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# DISSERTATION

Titel der Dissertation

„Proteins required for chromosome segregation during mitosis and meiosis“

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# Abstract

Accurate chromosome segregation depends on proper attachment of kinetochores to microtubules.

Merotelic kinetochore orientation which occurs when a single kinetochore is attached to microtubules emanating from opposite spindle poles has to be prevented. It is the major cause of aneuploidy in mitotic mammalian cells and it is the primary mechanism leading to chromosome instability in cancer cells. We identified a novel protein in *S. pombe* called Mde4 that forms a complex with Pcs1. Similarly to the *Δpcs1* mutant, in the absence of *mde4*, lagging chromosomes are frequently observed during mitosis. We developed an assay based on laser microsurgery to show that the stretched morphology of lagging kinetochores in *pcs1*Δ mutant cells is due to merotelic attachment. We further showed that Mde4 is regulated by Cdc2 and that Cdc2 activity prevents precocious localization of Mde4 to the metaphase spindle.

Meiosis is a specialized cell division that enables organisms to reproduce sexually by generating haploid gametes in two successive divisions. During meiosis, a single round of DNA replication is followed by two rounds of chromosome segregation, called meiosis I and meiosis II.

While meiosis II is similar to mitosis, the first meiotic division is fundamentally different. The formation of chiasmata, as a result of reciprocal recombination between homologous chromatids, and the orientation of sister kinetochores toward the same pole (mono-orientation) together ensure that maternal and paternal centromeres are pulled in opposite directions on meiosis I spindles. Segregation of chromosomes during meiosis I is triggered by separase cleavage of the cohesin's Rec8 subunit along chromosome arms. Cohesin in the vicinity of centromeres is protected from separase cleavage during meiosis I and holds sister chromatids together until anaphase II. We mapped Rec8 phosphorylation sites by mass spectrometry and showed that, in fission yeast, Rec8 phosphorylation is required for proper chromosome disjunction during meiosis. We further showed that the fission yeast casein kinase 1 (CK1) delta/epsilon isoforms Hhp1 and Hhp2 are required for full levels of Rec8 phosphorylation and for efficient removal of Rec8 at the onset of anaphase I. Our data are consistent with the model that Hhp1/Hhp2-dependent phosphorylation of Rec8 is required for separase-mediated cleavage of Rec8 during meiosis I. Moreover we applied a high-throughput

knockout technique to identify novel proteins required for proper segregation of chromosomes during meiosis. Using this approach, we identified a new protein, Dil1, which is required to prevent meiosis I homolog non-disjunction. Further analysis showed that Dil1 acts in the dynein pathway to promote oscillatory nuclear movement during meiosis and efficient pairing of homologous centromeres during meiotic prophase.

# Zusammenfassung

Die korrekte Segregation der Chromosomen in Mitose hängt vom richtigen Anheften der Mikrotubuli an die Kinetochore ab.

Man spricht von „merotelic kinetochor orientation“ wenn sich die Mikrotubuli der gegenüberliegenden Pole an ein einziges Kinetochor heften. Dieser Zustand sollte vermieden werden, da er die Hauptursache für Aneuploidie ist, die oft mit der Bildung von Krebs einhergeht. Wir konnten ein neues Protein, nämlich Mde4, das mit Pcs1 einen Komplex bildet, in der Spalthefe *S. pombe* identifizieren. Ähnlich wie in der *Δpcs1* Mutante kann man in Abwesenheit von Mde4 so genannte „lagging chromosomes“ beobachten. Im Rahmen dieser Arbeit konnten wir mittels einer neuen, auf Laser –Mikrochirurgie basierenden Methode zeigen, dass diese verbleibenden Chromosomen auf Grund von fehlerhafter merotelischer Anheftung an die Mikrotubuli zustande kommen. Weiters konnte gezeigt werden, dass Mde4 durch Cdc2 reguliert wird und Cdc2 eine frühzeitige Lokalisation von Mde4 an der Metaphase Spindel verhindert.

Die Meiose ist eine besondere Form der Zellteilung, in der haploide Keimzellen entstehen. Dies ist Voraussetzung für die sexuelle Fortpflanzung. Während der Meiose folgen auf eine einzige Replikation der DNA zwei Teilungen, die man als Meiose I und Meiose II bezeichnet. Während Meiose II der mitotischen Zellteilung sehr ähnlich ist, ist die erste meiotische Teilung grundlegend anders. Die Rekombination, gefolgt von der Bildung von Chiasmata und die Monoorientierung der Schwesterkinetochore, stellt sicher, dass die homologen Chromosomen zu entgegengesetzten Zellpolen gezogen werden. Die Spaltung der Cohesin Untereinheit Rec8 entlang der Chromosomenarme durch Seperase löst die erste meiotische Teilung aus. Cohesine in der Nähe der Centromere müssen geschützt werden um die Schwesterchromatide bis hin zur Anaphase II zusammenzuhalten und ihre korrekte Aufteilung in Meiose II zu garantieren. Mittels Massenspektrometrie haben wir alle Phosphorylierungsstellen in Rec8 identifiziert und gezeigt, dass die Phosphorylierung von Rec8 für die korrekte Chromosomenaufteilung während der Meiose erforderlich ist. Weiters konnte gezeigt werden, dass die Casein Kinase 1 (CK1) delta/epsilon Isoformen von *S. pombe*, Hhp1 und Hhp2, für die vollständige Phosphorylierung und die Spaltung von Rec8 mittels Seperase zuständig sind.

Darüberhinaus wurde ein „high-throughput-screen“ durchgeführt um neue Proteine, die an der Chromosomenaufteilung beteiligt sind, zu finden. So ist es gelungen das Protein Dil1 zu identifizieren. Dil1 verhindert eine Nicht-Auftrennung der homologen Chromosomen während der Meiose I. Es ist Teil des Dynein-Pathways und fördert die Paarung homologer Centromere während der meiotischen Prophase.

# 1. Introduction

## 1.1 General Introduction

Correct chromosome segregation is a crucial process during both, mitosis and meiosis. The DNA carrying the genomic information is duplicated and subsequently segregated to opposite poles. It has to be ensured that after cell division each daughter cell contains exactly the same number of chromosomes. This depends on proper attachment of kinetochores to microtubules. An error which often occurs during mitotic chromosome segregation is the so called merotelic attachment of kinetochores. Merotely happens when a single chromatid is attached to microtubules emanating from opposite spindle poles. These chromatids are often missegregated and therefore give rise to cells with too many or not enough chromosomes. These cells are called aneuploid cells and are the major hallmark of cancer cells.

Meiosis is a specialized form of cell division which is the basic prerequisite for sexual reproduction. A single round of DNA replication is followed by two consecutive rounds of chromosome segregation called Meiosis I and Meiosis II in order to form haploid gametes. During Meiosis I three major events have to take place in order achieve correct chromosome segregation. The homologues chromosomes have to find each other and have to be physically connected. This linkage is achieved by reciprocal recombination and the resulting chiasmata between homologous non-sister chromatids. Recombination is not only essential for the correct segregation but also for generating new combinations of alleles and genetic variation between the progeny of a given set of parents. The second unique and essential feature of meiosis I is the mono-orientation of sister chromatids which means that in contrast to mitosis both sisters have to segregate to the same pole. The third characteristic is the stepwise loss of sister chromatid cohesion. During meiosis I only the cohesin which is located at the arm regions is cleaved while the centromeric cohesin has to be protected until anaphase II in order to segregate the sister chromatids correctly. During the course of my work I tried to identify new factors and regulation pathways which play a role in these important processes.

## 1.2 *Schizosaccharomyces pombe* as a model organism

During the course of this work the fission yeast *S. pombe* was used as a model organism. *Schizosaccharomyces pombe* was first described in 1893 by P. Lindner and named from 'pombe', the Swahili word for beer, since it was originally isolated in millet beer from eastern Africa. In contrast to *Saccharomyces cerivisiae*, *S. pombe* is reproducing by fission alone and not by budding. *S. pombe* cells are usually cylindrical, with rounded ends. The haploid *S. pombe* genome is 13.8 Mb in size and is organized into three chromosomes, I (5.7 Mb), II (4.6 Mb) and III (3.5 Mb). (Wixon, 2002) It is sequenced and contains approximately 4979 genes.

Fission yeast can be maintained in haploid and diploid stage and provides all advantages of a unicellular organism. It has a doubling time of 2-4 hours and can be cultured under really simple conditions.

