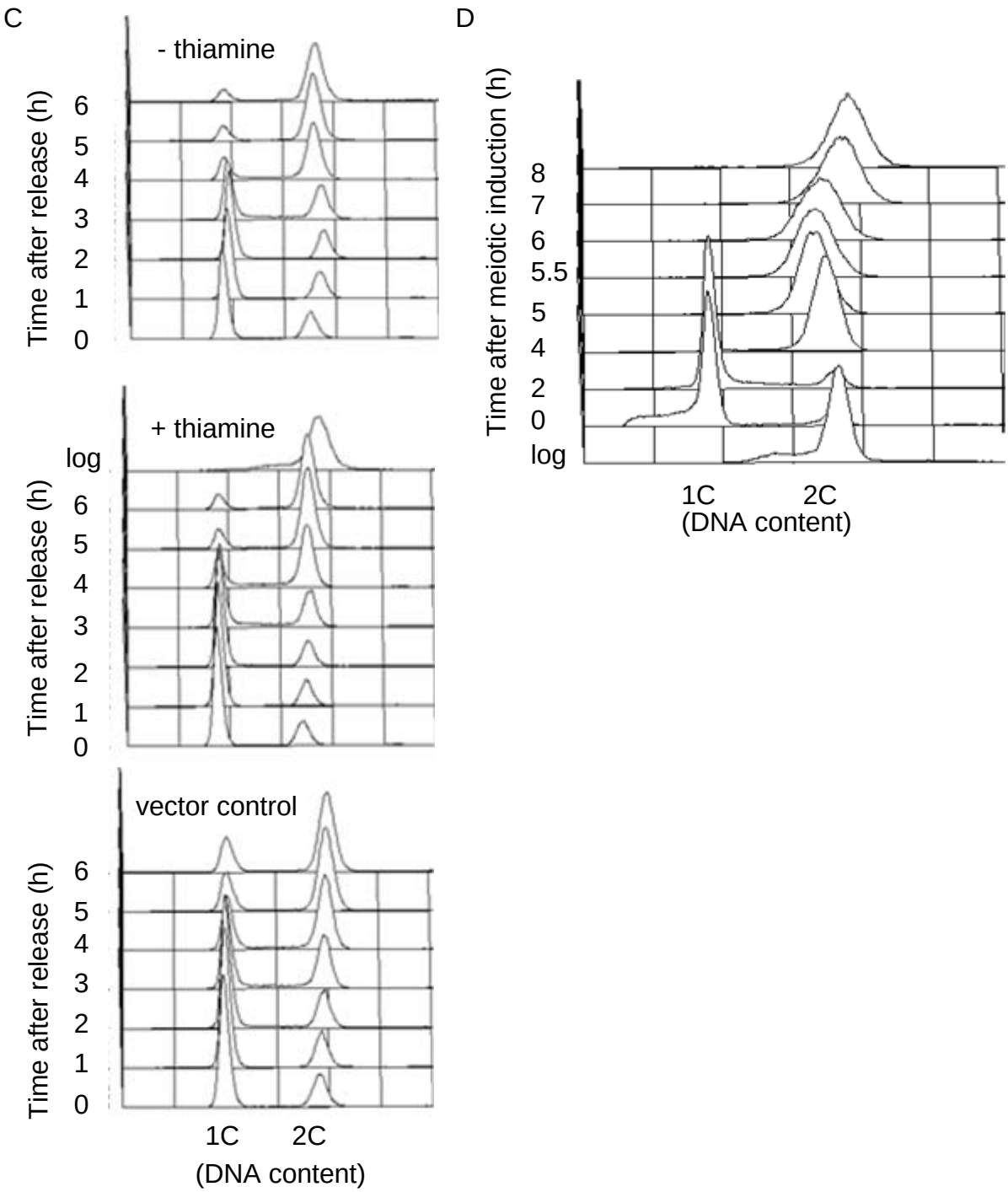

Figure 6.7 (Continued)
(C) Upper panel: flow cytometric analysis of mutant cells with *nmt1Xpr-mde3* (P3013) when thiamine was removed at the time of nitrogen starvation following protocol in (A). Middle panel: as upper panel, except thiamine was always in the culture to shut off the *nmt* promoter, i.e. following protocol in (A) – lower panel. Lower panel: upper panel, except cells bearing the empty plasmid (P3012) were used.
(D) *pat1-114 mde3Δ mes1pr-cdc18, cdt1* cells (P3009) were induced to carry out meiosis following protocol in Fig. 2.2, sampled and analyzed by flow cytometry following SYTOX Green staining.

cdc18 and *cdt1* during late meiosis does not give a more obvious re-replication phenotype (Fig. 6.7 D, compared to Fig. 4.3 A), which suggests that Mde3 is not a critical factor in meiotic DNA replication control.

6.2.4 Deleting *spo4* cannot enhance re-replication in MI-MII interval

The MI-MII interval in fission yeast is very short – only about 30 minutes. Also due to the reason that cells in cultures cannot be 100% synchronized, this makes it more difficult to carry out experiments which require fine tuning of protein activity during this interval. Therefore, I deleted *spo4* gene to prolong the MI-MII interval and facilitate more elaborate experiments, and also to see if entry into meiosis II is inhibitory to any ongoing DNA replication.

Spo4 is an Hsk1/Cdc7-like protein kinase and it is meiosis-specific, whose regulatory partner is Spo6. However, Spo4-Spo6 has no role in DNA replication, and deleting *spo4* did not affect pre-meiotic S phase. Instead, Spo4 is required for meiosis II entry; in meiosis, *spo4Δ* cells were arrested at the binucleate stage with cytoplasmic microtubules, indicating that they were in the interkinesis between meiosis I and meiosis II (Nakamura *et al*. 2002). This is not surprising as both *spo4* and *spo6* are only expressed during late meiosis, dependent on Mei4 (Nakamura *et al*. 2002).

Since Dfp1, Spd1 and Mde3 do not appear to play crucial roles in repressing re-replication induced by expression of Cdc18 and Cdt1 in late meiosis, the following experiment was designed to see if deleting *spo4* can make some difference. However, in the *pat1-114 mes1pr-cdc18, cdt1* background, a *spo4*Δ mutant does not exhibit more dramatic re-replication after meiosis I than seen in the *spo4*$^{+}$ cells (Fig. 6.8 A, compared to Fig. 4.3 A). Then I examined DNA replication in meiosis of *spo4*Δ cells capable of expressing Cdc18 and Cdt1, and also where Dfp1 was stabilized (by using the *dfp1M* mutant) after meiosis I. Meiotic re-replication in this strain is not more obvious than seen in the *spo4+* strain as well (Fig. 6.8 B, compared to Fig. 6.3 A). In summary, *spo4* deletion strategy is a useful tool to extend the short interkinesis between MI and MII, permitting more elaborate experiments, but the *spo4*Δ mutation itself seems to not be able to enhance the re-replication when licensing is induced.

6.2.5 Inhibiting protein synthesis in late meiosis can induce more re-replication

Xenopus oocytes treated with cycloheximide (CHX, an inhibitor of protein synthesis) 30 or 60 minutes after GVBD (germinal vesicle breakdown, which marks the onset of meiosis I) initiated DNA re-replication between 120 and 150 minute after GVBD (i.e. a time corresponding to the MI-MII interval), which

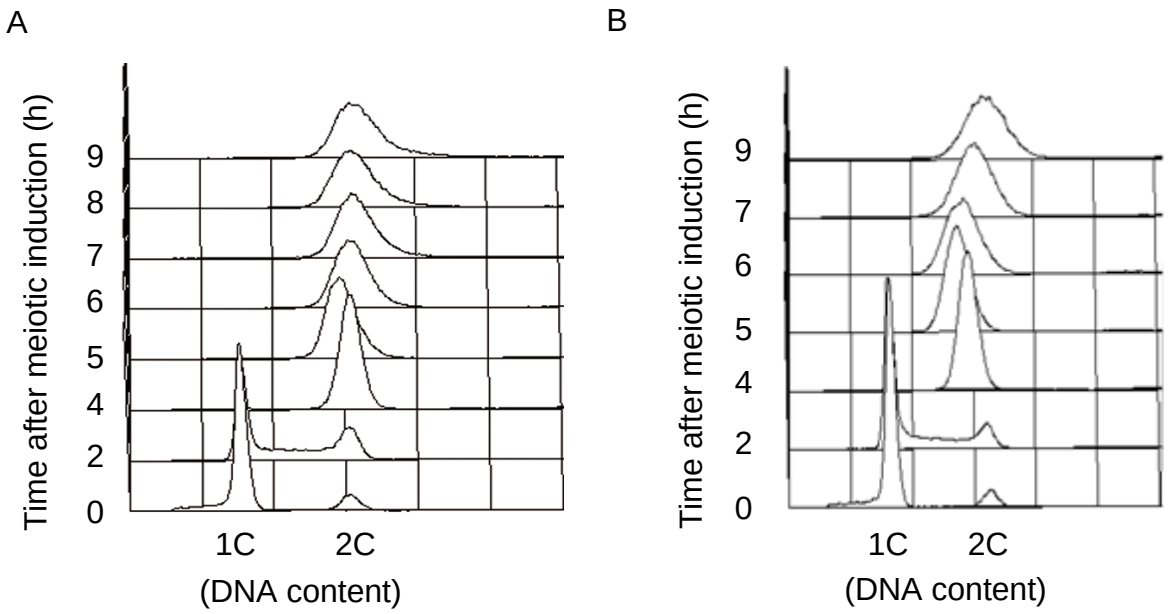

Figure 6.8 Deleting *spo4* does not enhance re-replication after MI in the *mes1pr-cdc18, cdt1* mutant.
(A) *spo4Δ* cells expressing Cdc18 and Cdt1 during late meiosis (P3030) were induced to undergo meiosis by Pat 1 inactivation as in Fig. 2.2, sampled at the time points shown, and DNA contents were analyzed by flow cytometry
(B) As (A) except *spo4Δ* cells with *dfp1M* mutation and *mes1pr-cdc18, cdt1* (P3011) were used.

could be detected by ^{32}P-dCTP incorporation (Furuno *et al*. 1994). Later results explained the reason that CHX treatment can induce re-replication because CHX prevents synthesis of Mos that helps to maintain intermediate CDK activity during MI-MII (Furuno *et al*. 1994). Considering the possibility that in fission yeast newly synthesized proteins in the MI-MII interval could be inhibitory to replication as well, cells were treated with CHX to prevent novel protein synthesis after meiosis I. Addition of CHX after meiosis I (@ 5.75 hrs after meiotic induction) does not induce more replication than no treatment (Fig. 6.9 A), but if CHX was added after meiosis I and then washed out one hour later, the 2C peak in the flow cytometry histogram trails to the right indicative of additional DNA replication (Fig. 6.9 C). Although the effect is rather slight, this suggests re-replication is evoked to a higher extent in this case. This phenotype is dependent on late meiotic expression of *cdc18* and *cdt1* as the effect on DNA replication is not seen if a cycloheximide pulse is applied to a *pat1-114 spo4*Δ strain (Fig. 6.9 D). The effects of CHX on protein synthesis was confirmed by western blotting; if CHX is added before the *mes1* promoter is on, expression of Cdc18 and Cdt1 during late meiosis is blocked (Fig. 6.9 E). Adding CHX at this time point prevents cells from entering MI as most cells were arrested with a single nucleus (Fig. 6.9 B), presumably due to inhibition of cyclin B (Cdc13) synthesis.

Stabilization of Dfp1 was also examined in the context of this experiment.

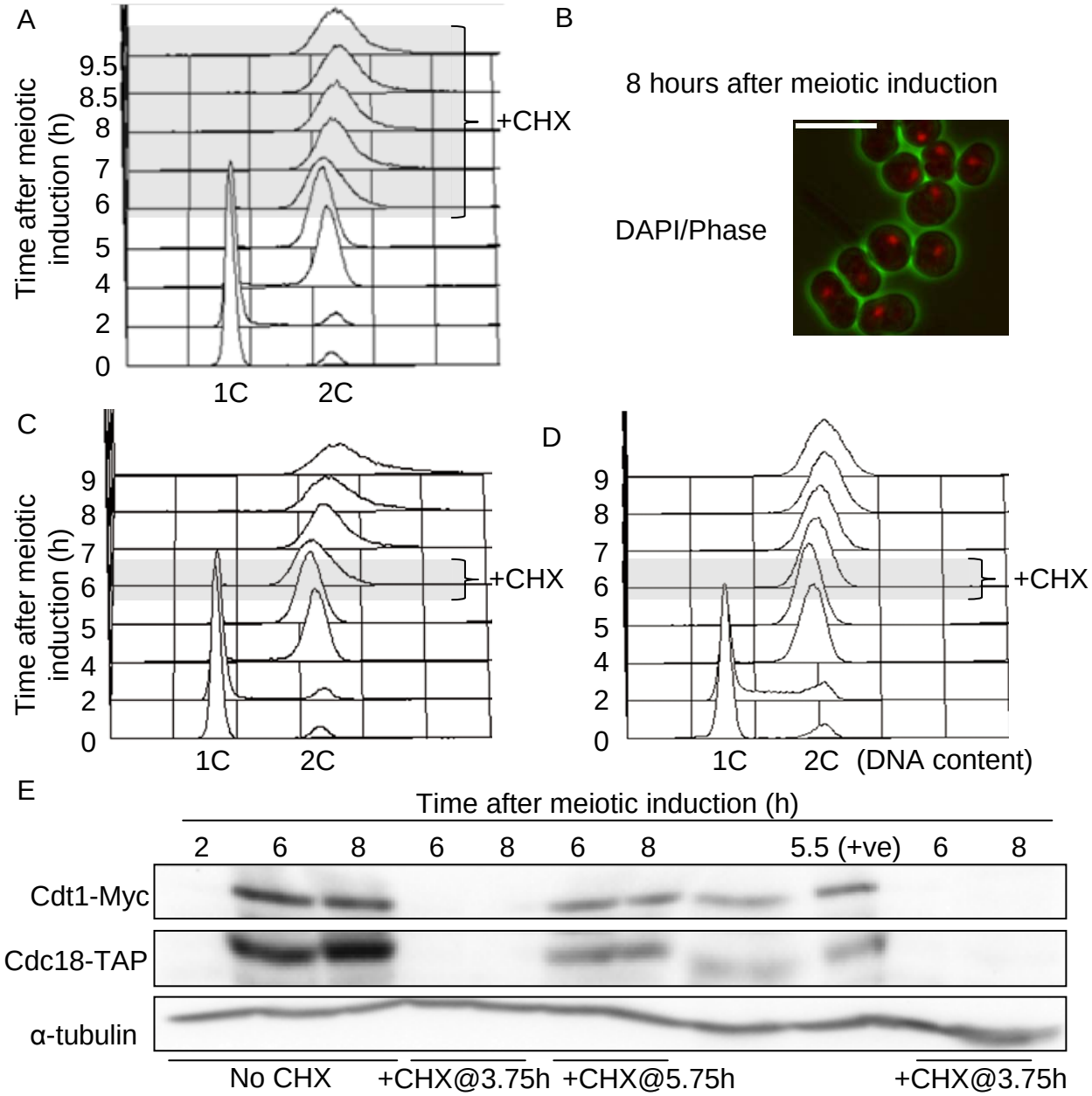

Figure 6.9 When Cdc18 and Cdt1 are expressed after meiosis I, inhibition of protein synthesis after MI induces more DNA re-replication.
(A) *spo4Δ* cells expressing Cdc18 and Cdt1 during late meiosis (P3030) were induced to undergo meiosis by Pat 1 inactivation as in Fig. 2.2, and CHX was added to the culture at 5.75 h after meiotic induction. DNA contents were analyzed by flow cytometry.
(B) as (A) except CHX was added to the culture at 3.75 h after meiotic induction. Image of cells at the time point shown. Bar=10 μm
(C) as (A) except that CHX was added to the culture at 5.75 h after meiotic induction and washed out one hour later.
(D) as (C) except that the strain used does not express Cdc18 and Cdt1 in late meiosis (*pat1-114 spo4Δ*, P3029).
(E) Western blotting analysis confirms that addition of CHX in meiotic G2 phase (@3.75h) prevents *mes1* promoter controlled expression of Cdc18 and Cdt1. When CHX was added after meiosis I (@5.75h), Cdc18 and Cdt1 can still be expressed by *mes1* promoter.