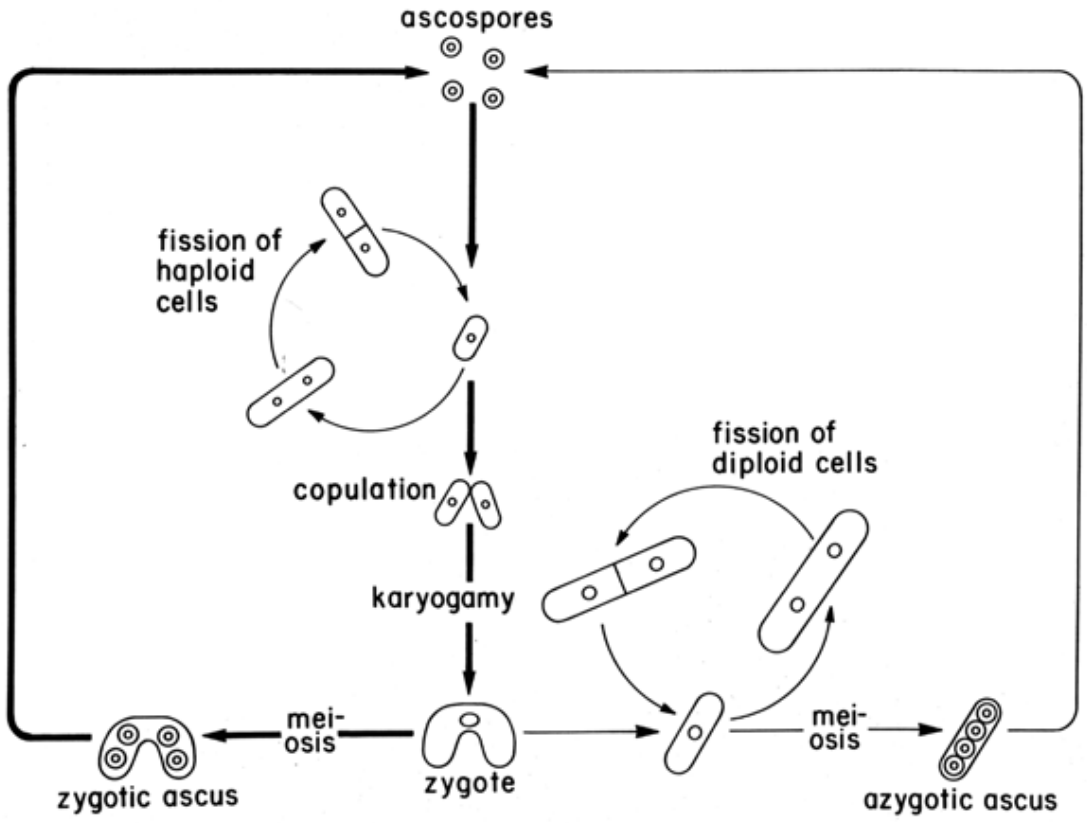



**Figure 1.1** Life cycle of *Schizosaccharomyces pombe* (Gutz, 1974). In: Handbook of Genetics, Vol. 1, Plenum Press, New York, London, pp 395-446

Like for *S. cerevisiae*, a big variety of classical genetic methods including mutagenesis, selection, suppressor and synthetic lethal analysis, tetrad dissection and random spore

analysis as well as a broad range of biochemical approaches can be implemented. Recently we have designed an efficient strategy to knock out genes in the fission yeast *Schizosaccharomyces pombe* on a large scale. Our technique is based on knockout constructs that contain regions homologous to the target gene cloned into vectors carrying dominant drug-resistance markers. Most of the steps are carried out in a 96-well format, allowing simultaneous deletion of 96 genes in one batch. Based on our knockout technique, we designed a strategy for cloning knockout constructs for all predicted fission yeast genes, which is available in a form of a searchable database (Rumpf *et al.*; Spirek *et al.*; Gregan *et al.*, 2006). This gives us a powerful tool for large scale screens. Since *S. pombe* is easy to synchronize in mitosis as well as in meiosis it is the perfect model organism for biochemical approaches like the purification of proteins together with their interacting partners. Tandem affinity purification (TAP) is a method that allows rapid purification of native protein complexes. We developed an improved technique to fuse the fission yeast genes with a TAP tag. We used this technique to design strategies for TAP-tagging of predicted *Schizosaccharomyces pombe* genes which are again available in a form of a searchable database (Cipak *et al.*, 2009). Even though the applicable tools for *S. pombe* and *S. cerevisiae* are similar, the biology of those two yeast species is very divergent. There are many genes and molecular pathways missing in *S. cerevisiae* which are present in *S. pombe* and higher eukaryotes. One such example is the kinetochore and underlying centromeric region. While the centromere DNA in *S. cerevisiae* consists of only 125bp the centromeres in *S. pombe* contain a long stretch (40 to 100 kb) of repetitive DNA sequences arranged into a large inverted repeat, most of which is required for full centromere function (Nakaseko *et al.*, 1986; Carbon & Clarke, 1990; Takahashi *et al.*, 1992; Hegemann & Fleig, 1993). Since in higher eukaryotes the centromeric regions often contain megabases of repetitive sequences, in this aspect, the fission yeast appears to be a better model to study the factors involved in chromosome segregation.

## 1.3 Mitosis

Mitosis is one form of cell division in eukaryotic cells. It is the process in which a parental cell divides in two genetically identical daughter cells. Unicellular organisms use this form of cell division as a basis for asexual reproduction. In multicellular

organisms however, the mitotic cell division is responsible for growth and differentiation. Mitosis is usually subdivided into several stages: During prophase chromatin becomes condensed and the centrosome which is responsible for the nucleation of microtubules is duplicated. With the assistance of motor proteins, the centrosomes are pushed to the opposite poles of the cell. Prophase is followed by a stage called prometaphase where the nuclear envelope is disassembled. This is true for most of the multicellular organisms which undergo a so called 'open mitosis'. In yeasts the nuclear envelope stays intact which is called 'closed mitosis'. At the centromere of each chromosome kinetochores are formed which build a platform for microtubule attachment. While kinetochore microtubules are searching for their attachment site, the mitotic spindle spanning from one centrosome to the other is established. Metaphase is reached when stable kinetochore-microtubule attachments are formed and the chromosomes align at the metaphase plate. At the onset of anaphase, proteins holding sister chromatids together are cleaved and kinetochore microtubules shorten in order to pull the chromosomes towards the centrosomes. In parallel, the mitotic spindle is elongated, thereby pushing the centrosomes to the very ends of the cell. As cells proceed to telophase the spindle disassembles and a new nuclear envelope is formed. At the same time cytokinesis takes place. In that stage the cell finally divides giving rise to two daughter cells, each containing one copy of the parental genome. Mitosis and cytokinesis together define the mitotic M-phase which is a short but important stage of the mitotic cell cycle.

## 1.4 The mitotic cell cycle

The mitotic cell cycle can be divided into two stages: M-phase which is comprised of mitosis and cytokinesis, and the interphase including $G_1$-, S- and $G_2$-phase. $G_1$ (G stands for gap) starts immediately after mitosis and cytokinesis. It is also referred to as growth phase and is characterized by high biosynthetic activity. During $G_1$-phase, cells are able to exit the cell cycle, enter a quiescent stage and stop dividing. This is common for fully differentiated cells of multicellular organisms. After $G_1$ the S-phase (S stands for synthesis) in which the DNA becomes replicated starts. During $G_2$ again a high level of biosynthesis occurs. $G_2$ is followed by M-phase which includes mitosis and cytokinesis. These complex events have to be regulated in a strict manner. This

regulation is achieved mainly by protein kinases and their regulatory subunits called cyclins which drive the cell through the cell cycle.

## 1.5 Chromosome segregation during mitosis

### *1.5.1 The kinetochore links microtubules to chromosomes*

When we talk about chromosome segregation during mitosis and meiosis it is important to understand the principles of microtubule attachment to kinetochores. Kinetochores are key cell division organelles which couple microtubules to specialized chromosomal regions known as centromeres. They consist of many different proteins organized to multiprotein complexes and control and power chromosome segregation. One key feature of kinetochores is that they govern the dynamic properties of microtubules consisting of α and β tubulin heterodimers associated to highly dynamic polymers. According to their proximity to DNA, kinetochore proteins can be classified into two main groups: the inner kinetochore proteins and the outer kinetochore proteins which include microtubule binding proteins. In addition, proteins involved in the spindle assembly checkpoint and correction of erroneous microtubule-kinetochore attachment localize to kinetochores. CENP-A, a specialized H3 histone variant is thought to specify centromere identity. Upon binding to DNA, the inner kinetochore proteins form a platform allowing the assembly of further protein complexes. Some of these proteins are present at kinetochores during most of the cell cycle and disassemble only during a brief period in S-phase to allow the replication machinery to pass. Plus-end tracking proteins (+TIPs), a class of microtubule associated proteins that is found at the plus ends of microtubules, and other microtubule-associated proteins (MAPs) belong to the group of outer microtubule binding proteins. Some of them strictly require microtubules for their associations with kinetochores. In order to deal with microtubule binding, kinetochores contain specialized machinery. Recent studies suggest that the KMN network represents the most important component of such a machinery (Santaguida & Musacchio, 2009). It consists of the Ndc80 complex, the Mis12 complex and MNL1. Despite binding of microtubules the KNM network provides a landing platform for the spindle assembly checkpoint (SAC) proteins (Musacchio & Salmon, 2007; Cheeseman & Desai, 2008). Beside the KMN network several protein complexes associated with kinetochores have been studied extensively during the last

years. The tripartite Ska complex plays a role in microtubule binding in higher eukaryots. In contrast the Dam1 could be identified exclusively in fungi. In the budding yeast *S. cerevisiae* it forms rings around microtubules and thereby plays a crucial role in the process of chromosome segregation (Westermann *et al.*, 2007) reviewed in (Santaguida & Musacchio, 2009). The overall structure of kinetochores is evolutionary conserved from yeasts to human.