Stabilization using the *dfp1M* mutation after meiosis I does not enhance the re-replication seen above induced by CHX pulse (Fig. 6.10 A and B).

6.2.6 Forcing expression of Cdc18, Cdt1 and Wee1 after meiosis I cannot evoke a complete round of replication

The additional re-replication induced by CHX addition and removal could be due to the oscillation of CDK activity, which could facilitate licensing and initiation of replication. However, other proteins could also be responsible for this effect. Thus I focused on manipulation of CDK (Cdc2) activity to see if this can achieve the same degree of re-replication as CHX treatment. CDK activity is maintained at an intermediate level after meiosis I, which is considered to be an important mechanism to repress replication, especially in *Xenopus* eggs: as reviewed above (section 1.5), inhibition of CDK residual activity via *wee1* expression after MI can induce the exit of meiosis and DNA re-replication (Nakajo *et al*. 2000). So it is also interesting to see how the two model systems (*S. pombe* and *Xenopus*) compare, by expressing *wee1* in *S. pombe* after meiosis I.

Wee1 was initially identified as a negative regulator of mitosis, whose loss of function induces cells to enter into M phase at a tiny size (Russell and Nurse 1987). In fact, during vegetative growth, G2 cell size control is implemented by

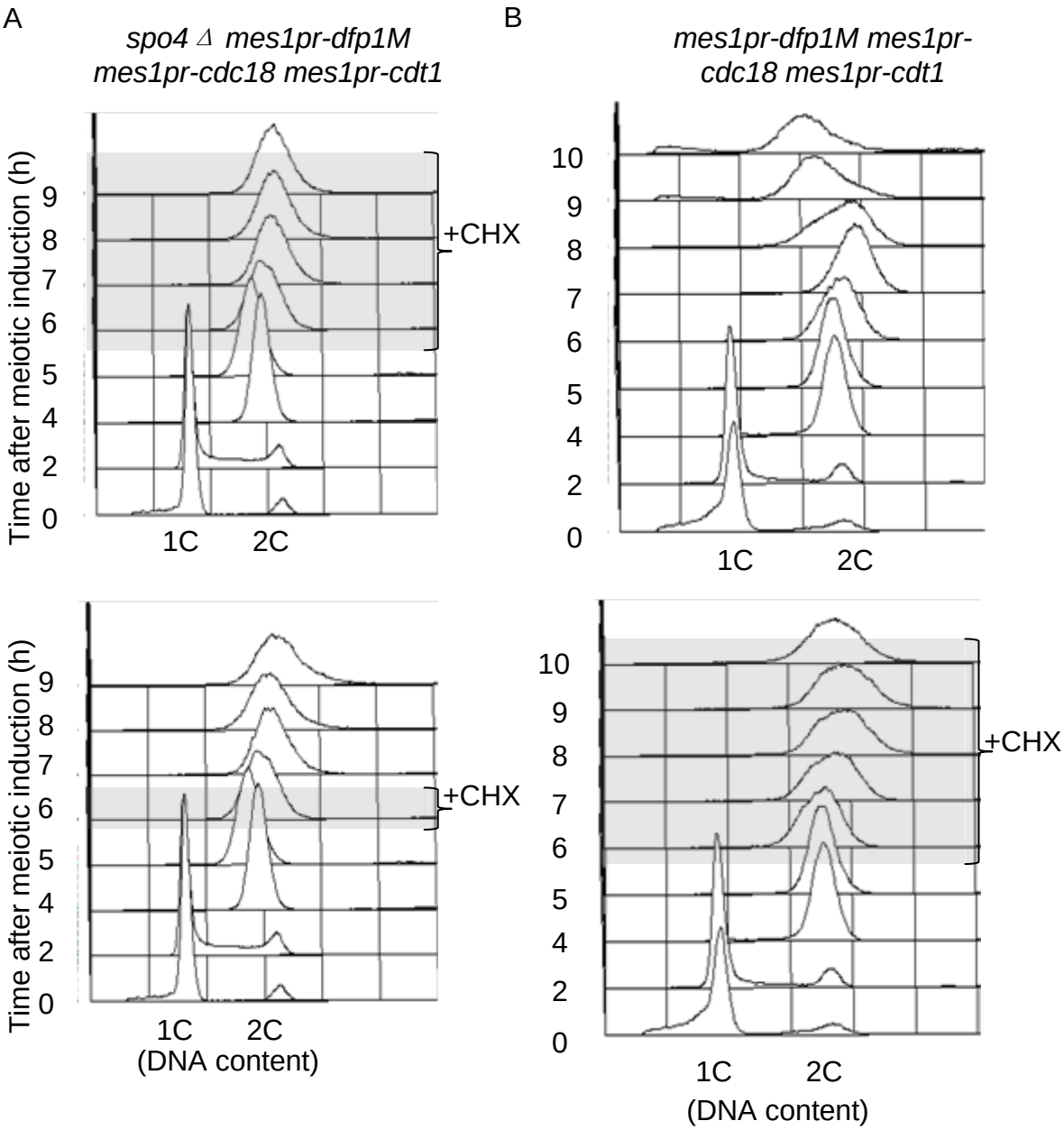

Figure 6.10 Further analysis of effects of CHX on DNA replication in late meiosis.

(A) *Spo4* cells with *dfp1M* mutation and expressing *cdc18* and *cdt1* during late meiosis (P3011) were induced to undergo meiosis (i) with CHX added at 5.75h after meiotic induction – upper panel; (ii) with CHX added at 5.75h and washed out 1 hour later – lower panel. Cells were sampled at the time points shown and DNA contents were analyzed by flow cytometry. See Fig. 6.8 B for flow cytometry analysis of DNA content of this strain during meiosis without CHX treatment.

(B) Cells expressing *dfp1M*, *cdc18* and *cdt1* from *mes1* promoter (P3020) were induced to undergo meiosis (i) without CHX – upper panel; (ii) with CHX added at 5.75h after meiotic induction – lower panel.

two protein kinases: Wee1 and Myt1. They repress CDK activity by phosphorylating Cdc2 on Tyr-15 (Wee1) and Thr-14 (Myt1) (Fattaey and Booher 1997). When *S. pombe* cells underwent meiosis, Wee1 levels also reduced following meiosis I (Dayamakin *et al*. 1992), as Cdc18 and Cdt1. However, when I examined the Tyr-15 phosphorylation status of Cdc2 during meiosis by western blotting using an antibody against this Tyr15-pi, I found that expressing *wee1* from a *mes1* promoter in late meiosis made no difference to the levels of Tyr-15 phosphorylation of Cdc2 (Fig. 6.11 A). Therefore, unlike the *Xenopus* egg system, the strategy using Wee1 in late meiosis to inhibit CDK does not work in *S. pombe*. Thus it seems that rather than focusing on the inhibitory kinase, a strategy to fine-tune activity of Cdc2 might be more straightforward.

6.2.7 Effects of inactivation and subsequent reactivation of CDK on meiotic re-replication after meiosis I

Previous experiments (see section 4.2.2) indicate that merely down regulating CDK activity after meiosis I using a *mes1* deletion strain does not lead to more DNA replication than in a *mes1*+ strain. Possibly, what is required to induce efficient replication is that CDK activity needs to be initially reduced to permit licensing and then increased to activate initiation. Thus, a Cdc2-'Shokat' (*cdc2-as*) mutant was used to control CDK activity *per se* (from J. Gregan)

A

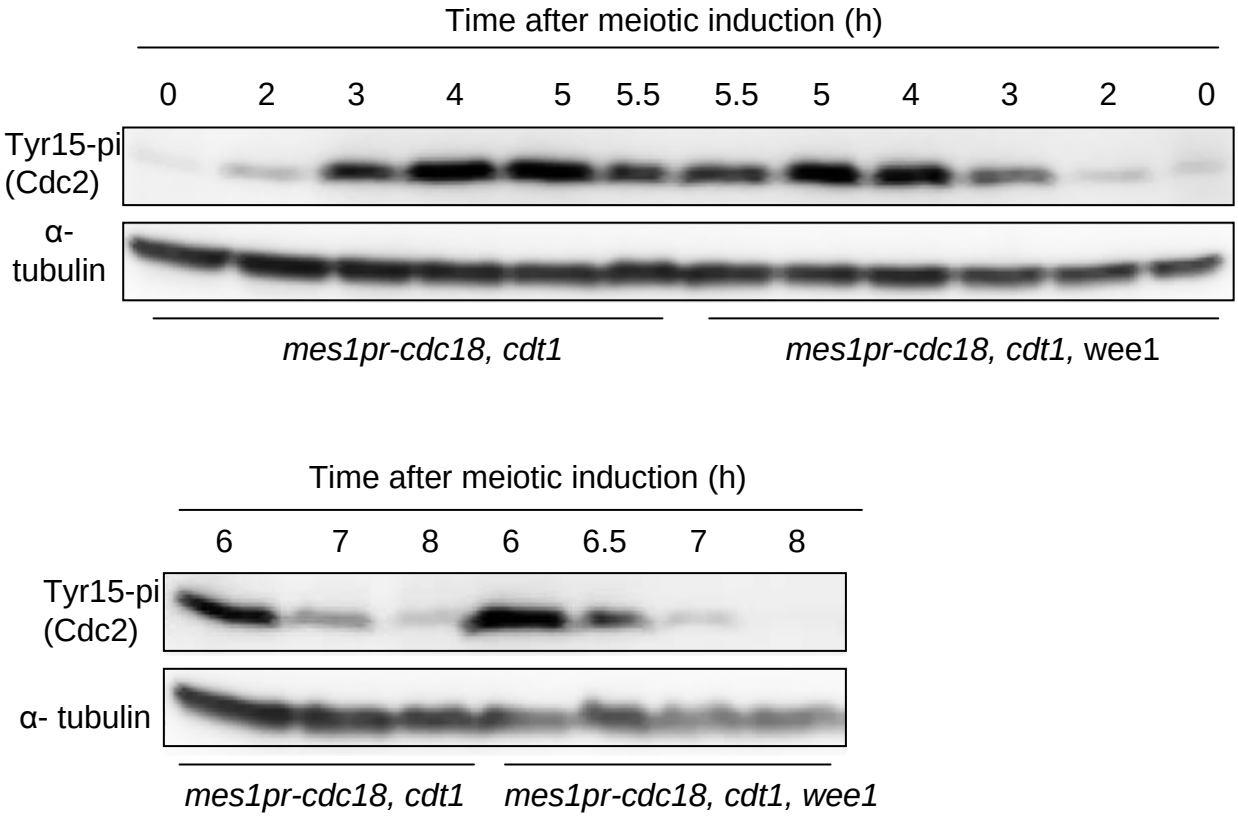

Figure 6.11 Phosphorylation status of Cdc2 Tyr-15 during late meiosis
(A) Cells forcing expressing *wee1*, *cdc18* and *cdt1* during late meiosis ("*mes1pr-cdc18, cdt1, wee1*" = P2476), or only expressing *cdc18* and *cdt1* during late meiosis ("+*mes1pr-cdc18, cdt1"* = P2453) were induced to meiosis and sampled at the time points shown, analyzed by western blotting after TCA extraction, using the antibody against Cdc2(Tyr-15)-pi. Levels of α-tubulin are the loading control.

(Bishop *et al*, 2000).

The activity of this Cdc2 mutant can be inhibited by an ATP analogue – 1-NM-PP1. However, Cdc2-as is not fully functional above 28ºC even in the absence of inhibitor: cells are viable but slightly elongated during vegetative growth at the higher temperature. Thus the *cdc2-as* mutant is similar to a leaky *cdc2-ts* mutant and cells expressing Cdc2-as may have a slightly longer G2 phase during the cell cycle. The *cdc2-as* strain is difficult to arrest in G1, and instead, they arrest in G2 phase after nitrogen starvation, as indicated by the 2C DNA content peak and the low septation index (Fig 6.12 and 6.14 C). Therefore meiosis in the *cdc2-as* strain was induced from G2 as previously described (Watanabe *et al*, 2001); here, the *mes1* promoter was activated after meiosis I as when meiosis is induced from G1 (Fig. 6.14 A). No replication after MI was detected when meiosis is induced from G2 and CDK activity was inhibited and then reactivated in a strain not expressing Cdt1 and Cdc18 at this time (Fig. 6.12 B). Meanwhile, when meiosis was induced from G2, the meiotic kinetics of the *mes1pr-cdc18, cdt1* strain and the control strain (both are *pat1-114 spo4*Δ *cdc2-as*) are similar to each other (Fig. 6.14 B), and the septation index remains low (Fig. 6.14 C).

When *pat1-114* cells with *mes1pr-cdc18 mes1pr-cdt1 spo4*Δ *cdc2-as* underwent meiosis, cells showed a slight increase in DNA content from 7.5 h

after meiotic induction, compared to a cdc2-as strain not expressing *cdc18* and *cdt1* (Fig. 6.12 A – left panel). DNA replication was slightly enhanced when cdc2-as was inhibited for one hour approximately from the time of meiosis I (Fig. 6.12 A – right panel).

In the previous experiments, cells were kept at 34^{O}C to inactivate Pat1, and this may have partially inactivated *Cdc2-as*. Therefore, in order to first inactivate CDK to allow efficient licensing and then restore CDK activity to stimulate initiation, I gradually decreased the temperature after induction of meiosis (Fig. 6.13 A). In this situation, re-replication after meiosis I was more dramatic and cells accumulated DNA up to 4C content (Fig. 6.13 B), although most cells still only partially re-replicated their DNA. More re-replication is evoked when CDK activity is allowed to oscillate (i.e. 1NMPP1 pulse treatment) than just inhibiting CDK (Fig. 6.13 C).

6.2.8 DNA combing analysis of replication in the MI-MII interval

One obvious question is which step in DNA replication is inhibited in MI-MII – licensing (i.e. loading of Mcm2-7 complexes onto origins), initiation or elongation? In the section 4.2.3, by analyzing MCM chromatin binding in meiosis I have shown that Mcm2 is only bound to DNA during pre-meiotic S phase as expected, but on Cdt1 and Cdc18 over-expression, the level of Mcm2 chromatin binding in MI-MII is similar to that seen earlier in meiosis. This