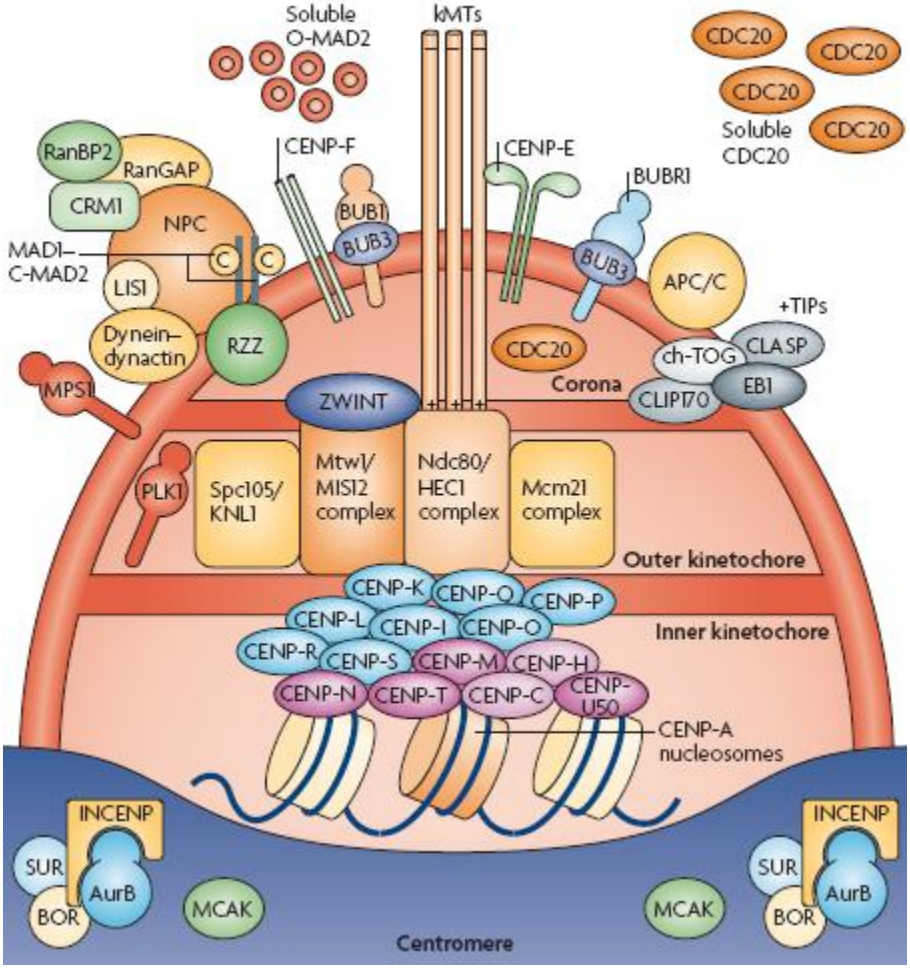



**Figure 1.2** The structure of kinetochores. The kinetochore consists of several protein complexes. The core complex is organized in two groups: the inner kinetochore complex and the outer microtubule binding complex. To regulate the stability of microtubule attachment Borealin (BOR), Survivin (SUR), Aurora-B (AurB), inner centromere protein (INCENP) and mitotic centromere-associated kinesin (MCAK) are located at the inner centromeric regions. The KMN network consisting of KNL1, Mis12 and Ndc80 builds a landing platform for the SAC proteins Mad1, Mad2, BubR1, Bub1 as well as Cdc20 and the APC/C which are present in order to prevent the precocious separation of sister chromatids (see also chapter 1.5.2) (figure taken from (Musacchio & Salmon, 2007)

### *1.5.2 Detection of mis-attachment and the SAC*

During mitosis the cell has to make sure that all sister kinetochores become bi-oriented. Therefore there must be mechanisms that are not only able to identify wrong attachments but also to correct them. There are different possibilities how sister kinetochores can be attached to microtubules. The correct amphitelic attachment in which two sisters are attached to microtubules emanating from the opposite poles is essential for faithful segregation of chromosomes. During mitosis also monotelic (only one sister is attached), merotelic (one sister is attached to microtubules from the two opposite poles) and syntelic (both sisters are attached to microtubules emanating from only one pole) kinetochore orientation can occur (**Figure 1.3** Different ways of kinetochore attachment. If the cell does not recognize and correct those incorrect attachments it is likely that chromosomes are mis-segregated, which may lead to aneuploidy.

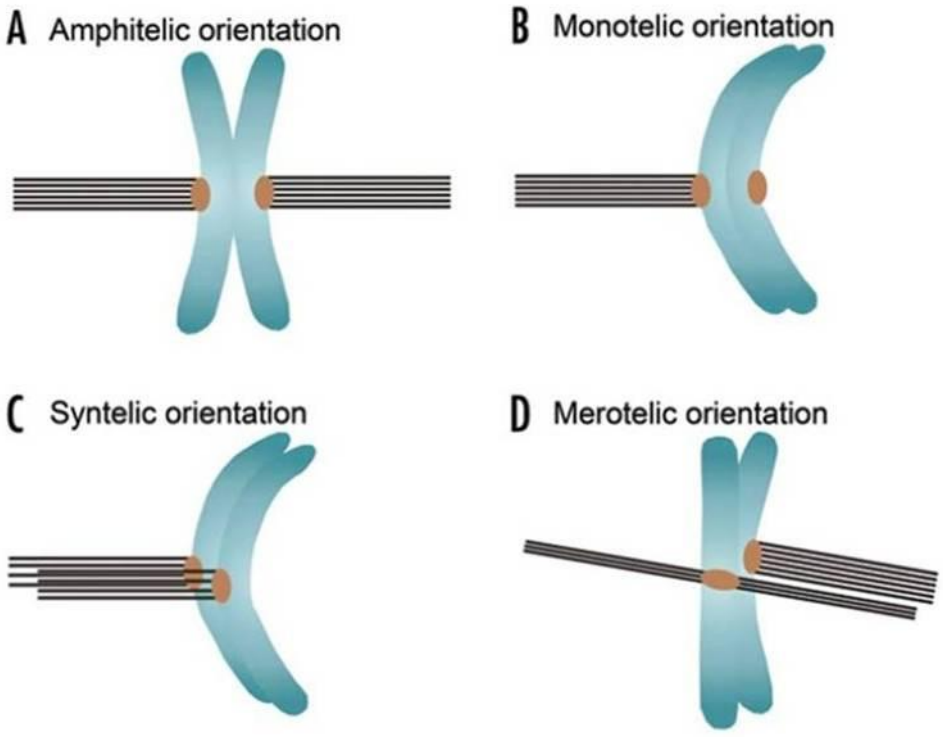



**Figure 1.3** Different ways of kinetochore attachment. During mitosis the cell has to make sure that all sister kinetochores are bioriented (amphitelic attachments). Monotelic, merotelic or syntelic attachments have to be prevented or repaired. Figure taken from (Cimini, 2007)

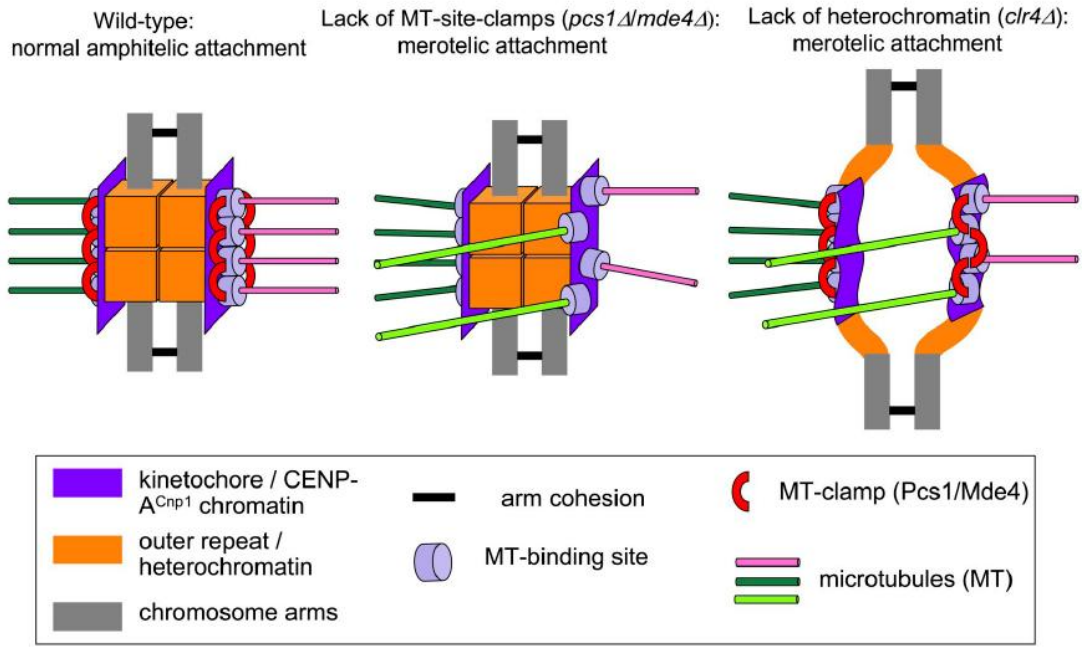
The spindle assembly checkpoint (SAC) is a pathway that monitors the attachment state of kinetochores. It delays anaphase until all kinetochores are attached to spindle microtubules. Several components of the SAC could be identified in previous screens. The proteins identified include Mad1, Mad2, Mad3 (BubR1 in humans), Bub1 and Bub3 (Hoyt *et al.*, 1991; Li & Murray, 1991). All those components are highly conserved among all eukaryotes. Mad2 and Mad3 directly bind and inhibit Cdc20, a co-factor of the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). Upon Cdc20 inhibition the onset of anaphase is blocked. When all kinetochores are amphitelically attached, the SAC is satisfied, Cdc20 is released and subsequently activates the APC/C. This leads to the polyubiquitylation of two key substrates, cyclin B and securin and triggers their degradation. Securin is the specific inhibitor for a protease called separase which is required to resolve sister chromatid cohesion (see also chapter 1.5.5). Proteolysis of cyclin B however leads to inactivation of the master mitotic kinase, CDK1, thereby promoting mitotic progression. But how can the SAC detect wrong kinetochore attachments? SAC proteins are known to be enriched at unattached kinetochores. Upon attachment of microtubules, kinetochores change their structural integrity which prevents binding of the core SAC proteins. This system allows the detection of unattached kinetochores (Khodjakov & Pines, 2010). During syntelic attachment, where all microtubule attachment sites are occupied, a different mechanism has to be applied. Syntelically attached kinetochores do not generate the tension that usually occurs when the two sisters are bi-oriented. At amphithelic kinetochores the increased tension and kinetochore to kinetochore distance stabilizes the spindle microtubule attachment to kinetochores. When this tension is low, like it is in syntelically attached sister kinetochores, the attachment is destabilized. After detachment of kinetochore microtubules the SAC recognizes unattached kinetochores and prevents anaphase. Destabilization of kinetochore attachment depends on Aurora B kinase. Aurora B kinase is located at the centromers and phosphorylates its kinetochore substrates which can destabilize microtubule attachments. Its opposing phosphatase PP1 localizes to the outer kinetochores (Liu *et al.*). Only if kinetochores come under tension they are pulled away from the inner centromeres and out of reach of Aurora B. However, they are still in the zone of PP1 and become dephosphorylated. This mechanism allows the dephosphorylation of Aurora B substrates and the

stabilization of kinetochore attachments. However merotelic attachment is not recognized by the SAC. Obviously merotelic attachment provides enough tension and sufficient occupancy to satisfy the spindle assembly checkpoint. However, correction mechanisms based on the Aurora-B kinase exist which are able to correct merotelic attachments (Peters, 2006; Musacchio & Salmon, 2007; Gregan *et al.*, 2011).

### *1.5.3 Pcs1/Mde4 and heterochromatin are required to prevent merotelic kinetochore attachment*

Pcs1 is the fission yeast ortholog of the monopolin subunit Csm1 in *S. cerevisiae.* While the function of the monopolin complex in *S. cerevisiae* is to clamp together microtubule attachment sites on the two sister kinetochores during meiosis I (Rabitsch *et al.*, 2003)(see also chapter 1.7.3), *S. pombe* cells lacking Pcs1 show no apparent defect in chromosome segregation during the first meiotic division. Pcs1 binds tightly to a protein called Mde4 which shares similar sequence features with another *S. cerevisiae* monopolin protein called Lrs4. Both, Mde4 and Pcs1, localize to the inner centromeric regions. The absence of either Mde4 or Pcs1 leads to a high number of lagging chromosomes during late anaphase resulting in sister chromatid nondisjunction during mitosis and meiosis II. The kinetochores of lagging chromosomes show typical features of merotelically attached kinetochores. Under the microscope, they appear laterally stretched and DAPI quantification provides evidence that the lagging chromosomes are indeed single chromatids. Although the Csm1/Lrs4 and the Pcs1/Mde4 complexes act during different stages, it has been suggested that they have a similar role, namely clamping together microtubule attachment sites. Due to the fact that in *S. cerevisiae* only a single microtubule is attached to one kinetochore, the only situation when they have to deal with multiple attachment sites, is during meiosis I when the two sister kinetochores have to act as one unit. In *S. pombe* and in higher eukaryotes several microtubules are attached to one kinetochore, hence the cells need an additional mechanism clamping together microtubule attachment sites also in mitosis and meiosis II (Gregan J, 2007). A second mechanism that helps to prevent merotelic kinetochore attachment in fission yeast is the Clr4/Swi6 dependent structure of centromeric heterochromatin (Pidoux & Allshire, 2005). Cells lacking Clr4 show a similar phenotype as has been observed in *pcs1*$\Delta$ or

*mde4*Δ mutants. It has been suggested that heterochromatin contributes to a higher order structure that provides rigidity to each individual centromere/kinetochore and that this ensures that microtubule binding sites can be properly oriented. Absence of centromeric heterochromatin might cause increased kinetochore flexibility, making it more prone to interaction with microtubules coming from opposite poles (Gregan J,




2007).

**Figure 1.4** Mechanisms preventing merotelic kinetochore orientation. The model suggests that the Pcs1/Mde4 complex clamps together microtubule attachment sites on one kinetochore while the heterochromatin is required to provide rigidity to each kinetochore. Both mechanisms are essential in order to prevent merotelic attachment. Figure taken from (Gregan J, 2007)

### *1.5.4 Sister chromatid cohesion*

One major mechanism ensuring the bi-orientation of sister chromatids is sister chromatid cohesion. Upon bi-orientation, physical linkage between sister chromatids counteracts the pulling forces of the microtubules thereby creating tension needed for stabilization of microtubule-kinetochore attachments. Only when all chromatids are under tension and are aligned at the metaphase plate, cohesion is destroyed and the sisters can segregate to the opposite poles (Tanaka *et al.*, 2000; Nasmyth, 2001). Sister chromatid cohesion is mediated by a multi subunit complex called cohesin. The core cohesin complex consists of a heterodimer formed between the SMC proteins Smc1

and Smc3, associated with the non SMC proteins Scc1 (alternatively the meiosis specific α-kleisin subunit Rec8 (Klein *et al.*, 1999; Watanabe & Nurse, 1999)) and Scc3 (Guacci *et al.*, 1997; Michaelis *et al.*, 1997). SMC proteins are composed of a 50 nm long intramolecular antiparallel coiled coil with a hinge domain at one end and one half of an ABC-type ATPase domain at the other. Smc1 and Smc3 associate via their hinge domains to form a V-shaped structure. The two ATPase domain containing heads are linked via Scc1 forming a ring with a diameter of about 40 nm (Haering *et al.*, 2002; Gruber *et al.*, 2003; Nasmyth & Haering, 2009). The hydrolysis of ATP is important for a stable chromosome-cohesin association (Arumugam *et al.*, 2003). In addition accessory proteins Pds5, Wapl and Sororin bind loosely to the core complex (Panizza *et al.*, 2000; Kueng *et al.*, 2006; Nishiyama *et al.*, 2010).

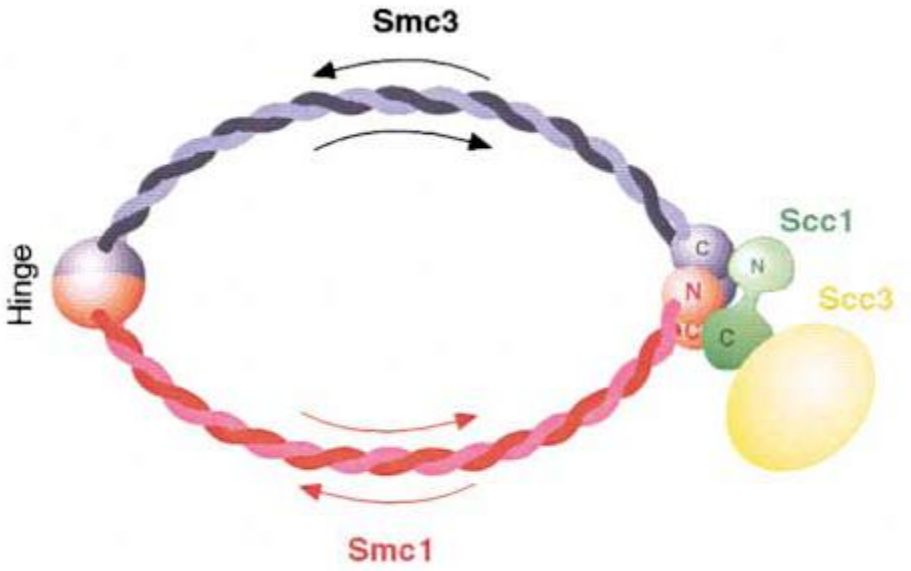



**Figure 1.5** Structure of the yeast cohesin complex. The cohesin core complex consits of the Smc1/Smc3 heterodimer which is bound to Scc1 and thereby forms a huge tripartite ring. (Haering *et al.*, 2002)

Cohesin is loaded onto chromosomes prior to S-phase and remains until anaphase. The separate Scc2/4 complex and Eco1 are required for the loading of cohesin but not for its maintenance (Ciosk *et al.*, 2000; Ivanov *et al.*, 2002). After endless discussions how cohesin holds together sister chromatids, the ring model finally made its point. It predicts that cohesin holds sister chromatids together by trapping them within the cohesin ring. It has been suggested that a transient dissociation of Smc1 and Smc3 at

the hinge region is required for the entry of the DNA into the cohesin ring (Gruber *et al.*, 2006).

### *1.5.5 Removal of sister chromatid cohesion*

The dissociation of cohesin has to be tightly regulated and takes place in two independent steps. One takes place during anaphase and involves the cleavage of the cohesin $\alpha$-kleisin subunit Scc1. The other one triggers the dissociation of a large bulk of cohesin during prophase and pro-metaphase from chromosome arms without cleavage. This process is called the prophase pathway. The exact function of the prophase pathway is not known so far. It could be shown that the phosphorylation of Scc3 by AuroraB and Polo-like kinase is essential for the removal during the early stages of mitosis (Hauf *et al.*, 2005). However, the prophase pathway does not seem to play a role in chromosome segregation and it is not evident in yeast mitosis.