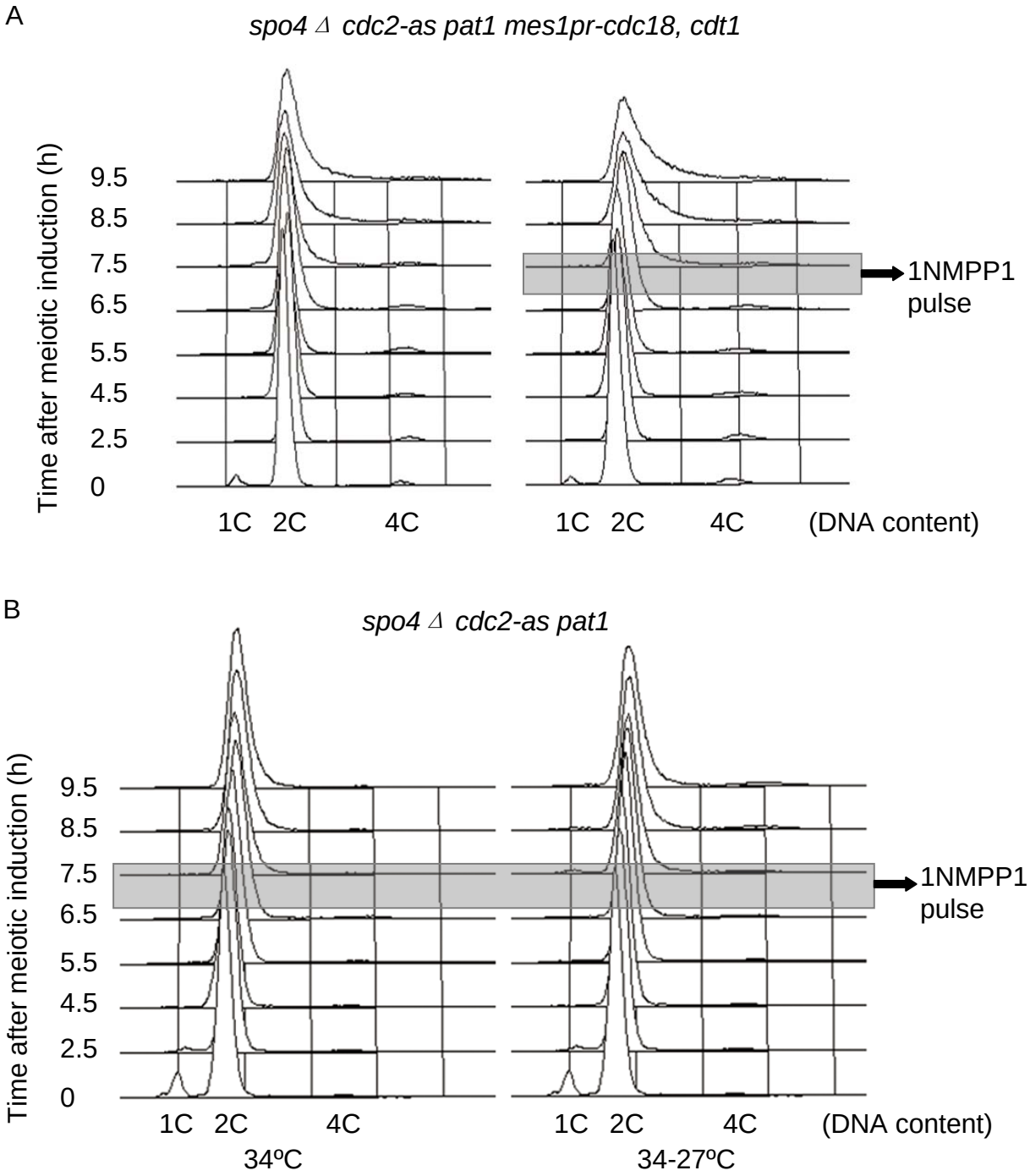

Figure 6.12 Using an ATP analogue 1NMPP1 to inhibits Cdc2 activity in late meiosis by using a *cdc2-as* (Cdc2-"shokat") mutant.
(A) Left panel: *cdc2-as spo4 Δ pat1* cells capable of expressing *cdc18* and *cdt1* during late meiosis (P3028) were arrested in G2 phase, induced to undergo meiosis by inactivation of Pat1, then analyzed for DNA content. Right panel: shows cells treated with 1NMPP1 at 6.75 hours after meiotic induction, and the inhibitor was washed out one hour later.
(B) DNA content of *spo4 Δ cdc2-as pat1* cells (i.e. not expressing Cdc18 and Cdt1in late meiosis, P3027) undergoing meiosis. Cells were induced to enter meiosis and pulsed with 1NMPP1 as in (A), either at 34ºC (left panel) or reducing the temperature to 27ºC after meiotic induction as in Fig. 6.13 (A) (right panel).

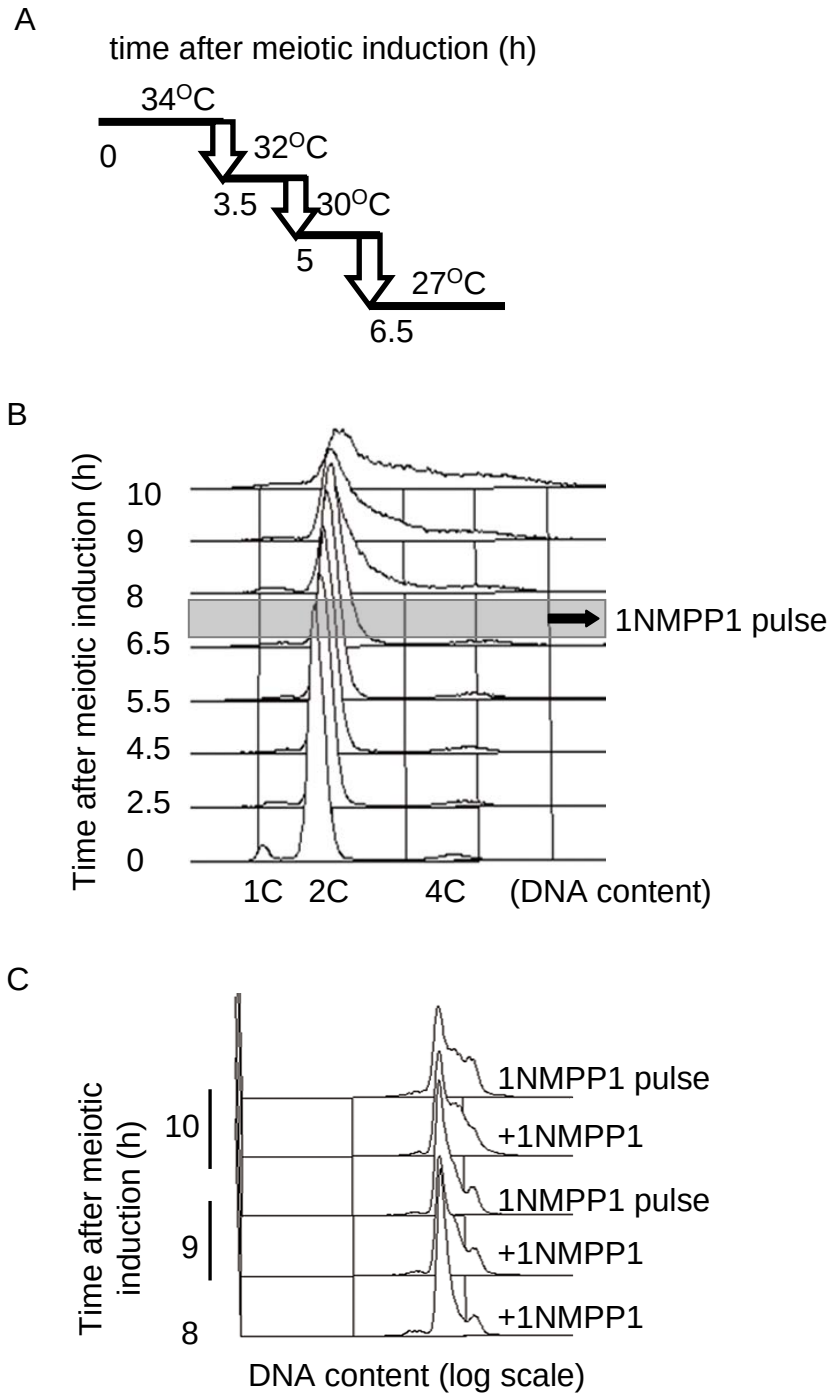

Figure 6.13 Inactivation and subsequent activation of Cdc2 in late meiosis enhances an extra round of DNA replication in strains expressing *cdt1* and *cdc18*.

(A) Illustrating that how temperature was reduced after meiotic induction.

(B) *cdc2-as spo4Δ mes1-cdt1,cdc18 pat1-114* (P3028) cells were induced to enter meiosis by inactivating Pat1 at 34^{O}C and then the temperature was reduced as shown in (A). 1NMPP1 was added at 6.75h and washed one hour later. Samples were taken at the time points shown, and processed for analysis of DNA content by flow cytometry.

(C) Comparison of cells pulsed with 1NMPP1 for one hour, with cells where the inhibitor was not washed out.

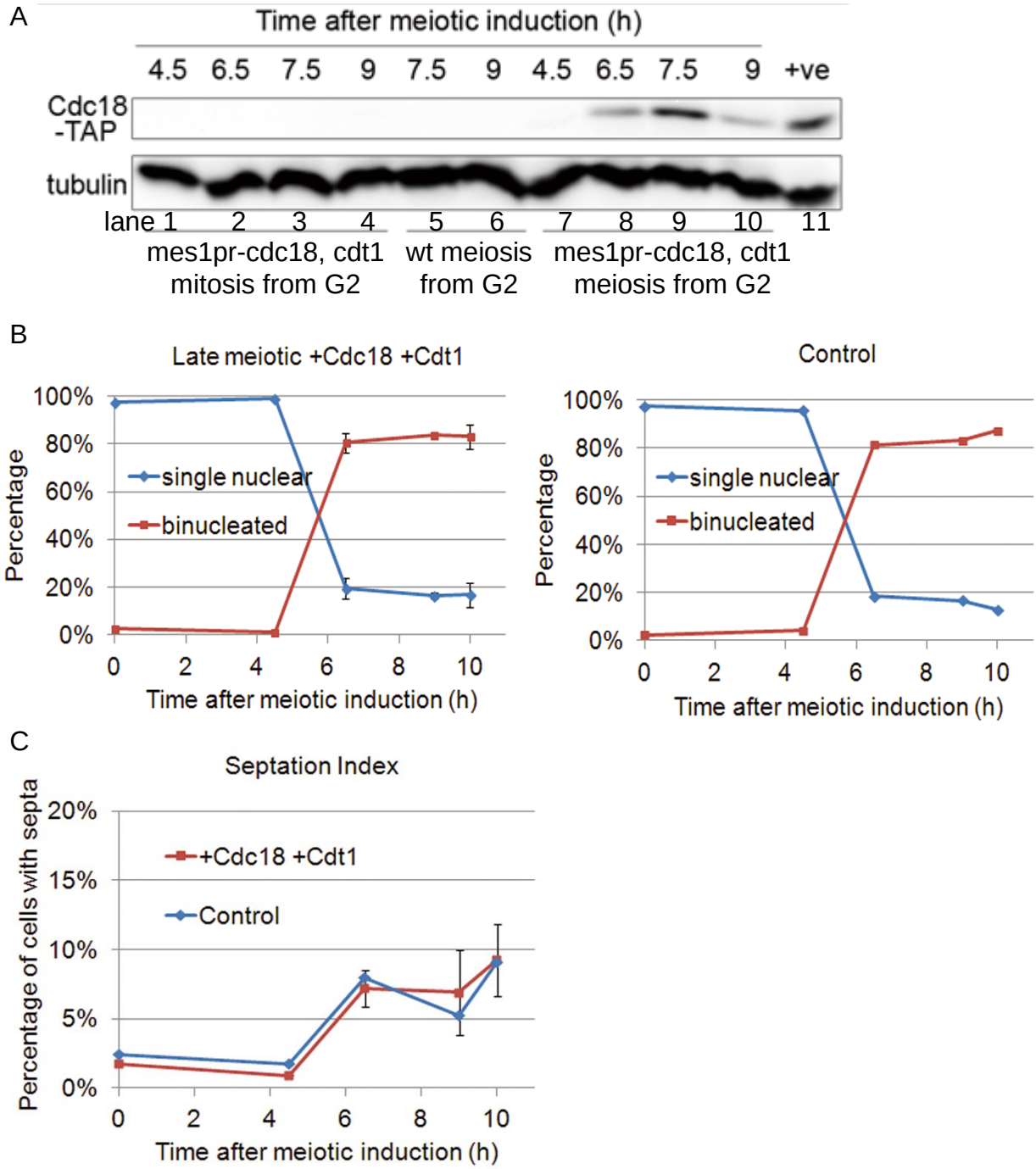

Figure 6.14 Monitoring meiosis progress of the *cdc2-as* strain.
(A) Western blot showing level of Cdc18 in **(i)** a *mes1pr-cdt1, cdc18* (*cdc2-as spo4⊿ pat1-114*) strain (P3028) where meiosis was induced from G2 as in Fig.5.7 (D) – lanes 7~10; **(ii)** a control strain (P3027, *cdc2-as spo4⊿ pat1-114*) when meiosis was induced similarly as (i) – lanes 5 and 6; and **(iii)** the same *mes1-cdt1, cdc18* strain as in (i) (P3028) but cells were released into mitosis after G2 arrest – lanes 1~4. +ve = a *mes1pr-cdt1, cdc18* strain (P2450, *pat1-114 cdc2wt*), 5.5 h after meiotic induction (meiosis from G1).
(B) Kinetics of nuclear divisions in a meiosis induced from G2 by Pat1 inactivation and temperature was reduced as shown in Fig. 6.10 - (C). left panel: strain P3028; right panel: strain P3027.
(C) As (B) except the septation index was monitored during meiosis.

suggests that the licensing step may not be rate limiting. Thus initiation or elongation may be the problem. One way to investigate this is to use DNA (molecular) combing, where halogenated nucleosides such as BrdU are incorporated into replicating DNA, and the replicated regions are detected by stretching the DNA onto glass slides followed by antibody detection (Lemaitre *et al*., 2005; Michalet *et al*., 1997). Therefore, if initiation but not elongation is inefficient I would expect to see infrequent islands of replication separated by large zones of unreplicated DNA. In this case, the re-replication observed is due to synthesis of a small amount of long DNA fragments. If elongation but not initiation is a problem, I should see frequent sites at which incorporation is occurring, i.e. lots of short DNA patches should be produced. So the key issue here is to investigate the distribution of newly synthesized DNA fragments induced by expressing Cdc18 and Cdt1 after meiosis I.

In order to incorporate exogenous thymidine analogues, yeast cells should be modified to express TK and hENT1. Meanwhile, to eliminate influences of meiotic recombination, all the strains used for DNA combing experiments were *rec12Δ*. The procedure for labelling DNA with BrdU is similar to the EdU labelling procedure in section 4.2.2. *Pat1-114* cells were arrested in G1 by nitrogen starvation and then released into synchronized meiosis. BrdU was added to cultures before cells have carried out meiosis I at a concentration of 3μM, cells were harvested 1.5 hours later, and then processed for DNA combing (see protocol in section 2.8).

In wild type (*rec12Δ*) cells, most DNA fibres are negative for BrdU

incorporation as expected (Fig. 6.15 A); the rarely seen BrdU incorporation patches could be due to mitochondrial DNA replication or cells still in pre-meiotic S phase, as the synchrony might not perfect. By respectively measuring the total length of DNA fibres and BrdU positive fragments in the same field (at least 200 DNA fibres were measured) and dividing the latter by the former, percentage of DNA fragments that are synthesized after meiosis I can be estimated, in this case, it is only 1%.

When Cdc18 and Cdt1 are expressed after meiosis I, more DNA fibres are positive for BrdU incorporation, indicative of re-replication during late meiosis (Fig. 6.15 B). The percentage of re-replication is 11% for the Cdc18 and Cdt1 over-expression strain and 13% when Cdc18 and Cdt1 are expressed at their physiological levels, which is very similar to estimates made by comparing flow cytometry histograms of EdU incorporation (Fig. 4.8 C). Short DNA fragments are synthesized during the re-replication after meiosis I (median 17-18 kb) (Fig. 6.15 C), which is only twice the track length seen when the elongation step of a normal S phase is inhibited with HU (Patel *et al*, 2006). It suggests that elongation is restricted in MI-MII.

In addition, the percentage of origins used was estimated as follows. *S. pombe* genome size is 13.8 Mb. As approximately 10% of the genome has been re-replicated, the re-replicated DNA is 1.38Mb. The mean track length is 23kb, so number of origins used in the re-replication is 1.38Mb/ 23kb = 60. This represents about 15% of ‘strong’ replication origins as Heichinger *et al* suggested that there are 401 ‘strong’ origins used in pre-meiotic S phase or