Since this thesis deals with the mechanisms governing chromosome segregation, the more important process is the removal of cohesin during anaphase. After all kinetochores come under tension at the metaphase to anaphase transition a protein called separase is activated and removes cohesin by cleavage of its $\alpha$-kleisin subunit Scc1. This is the point of no return (Uhlmann & Nasmyth, 1998; Uhlmann *et al.*, 1999). To make sure that separase is only active when all sister chromatids are bioriented, a stringent regulation is absolutely necessary. During a large part of the cell cycle separase is inactivated through its binding to a chaperone called securin (Uhlmann *et al.*, 2000). When all sisters are bi-oriented and under tension the SAC is satisfied. Subsequently, CDC20 is released and activates the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). The latter is ubiquitinylating securin which leads immediately to its degradation by the 26S proteasome (Peters, 2006; Musacchio & Salmon, 2007). Freed of its inhibitor separase is now able to cut cohesin's Scc1 subunit. Subsequently cohesin is removed from chromosomes which triggers sister chromatid disjunction (Uhlmann *et al.*, 1999; Waizenegger *et al.*, 2000).

## 1.6 Meiosis

During sexual reproduction haploid gametes fuse to diploid zygotes which can develop into embryos and consequently adult organisms. Therefore sexually reproducing organisms have to produce haploid gametes from diploid progenitor cells in a

specialized cell division called meiosis. In meiosis a single round of DNA replication is followed by two successive rounds of chromosome segregation called meiosis I and meiosis II. In contrast to mitosis, during the first meiotic division homologs are segregated to opposite poles. To do so, the homologous chromosomes must be joined before segregation. This linkage of homologs is achieved through reciprocal recombination and the formation of chiasmata. This is not only important for linkage and correct segregation of homologs during the first meiotic division, it is also the process in which maternal and paternal genetic material is exchanged. Thus reciprocal recombination generates new combinations of alleles and genetic variation between the progeny of a given set of parents (Petronczki et al., 2003).

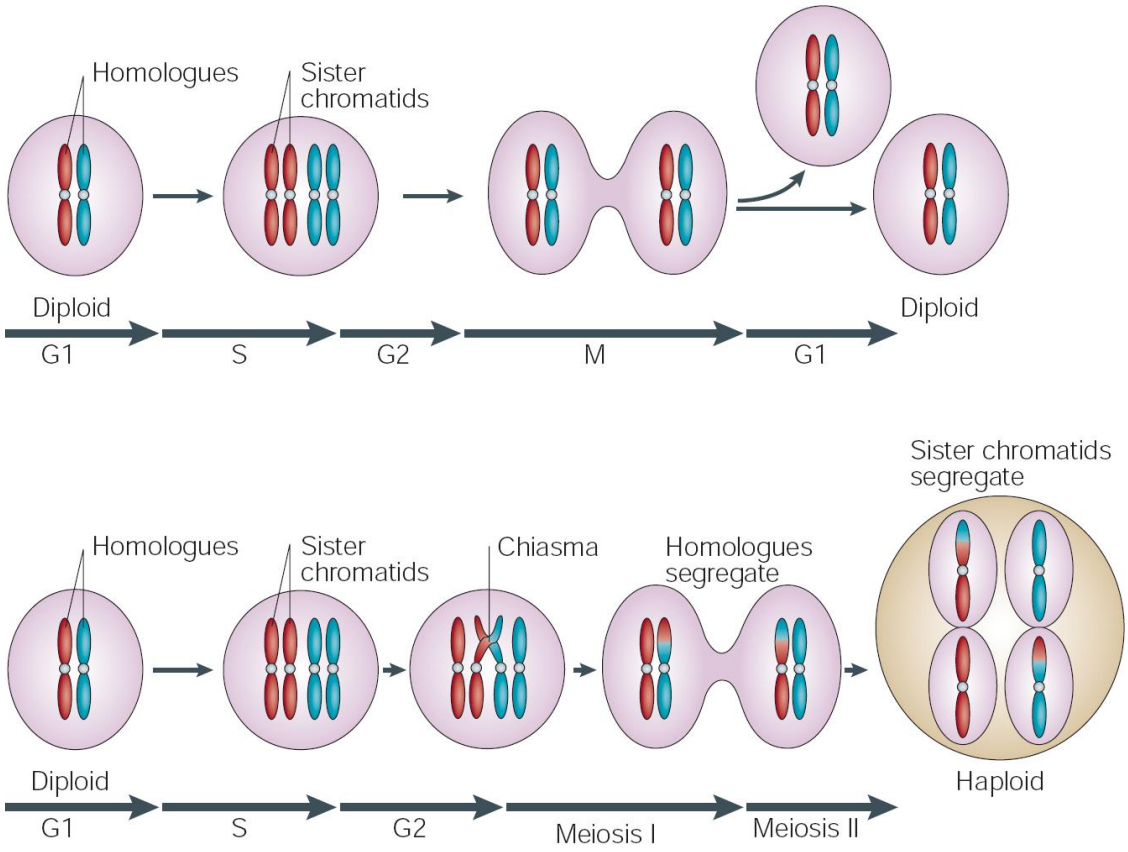



**Figure 1.6** Mitosis versus Meiosis. In both mitosis and meiosis only one round of DNA replication takes place. In mitosis sisters segregate and diploid daughter cells are produced, while in meiosis DNA replication is followed by two rounds of chromosome segregation, leading to the generation of haploid gametes (Marston & Amon, 2004)

In order to segregate sisters together during meiosis I, they have to be attached to microtubules emanating from one spindle pole. In budding yeast this monoorientation is ensured by the presence of the monopolin complex (Toth *et al.*, 2000; Rabitsch *et*

*al.*, 2003). Only when all homolog pairs come under tension, the cohesion along the chromosome arms is destroyed, chiasmata are resolved and the homologs can segregate to the opposite poles. However, centromeric cohesion has to be protected in order to segregate the sisters correctly during the second meiotic division. Meiosis II is similar to mitosis in that sisters have to be attached to microtubules emanating from opposite spindle poles. The centromeric cohesion is important to counteract the pulling forces of the spindle and thereby creating tension. Again, when all sisters come under tension, the second round of separase activation leads to loss of the centromeric cohesion and the sister chromatids can segregate to opposite poles (Salah & Nasmyth, 2000)(reviewed in (Petronczki *et al.*, 2003). Thus the stepwise loss of cohesion during meiosis is a crucial event which has to take place in order to segregate chromosomes correctly (Riedel *et al.*, 2006).

## 1.7 Segregation during meiosis

### *1.7.1 Pairing of homologous chromosomes*

During meiotic prophase homologous chromosomes have to find each other and associate along their entire length. In some organisms this step is the prerequisite for recombination and thus for a successful meiotic division. Various strategies for homologous chromosome pairing have been developed. In a wide variety of organisms a characteristic arrangement of chromosomes called the 'bouquet' arrangement can be observed. It occurs due to the clustering of telomeres which are attached to a restricted area of the nuclear envelope. In the fission yeast *S. pombe* the 'bouquet' emerges particularly strong. The characteristic elongated nucleus also referred to as 'horsetail nucleus' is moving forward and backward during prophase. The driving force of the horsetail movement is created by a dynein protein motor on microtubules and transferred to telomeres on the nucleoplasmic side via conserved SUN and KASH domain proteins called Sad1 and Kms1. These proteins build a bridge spanning the nuclear envelope (Starr, 2009; Ding *et al.*, 2010). Due to the nuclear oscillation chromosomes are shuffled until they find their correct partner. However, it is still not completely understood how the chromosomes can recognize their homologous partners. In most organisms like budding yeast, mice and humans, double strand breaks (DSB) are the prerequisite for pairing of homologous chromosomes while in

other species matching can occur by DSB-independent means. In *C. elegans* for example, a set of four zinc finger proteins binding with so-called pairing centers play the key role in the recognition of homologous chromosomes. In other organisms including *S. pombe* the centromere heterochromatin is essential for pairing (Ding *et al.*, 2004; Gerton & Hawley, 2005; Ding *et al.*, 2010). Pairing is followed by synapsis. In most eukaryotes the synaptonemal complex (SC) is formed during meiotic prophase. It consists of two parallel lateral elements attached to the chromatid loops and a transversal element connecting the lateral elements. In *S. pombe* however, the typical tripartite SC is missing. Instead, non-continuos structures called linear elements, resembling the axial elements of the SC, are formed. The linear elements are deemed to be the minimal structural requirement for efficient pairing and recombination of meiotic chromosomes (Loidl, 2006).