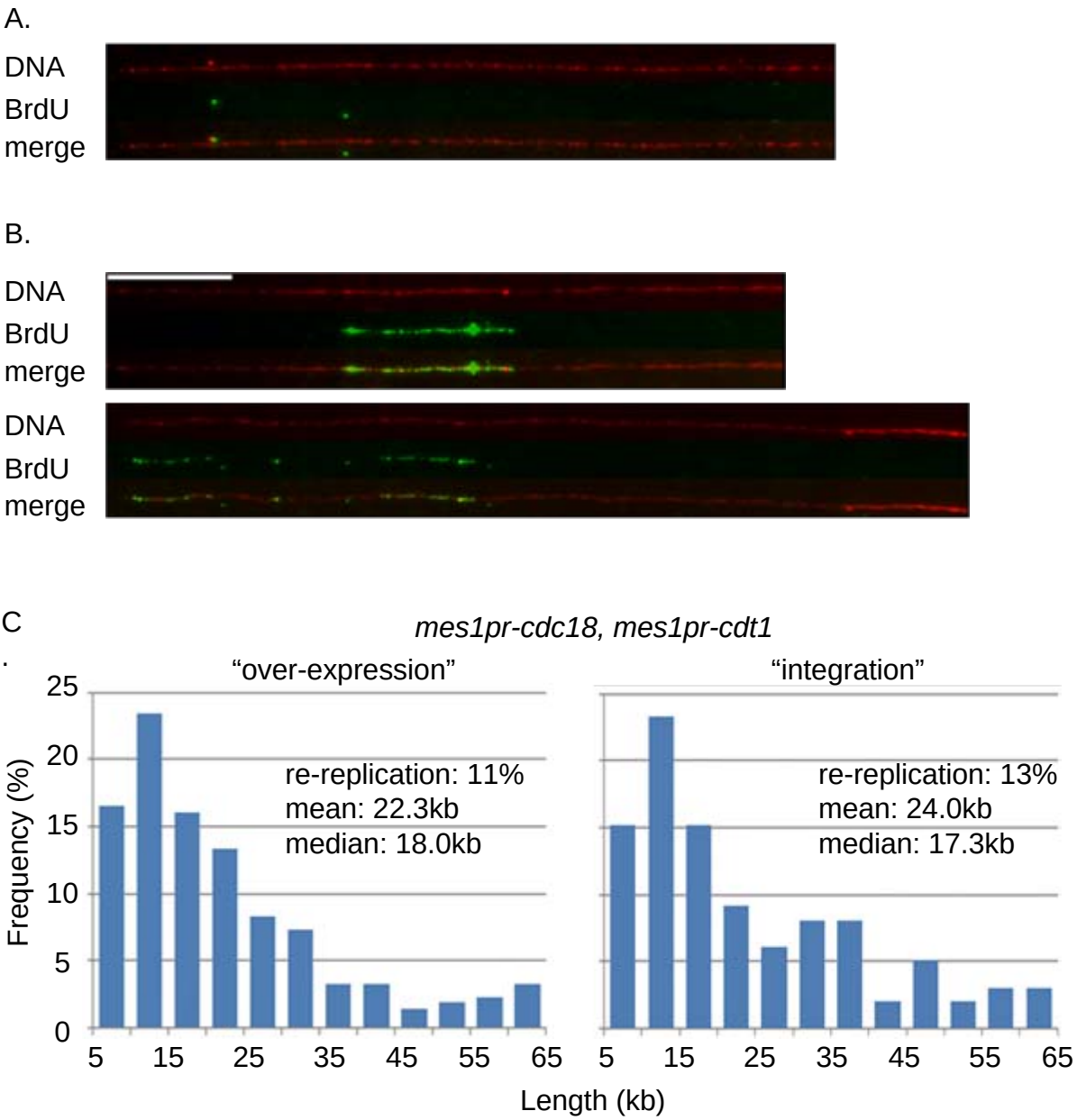

Figure 6.15 DNA combing reveals that elongation is restricted during the re-replication in late meiosis. BrdU was added around the time of MI and cells were harvested and processed 1.5 hours later.
(A-B) Representative images of the combed DNA fibres from
(A) negative control / wild-type cells (*pat1-114 rec12 Δ TK hENT1*, strain p3023), and
(B) *pat1-114 rec12 Δ TK hENT1* cells capable of over-expressing Cdc18 and Cdt1 during late meiosis (strain p2463). Red: DNA; Green: BrdU. Scale Bar=20kb (10 μm).
(C) Distribution of BrdU track lengths in cells over-expressing Cdc18 and Cdt1 during late meiosis (left panel, strain p2463) and cells expressing Cdc18 and Cdt1 in late meiosis at physiological levels (right panel, strain p2458). The average bubble length of the "over-expression" mutant is 22.3kb with a median of 18.0kb whilst for the "integration" mutant it is 24.0 kb with a median of 17.3kb.

mitotic S phase, defined by microarray analysis (Heichinger *et al*, 2006). It coincides with the estimation from Heichinger *et al* that origin utilization efficiency is 15% in pre-meiotic S phase, so that about 59 origins are used (Heichinger *et al*, 2006). This suggests that initiation is not restricted in late meiosis if Cdc18 and Cdt1 are expressed, compared to pre-meiotic S phase.

6.3 Discussion

In this Chapter I have demonstrated that additionally stimulating initiation by temporary inactivation and then reactivation of CDK can result in slightly more re-replication. However, as elongation is still restricted, I do not see a complete round of DNA replication. In addition, although I have found that protein levels of Dpf1 and Spd1 show an interesting pattern in meiosis (see Chapter 5), which may indicate their involvements in preventing DNA synthesis during late meiosis, in this Chapter my results suggest that they seem to be irrelevant, or at least they are not critical factors. In addition, I carried out some experiments similar to those carried out in other model organisms such as *Xenopus* and budding yeast, involving Wee1 and Mde3/Ime2. These studies gave indications for an involvement in replication control in other organisms (Furuno *et al*. 1994, Nakajo *et al*. 2000, Iwabuchi *et al*. 2000, Holt *et al*. 2007) but no such link was shown here in fission yeast meiosis (section 6.2.3 and 6.2.6), possibly indicating the differences between meiotic control in these model organisms.

In Chapter 4, I have shown that there is neither a DNA replication nor DNA damage checkpoint regulating MII entry in *S. pombe*. In other words, once meiosis I has occurred, there are no controls to stop meiosis II even if DNA damages occur. Owing to this situation, which is unlike the *Xenopus* egg system that is naturally arrested at MII metaphase (note that this arrest is not a DNA damage/replication checkpoint), the MI-MII interval is short in fission yeast, which might represent a temporal constraint for inducing re-replication. Thus I explored the use of *spo4*Δ mutant to relieve this time limitation, by arresting cells before MII (Nakamura *et al*. 2002). Meanwhile, use of this mutation may also help to avoid the problem that cells may not be synchronized very well as they traverse the MI-MII interval. In addition, a previous report suggests that although Spo4 is a Cdc7/Hsk1 like kinase, its primary role is not in DNA replication (Nakamura *et al*. 2002); hence deleting the *spo4* gene should not have negative effects on DNA synthesis during late meiosis.

Combining *spo4*Δ with late meiotic expression of Cdc18 and Cdt1, however, does not lead to more re-replication than that observed in a *spo4*$^{+}$ strain however. Manipulation of CDK activity, however, does suggest that CDK activity plays an important role in preventing untimely DNA replication during MI-MII interval. Ideally, efficient DNA replication may require first inactivation of

CDK to permit licensing and then increasing CDK activity to promote initiation. Meanwhile considering the existence of various meiotic-specific cyclins (including Rem1 and Crs1) (Malapeira *et al*. 2005, Averbeck *et al*. 2005), these might be relevant to fine tuning of CDK activity. For example, Mes1 regulates CDK activity by preventing APC/C mediated proteolysis of Cdc13, but there are no reports about its role in regulating other cyclins such as Rem1 and Crs1 (Izawa *et al*. 2005; Kimata *et al*. 2011). In this chapter, oscillation of CDK activity is achieved by regulation of Cdc2 itself, using a drug-sensitive Cdc2-'Shokat' mutant. After meiosis I, when a *spo4*Δ mutant is used to block cells in the MI-MII interval, and when Cdc18 and Cdt1 are present, and CDK is firstly inactivated and then activated, re-replication up to more than 4C can be induced. However, there is still an incomplete round of replication. The reactivation of CDK (Cdc2/Cyclin) is also of prime importance to repress replication between MI and MII in *Xenopus* oocytes, although to what extent the re-replication occurs by repressing CDK after MI in *Xenopus* eggs is unclear as the detection of re-replication is done by ^{32}P-dCTP incorporation and was not quantified (Furuno *et al*. 1994, Nakajo *et al*. 2000, Iwabuchi *et al*. 2000).

In fission yeast, degradation of Cdc18 after S phase is regulated by Cdc2 (Lemaître *et al*. 2004, Lindner *et al*. 2002, Baum *et al*. 1998). However, in budding yeast, proteolysis of Cdc6/Cdc18 in mitotic S phase is also Cdc2

dependent (Piatti *et al*. 1995), but its degradation during late meiosis depends on Ime2, a meiosis-specific CDK orthologue, which is considered to be functional redundantly with CDK in meiosis (Ofir *et al*. 2004). A previous publication suggests that licensing is restricted during MI/MII interval, partially due to phosphorylation of pre-RC components (Cdc18, Mcm6 and Orc6) by Ime2 (Holt *et al*. 2007). Thus I have examined the role of Mde3 (Ime2 orthologue) in fission yeast meiosis, but found that deleting Mde3 in addition to expressing Cdc18 and Cdt1 during late meiosis cannot enhance the re-replication phenotype.