### *1.7.2 Recombination*

Homologous recombination is the process in which the polymorphic information on maternal and paternal chromosomes is exchanged thus creating genetic diversity in the offspring. Moreover, the linkage between homologs is essential for the correct chromosome segregation during the first meiotic division. The process of recombination, the involved proteins and its DNA intermediates have been studied extensively during the past. It is well described in the budding yeast *Saccharomyces cerevisiae* and various data suggest that the key players are conserved throughout all eukaryotes (Szekvolgyi & Nicolas, 2010). Meiotic recombination starts with the formation of DNA double-strand breaks (DSBs) generated by the evolutionary conserved Spo11 endonuclease (Keeney *et al.*, 1997). A Spo11 dimer cuts the DNA by a topoisomerase-like transesterification reaction. After removal of Spo11 covalently bound to short oligonucleotides, the DSBs are processed by the Mre11-Rad50-Xrs2 and the Com1-Sae2 complexes in order to produce 3' single stranded DNA overhangs. The Mre11-Rad50-Xrs2 complex also plays a role in DSB formation (Borde & Cobb, 2009; Szekvolgyi & Nicolas, 2010). After formation of 3' single stranded DNA overhangs, strand invasion, mediated by Rad51 and Dmc1, takes place. These two proteins are not redundant and their specific substrates and the way they are coordinated are not fully understood so far (Bishop *et al.*, 1992; Szekvolgyi & Nicolas, 2010). Strand invasion results in the formation of joint molecules which can either occur as single-end

invasion (SEI) intermediates or double Holiday junction (dHJ) intermediates; depending on whether only one or both 3' ends are engaged. The final step of meiotic recombination is the resolution of these intermediates. The catalyzing enzymes are still poorly characterized. The outcome of the reaction depends on the cleavage pattern. The cleavage can be vertical and horizontal. Only asymmetric cleavage leads to a crossover (CO) product which contains maternal and paternal information. This means that one junction has to be cleaved vertically while the other is cleaved horizontally. Cleavage in a symmetric manner leads to the formation of non-crossover (NCO) products.

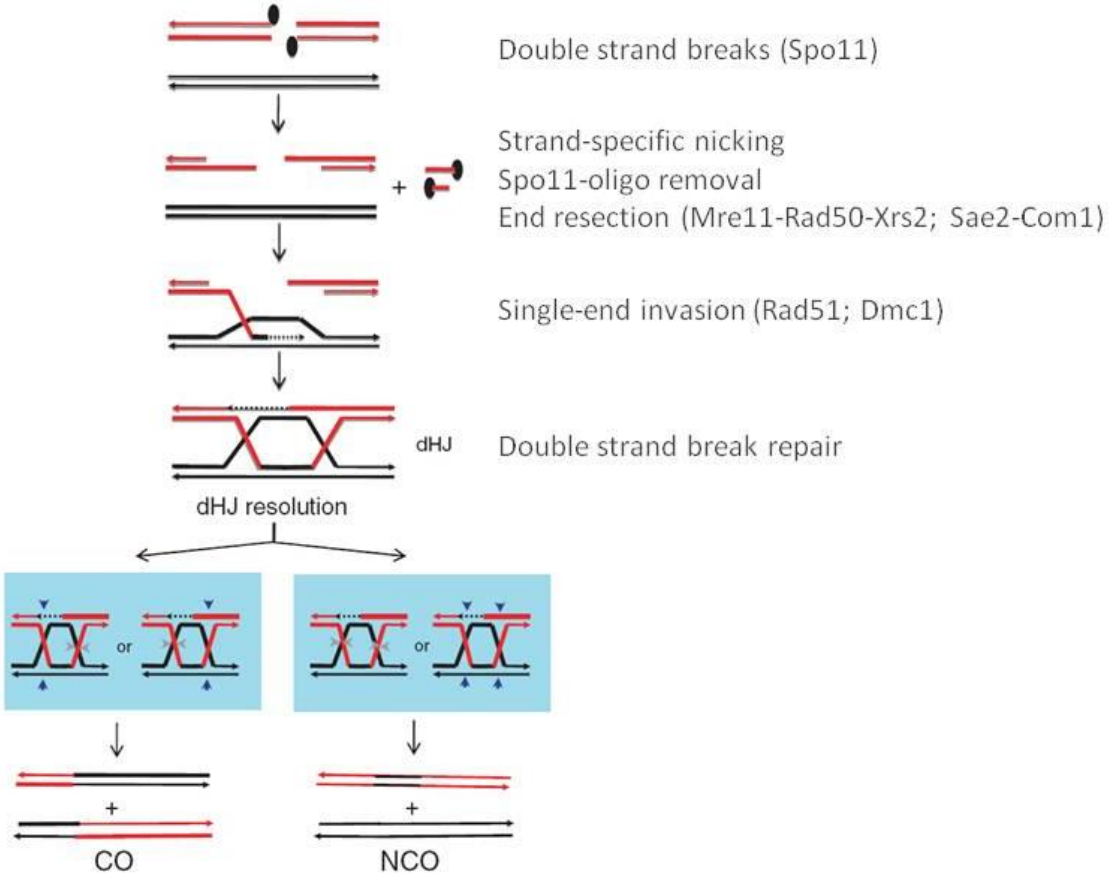



**Figure 1.7** The major events of meiotic recombination. Spo11 is required for the formation of double strand breaks. After the removal of Spo11, DSBs are further processed and 3' single stranded DNA overhangs are generated. Strand invasion is mediated by Rad51 and Dmc1 and leads to the formation of double holiday junctions. The cleavage pattern during the resolution step defines whether crossovers or non crossovers are formed. Modified from (Szekvolgyi & Nicolas, 2010)

### *1.7.3 Monoorientation of sister kinetochores*

A unique feature of chromosome segregation during the first meiotic division is that the two sister chromatids have to be attached to spindle microtubules emanating from the same pole. Thus the two kinetochores of the sisters have to act as one microtubule attachment site. In budding yeast the monopolin complex ensures this monoorientation of sisters. It consists of four subunits called Csm1, Lrs4, Mam1 and Hrr25 (Toth *et al.*, 2000; Rabitsch *et al.*, 2003; Petronczki *et al.*, 2006). In cells lacking one of those subunits sisters are frequently bioriented during meiosis I. The proteins Csm1 and Lrs4 form a high affinity subcomplex and are also expressed in vegetative cells. In those cells, Csm1 and Lrs4 are located in the nucleolus. Before the first meiotic division Csm1 and Lrs4 are released from the nucleolus and localize to the kinetochores (Rabitsch *et al.*, 2003; Brito *et al.*, 2010). Two pairs of Csm1 interact with one pair of Lrs4 to build a V-shaped structure (Corbett *et al.*, 2010). A large scale yeast two-hybrid screen provided evidence that the kinetochore proteins Ctf19 and Dsn1 are potential binding partners of Csm1 (Wong *et al.*, 2007). While Ctf19 is poorly conserved in higher eukaryotes, Dsn1 is part of the highly conserved MIND/Mis12 complex (Cheeseman & Desai, 2008). Thus the Csm1/Lrs4 complex might directly crosslink kinetochore components to regulate kinetochore-microtubule attachments (Corbett *et al.*, 2010). The third protein of the monopolin complex is Mam1. Mam1 is upregulated during meiosis I. It appears on kinetochores during pachytene and remains until metaphase (Toth *et al.*, 2000). Recently it could be shown that in budding yeast the Csm1/Lrs4 complex together with condensins plays an important role in loading of Mam1 to kinetochores. It has been proposed that they establish a Mam1 binding platform. Nevertheless, it is not clear if condensins play a role in mono-orientation in other eukaryotes (Chan *et al.*, 2004; Resnick *et al.*, 2009; Brito *et al.*, 2010). The last component of the monopolin complex is Hrr25; the highly conserved casein kinase 1 $\delta/3$ of budding yeast. It binds at the centromeric regions of chromosomes during meiosis I. There, its interaction with Mam1 as well as its kinase activity is important for the mono-orientation of sisters during meiosis I. The identification of a highly conserved CK1 as a subunit of the monopolin complex strengthens the possibility that the mechanisms for mono-orientation are evolutionary conserved (Petronczki *et al.*, 2006).

Using a genetic screen designed to isolate mutants that are defective in mono-orientation Yokobayashi *et al.* could identify the *S. pombe* protein Moa1. Moa1 acts at the centromeres together with the meiosis specific cohesin subunit Rec8 and promotes a side by side structure of kinetochores during meiosis I (Yokobayashi & Watanabe, 2005).

### *1.7.4 Protection of centromeric cohesion*

In order to segregate chromosomes properly during meiosis, sister chromatid cohesion has to be lost in a stepwise manner. While cohesin at the arm regions is cleaved during meiosis I and thereby ensures the resolution of chiasmata and the segregation of homolog pairs (Kudo *et al.*, 2006), cohesion close to the centromeres has to be protected throughout meiosis I. During meiosis II the remaining cohesin rings are required to counteract the pulling forces of the microtubules, thus ensuring bi-orientation of sister kinetochores. Only when all kinetochores come under tension the checkpoint is satisfied and the sisters can finally segregate to the opposite poles. The presence of the meiosis specific $\alpha$-kleisin subunit Rec8 is a prerequisite for the sequential loss of cohesion. Rec8 replaces Scc1 in the cohesion ring during the meiotic cell cycle and can be cleaved by separase at either one of its two recognition sites (Klein *et al.*, 1999; Watanabe & Nurse, 1999; Buonomo *et al.*, 2000; Kudo *et al.*, 2009). Regulation of the stepwise cleavage was studied extensively during the last years. With the discovery of shugoshin, an ortholog of *Drosophila melanogaster* MEI-S332, a new class of proteins was identified. MEI-S332 and Sgo1 are expressed during both meiosis and mitosis. In contrast to *Drosophila melanogaster*, *S. pombe* possesses two paralogues, Sgo1 and Sgo2. While Sgo2 is expressed in both, meiotic and mitotic cells, the expression of Sgo1 is specific for meiosis where it is required for the protection of centromeric cohesin. It could be shown that in the absence of Sgo1 chromosomes segregate normally during meiosis I but during meiosis II sisters segregate randomly. This phenotype occurs due to the precocious loss of centromeric cohesin during meiosis I (Kitajima *et al.*, 2004; Rabitsch *et al.*, 2004). Newer findings suggest that the protein kinase Bub1 phosphorylates histone H2A and thereby creats a mark which is essential for the Sgo1 localization at the centromeres (Kawashima *et al.*, 2010). Another pathway involves the heterochromatin protein Swi6 which plays a crucial role

in the recruitment and/or the maintenance of shugoshin at centromeres (Yamagishi *et al.*, 2008). Shugoshin finally recruits the conserved protein phosphatase 2A to the centromeric regions where it protects centromeric cohesin from separase activity probably by dephosphorylating its meiosis specific subunit Rec8 (Kitajima *et al.*, 2006; Riedel *et al.*, 2006; Ishiguro *et al.*, 2010; Katis *et al.*, 2010; Rumpf *et al.*, 2010).