Finally, using DNA combing, I found that when the efficiency of licensing was increased by over-expression of Cdc18 and Cdt1, the fraction of DNA re-replicated was similar to when these proteins were expressed at physiological levels. It may reflect an interpretation that increased efficiency of origin licensing may artefactually reduce initiation efficiency, via CDK inhibition by Cdc18 (Greenwood *et al*, 1998). Furthermore, many short DNA fragments are synthesized during the re-replication induced by expressing Cdc18 and Cdt1 after meiosis I, thus elongation is inhibited in MI-MII. When Cdc18 and Cdt1 are expressed in late meiosis to either physiological or over-expression levels, about the same numbers of origins were active in late meiosis (~60 origins) as in pre-meiotic S phase (~59 origins), suggesting that initiation is not restricted.

Chapter 7

Conclusions and further works

In contrast to *Xenopus* oocytes (and possibly other vertebrate oocytes), in which ability to replicate DNA is regained in metaphase I (shortly after germinal vesicle breakdown) by synthesis of Cdc6/Cdc18 (Lemaître *et al*. 2002), fission yeast cells undergoing meiosis lose replication competence by down-regulation of two pre-RC components Cdc18 and Cdt1 after pre-meiotic S phase. Forcing expression of Cdc18 and Cdt1 during the MI-MII interval to levels similar to those in pre-meiotic S phase is enough to lead to some DNA re-replication after meiosis I, which is detectable by EdU incorporation. The fraction of the genome replicated under this condition is not dramatic (10~15%), which reflects existence of other mechanisms inhibiting replication in addition to preventing licensing by absence of Cdc18 and Cdt1. DNA combing analysis suggests that elongation is restricted even when MCM chromatin binding occurs at similar levels to those seen in pre-meiotic S phase. It seems that fission yeast has robust mechanisms to prevent DNA replication during the interval between meiosis I and meiosis II, so even when prevention of licensing is deregulated, re-replication cannot be induced massively.

In the mitotic cell cycle, redundant or overlapping blocks to MCM chromatin loading are thought to provide the main mechanism to prevent re-replication in S and G2 phases, but blocks to elongation may operate under some circumstances. For instance, in the case of polytene chromosome re-replication in *Drosophila* salivary glands, the SUUR chromatin protein appears to impede replication fork progression (Belyaeva *et al*. 1998, Sher *et al*. 2012). Also, checkpoint-induced p21 may inhibit DNA synthesis via interaction with PCNA (Chen *et al*. 1995). Restriction of DNA replication at the elongation step has the advantage that a single initiation event would only generate a short re-replicated region which could be repaired, or displaced during the next S phase, without causing genome instability. It is unclear how elongation is limited in the meiosis I-II interval, but this could involve regulation of replication fork components, or chromatin modifications. Analysis of Spd1 involvement in RNR regulation did not imply that dNTP supply is a limiting factor for DNA synthesis. Also both RNR subunits Cdc22 and Suc22 were pancellular during late meiosis. This suggests that there is no RNR down regulation via changes in nuclear-cytoplasmic localization of RNR subunits in late meiosis as in the mitotic cell cycle (Nestoras *et al*. 2010). However, none of these studies have measured RNR activity directly. In addition to inhibitory chromatin modifications, it is possible that chromatin remains in a partially condensed state during the meiosis I-II interval (John, 1990), which could provide an environment unfavourable to replication fork movement.

Cells that have partially re-replicated their genome in the MI-MII interval can still proceed into meiosis II and spore formation, though spore viability is dramatically reduced. This indicates that there is no DNA replication checkpoint regulating entry into MII in fission yeast. Meanwhile, when DNA is damaged after meiosis I using DSB-inducing bleomycin, cells also enter meiosis II with similar kinetics to cells without DNA damage, suggesting that there is no DNA damage checkpoint after meiosis I as well. The DNA replication checkpoint in pre-meiotic S phase monitors progress of DNA replication, and blocks entry into MI if errors (such as stalled replication forks induced by RNR inactivation) occur (Murakami and Nurse 1999, Murakami and Nurse 2000). Therefore absence of a DNA replication checkpoint may not be surprising as normally after meiosis I no replication is expected. However, it has previously been shown that there does not seem to be a DNA damage checkpoint arresting entry into meiosis I at least in fission yeast (Pankratz and Forsburg, 2005). So the observation that there appears to be no DNA damage checkpoint between MI and MII as well may reflect a high tolerance of DNA damage in fission yeasts. This may also imply that meiotic cells may be particularly prone to DNA damage after meiosis I, since meiotic recombination is probably not active to repair any DSBs.

The regulation of DNA replication in late meiosis appears to be simpler in

Xenopus, compared to fission yeast, as in oocytes just CDK depression leads to replication in the meiosis I-II interval, and all the factors required for Mcm chromatin binding and later stages of DNA replication are present. However, the degree of re-replication in *Xenopus* eggs on CDK inactivation has not been quantitated, and it is possible that replication may still be constrained by other mechanisms.

In *Xenopus* eggs, re-replication occurs after MI when repression of licensing (activated CDK) is released. In *S. pombe*, availabilities of essential components for DNA replication are restricted (Cdc18 and Cdt1) during late meiosis, which blocks DNA synthesis. Also the elongation step of replication is inefficient when the licensing step is subverted, although the mechanism of this is obscure. This may reflect the different destinies of eggs and unicellular organisms like yeast after meiosis. After fertilization, eggs will immediately enter the mitotic cell cycle, thus the cell needs to be “set up” for S phase. In contrast, yeast will form spores after meiosis to cope with lack of nutrients and spores may remain dormant for extended periods. This may be relevant to the strict regulation of DNA replication seen in late meiosis in fission yeast.

It is possible that the yeast strains described here that are partially de-regulated for replication in meiosis I-II will be useful for uncovering mechanisms restricting DNA replication in the MI-MII interval by obtaining

mutants which replicate DNA more efficiently. On the other hand, genome-wide analysis/ quantitative proteomics methods such as SILAC (stable isotope labeling by/with amino acids in cell culture) may be able to further reveal if there are other proteins essential for replication are absence in the MI-MII interval (Ong and Mann 2006, Kubota *et al*. 2011).

Mechanisms restricting elongation steps may also be related to chromosome condensation or histone modifications. It is possible that chromatin remains in a partially condensed state during the meiosis I-II interval. Electron microscopy can be used to check the state of chromatin condensation in MI-MII interval (Jurgensmeier *et al*. 1997, Ink *et al*. 1997). However, neither any chemicals nor enzymes have been reported to be used for large-scale chromatin decondensation so far. So if condensation of DNA is identified, further experiments will not be straightforward. Chromosome decondensation is related to active transcription. So one strategy is to activate transcription at a specific locus, and just focus on this area, i.e. comparing re-replication in this area before and after the transcription activation by single molecular analysis. On the other hand, chromatin assembly is coupled with completion of replication. In late meiosis, it may not be able to correctly package chromatin due to limited histones, which might prevent synthesis of long DNA fragments/ movement of replication forks. This would be another interesting area for more investigations.

Although RNR subunits are pancellular in late meiosis (section 5.2.3) and deletion of *spd1* in addition to expressing Cdc18 and Cdt1 after MI does not enhance the re-replication (section 6.2.2), there is no direct evidence that dNTPs are present at sufficient levels in late meiosis for efficient DNA synthesis. RNR activity in meiosis has not been examined. Restricted dNTP supply due to other RNR inhibitors such as Spd2 is possible. Therefore, one experiment that could be done in the future is to quantitatively determine cellular dNTP levels during meiosis (the technique was described in (Shewach 1992), especially comparing dNTP level in MI-MII interval to that in pre-meiotic S phase. Alternatively, providing exogenous deoxynucleosides in addition to expressing Cdc18 and Cdt1 in late meiosis, to see if this can allow a more complete round of replication might be another way to answer this question. In this case, however, strain modifications will be required to allow *S. pombe* to take up nucleosides and metabolize them to dNTPs.

It would also be interesting to test the assumption that partial re-replication in haploid gametes is highly deleterious, or whether post-meiotic cell cycle can cope with meiotic errors causing minor replication of chromosomal regions in the interval between meiosis I and II. In *S. pombe*, re-replication after meiosis I appears to reduce spore viability, but the mechanism of this is unknown. It may be due to disturbance of MII or defects in later sporulation processes.

It seems that, at least in fission yeast, cells have relaxed surveillance of DNA damage during meiosis. It will be interesting to determine if there is any physiological significance to the absence of a damage checkpoint, such as sensitivity to DNA damage, and whether this has any relevance to the situation in higher eukaryotes.

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