All those observations suggest that the phosphorylation of Rec8 is required for its efficient cleavage by separase. The identification of the kinase which is required for the phosphorylation of Rec8 was one major part of this thesis. It is known that the polo-like kinase promotes the cleavage of the mitotic cohesin subunit Scc1 in yeast cells (Alexandru *et al.*, 2001) but mutating the polo-like kinase consensus sites on Rec8 has only little effect on its cleavage suggesting that the polo-like kinase is not the sought-after candidate (Brar *et al.*, 2006).

## 2. Publication: Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast mutants lacking Pcs1 and Clr4.

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Running title: Mde4 phosphorylation prevents merotely.

Keywords: chromosome segregation, mitosis, kinetochore, merotely, fission yeast

**Summary**

In order to segregate chromosomes properly, the cell must prevent merotelic kinetochore attachment, an error that occurs when a single kinetochore is attached to microtubules emanating from both spindle poles. Merotelic kinetochore orientation represents a major mechanism of aneuploidy in mitotic mammalian cells and it is the primary mechanism of chromosome instability in cancer cells. Fission yeast mutants defective in putative microtubule-site clamp Pcs1/Mde4 or Clr4/Swi6-dependent centromeric heterochromatin display high frequencies of lagging chromosomes during anaphase. Here, we developed an assay based on laser microsurgery to show that the stretched morphology of lagging kinetochores in *pcs1Δ* and *clr4Δ* mutant cells is due to merotelic attachment. We further show that Mde4 is regulated by Cdc2 and that Cdc2 activity prevents precocious localization of Mde4 to the metaphase spindle. Finally, we show that Pcs1/Mde4 complex shares similar features with the conserved kinetochore complex Spc24/Spc25 suggesting that these two complexes may occupy a similar functional niche.

**Introduction**

For accurate segregation of chromosomes, sister kinetochores must attach to microtubules emanating from opposite spindle poles (amphitelic attachment) (Biggins & Walczak, 2003; Westermann *et al.*, 2007; Santaguida & Musacchio, 2009). Merotelic kinetochore orientation is an error in which a single kinetochore binds microtubules emanating from both spindle poles (Cimini, 2007; Cimini, 2008). Merotelic attachments frequently occur in the early stages of mitosis, but most are corrected before anaphase onset (Cimini *et al.*, 2003; Cimini *et al.*, 2004). However, because merotelic attachments are not detected by the mitotic checkpoint (Cimini *et al.*, 2002; Cimini *et al.*, 2004), they can persist until anaphase, causing chromatids to lag on the mitotic spindle and hindering their poleward segregation (Cimini *et al.*, 2001; Cimini *et al.*, 2003). Thus, despite correction mechanisms, merotelic kinetochore orientation represents a major mechanism of aneuploidy in mitotic mammalian cells (Cimini *et al.*, 2001; Torosantucci *et al.*, 2009). Moreover, recent studies have shown that merotely is the primary mechanism of chromosome instability (CIN) in cancer cells (Martinez & van Wely; Thompson & Compton, 2008; Bakhoum *et al.*, 2009; Ganem *et*

*al.*, 2009; Silkworth *et al.*, 2009). Our previous work implicated Pcs1/Mde4 complex and Clr4/Swi6-dependent centromeric heterochromatin in preventing merotelic attachments (Rabitsch *et al.*, 2003; Gregan *et al.*, 2007). We proposed that Pcs1 and Mde4 may prevent merotelic kinetochore orientation by clamping together (or cross-linking) microtubule attachment sites. This model makes two important predictions. First, lagging kinetochores in cells lacking Pcs1 or Mde4 should be merotelically attached, and second, Pcs1 and Mde4 should localize to kinetochores at the time when stable kinetochore-microtubule attachments are formed. Here, we use laser microsurgery to show that lagging kinetochores in *pcs1Δ* and *clr4Δ* mutant cells are merotelically attached and we use in situ chromatin binding assay and chromatin immunoprecipitation to show that Pcs1 is localized to kinetochores in metaphase-like cells. Our further analysis shows that Cdc2-dependent phosphorylation of Mde4 is important for its proper localization during metaphase and that Pcs1/Mde4 complex shares similar features with Spc24/Spc25, a conserved family of eukaryotic kinetochore proteins.

**Results and Discussion**

**Laser microsurgery shows that lagging kinetochores in *pcs1*- and *clr4*- mutants are merotelically attached.**

Our recent observation that lagging chromosomes in *pcs1Δ*, *mde4Δ* and *clr4Δ* mutants have stretched kinetochores (Gregan *et al.*, 2007) is consistent with the notion that these kinetochores are merotelically attached. However, we could not exclude the possibility that chromosomes in these mutants are lagging because they are not attached to microtubules and the stretched appearance of lagging kinetochores is caused by other means than pulling forces of microtubules. We therefore decided to develop an assay that would allow us to distinguish between these two possibilities. Pulling forces of microtubules emanating from opposite spindle poles induce lateral stretching of merotelically attached kinetochores. Therefore, one of the characteristic features of merotelic kinetochores is their stretched morphology (Khodjakov *et al.*, 1997; Pidoux *et al.*, 2000; Cimini *et al.*, 2001; Gregan *et al.*, 2007). We argued that if we

sever microtubules attached to a stretched merotelic kinetochore on one side, the kinetochore should resume normal globular shape and move to the spindle pole with intact microtubules (Figure 1A). On the other hand, if the stretched kinetochore morphology was not due to merotelic attachment, severing of microtubules should not affect kinetochore morphology. We used Nuf2-GFP to label kinetochores and mCherry-tubulin to visualize the spindle. A picosecond pulsed laser coupled to a laser scanning confocal microscope was used to sever microtubules in *pcs1Δ* and *clr4Δ* mutant cells.

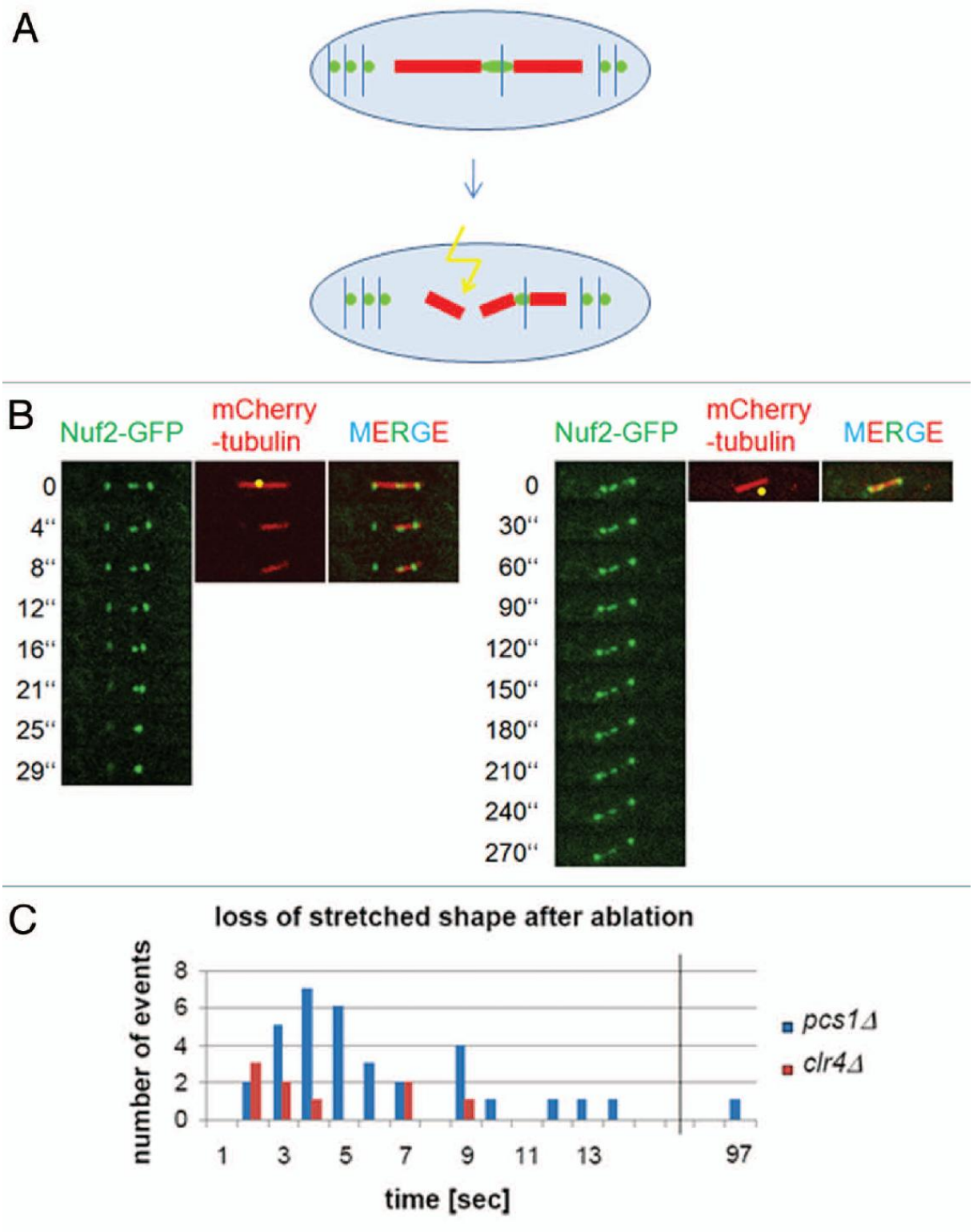


**Figure 1.** Stretched shape of lagging kinetochores is lost after severing microtubules on one side. (A) A scheme of the experiment. Pulling forces of microtubules emanating from opposite spindle poles induce lateral stretching of merotelically attached kinetochores. After severing microtubules attached to a stretched merotelic kinetochore on one side, the kinetochore should resume normal globular shape. (B) *pcs1Δ* mutant cells expressing mCherry-tubulin and Nuf2-GFP to label spindle

microtubules and kinetochores respectively, were imaged before and after laser ablation. The laser ablated area is indicated by a yellow dot. In the control experiment on the right, laser was focused outside the spindle. (C) The loss of stretched shape of lagging kinetochores after laser ablation was quantified in *pcs1Δ* and *clr4Δ* mutant cells.

After laser severing, spindle poles moved toward one another suggesting that spindles were efficiently cut, as previously reported (Khodjakov *et al.*, 2004; Tolic-Norrelykke *et al.*, 2004; Sacconi *et al.*, 2005; Maghelli & Tolic-Norrelykke, 2008). Importantly, the lagging kinetochores recoiled and resumed their normal globular shape shortly after laser ablation (Figure 1B, Figure 1C) and invariably joined the opposite spindle pole with intact microtubules. In the control experiment, ablating a spot next to the spindle did not affect the stretched appearance of lagging kinetochore. During the course of this work, a similar strategy has been used to analyze mutants defective in sister-chromatid cohesion (Courtheoux *et al.*, 2009).

Thus, our observation that the intra-kinetochore stretching in *pcs1Δ* and *clr4Δ* mutant cells was abolished by laser severing of spindle microtubules demonstrates that stretching of lagging kinetochores is mediated by forces exerted by spindle microtubules. Importantly, it excludes the possibility that chromosomes in these mutants are lagging because they are not attached to microtubules, or attached only weakly and provides the best evidence so far that kinetochores of lagging chromosomes in cells lacking Pcs1/Mde4-dependent clamps and in cells with defective centromeric heterochromatin are merotelically attached.

Importantly, we have developed an assay which is not only a tool to detect merotely, but it also provides a possibility to study structural and mechanical properties of the kinetochore in live cells. Mounting evidence suggests that the mechanical properties of the kinetochore make fundamental contributions to faithful segregation of chromosomes (Loncarek *et al.*, 2007; Lee *et al.*, 2008; Maresca & Salmon, 2009; Uchida *et al.*, 2009; Wan *et al.*, 2009). However, such kinetochore properties have not been experimentally analyzed. Our observation that stretched kinetochores resumed their normal globular shape shortly after severing of the spindle suggests that the kinetochore region labeled by the Nuf2-GFP has elastic properties and paves the way for the future analysis of mechanical properties of the kinetochore in live cells.

**Pcs1 localizes to the central region of centromeres and forms a complex with Mde4 in *nda3*-arrested cells.**

We have recently proposed that Pcs1 and Mde4 may clamp together microtubule attachment sites (Rabitsch *et al.*, 2003; Gregan *et al.*, 2007). If Pcs1 and Mde4 prevent merotelic kinetochore orientation by clamping together microtubule attachment sites, they should localize to kinetochores around metaphase, when stable kinetochore-microtubule attachments are formed. Both Pcs1 and Mde4 localize to kinetochores during most of the cell cycle as evidenced by co-localization of GFP-tagged Pcs1 or Mde4 with known kinetochore proteins.

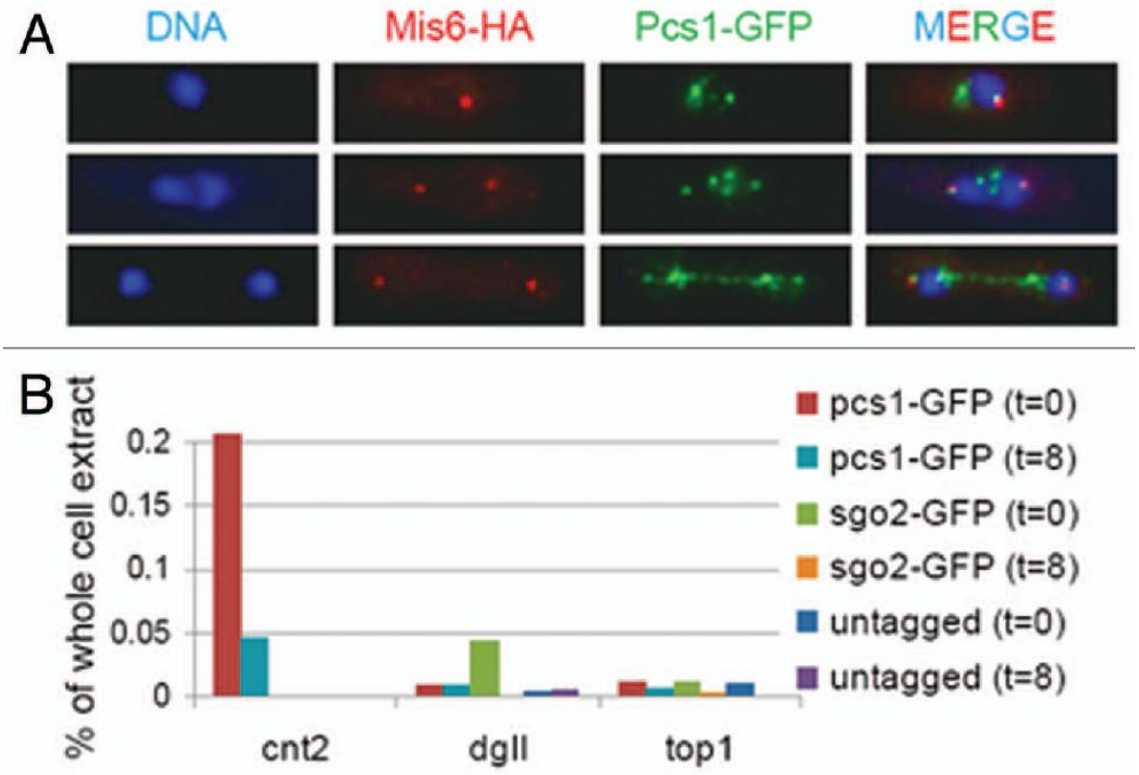


**Figure 2.** Analysis of Pcs1 localization. (A) Cells expressing Pcs1-GFP and Mis6-HA (JG15031) were permeabilized by zymolyase digestion, extracted with detergent, fixed and stained with antibodies against HA and GFP. Nuclei were visualized by Hoechst staining. (B) Untagged cells (JG12013) or cells carrying Pcs1-GFP (JG14985) or Sgo2-GFP (JG12239) were harvested at the indicated time-points (0 and 8 minutes) after release from *nda3-KM311*-arrest. Chromatin binding of the GFP-tagged proteins was analyzed by chromatin-immunoprecipitation followed by quantitative PCR using oligonucleotide primers specific for the centromeric central region (cnt2), outer centromere (dgII) and chromosome arm (top1).

However, the kinetochore localization was difficult to address during early mitosis because Pcs1-GFP and Mde4-GFP signals are dispersed all over the nucleus (Rabitsch *et al.*, 2004; Gregan *et al.*, 2007). In order to remove the soluble fraction of Pcs1-GFP which may prevent detection of kinetochore-bound Pcs1-GFP, we used an in situ chromatin binding assay (Kearsey *et al.*, 2005). Detergent extraction successfully removed soluble Pcs1-GFP from nuclei and revealed Pcs1-GFP foci in metaphase/anaphase cells. Some of the Pcs1-GFP foci co-localized with the kinetochore protein Mis6 (Figure 2A). Interestingly, we observed co-localization of Pcs1-GFP with Mis6-HA in 90% of early anaphase cells, but only in 59% of late anaphase cells. This suggests that Pcs1 localization at kinetochores decreases during anaphase.

To determine more precisely the centromeric region to which Pcs1 binds, we arrested cells in a metaphase-like stage using a cold-sensitive tubulin allele (*nda3-KM311*) (Toda *et al.*, 1983) and analyzed the localization of Pcs1-GFP by chromatin immunoprecipitation. In *nda3*-arrested cells, Sgo2-GFP was enriched at the outer centromeric repeats, as previously reported (Kitajima *et al.*, 2004), while Pcs1-GFP was enriched at the central centromeric region (Figure 2B, t=0). Eight minutes after release from the *nda3*-arrest, when 60% of cells were in anaphase, Pcs1-GFP was still enriched at the central centromeric region (Figure 2B, t=8), although the enrichment was lower as compared to *nda3*-arrested cells. We conclude that Pcs1 localizes to central region of centromeres in metaphase-like cells, and its centromeric enrichment is progressively reduced as cells undergo anaphase.

We have previously shown that Pcs1 forms a complex with Mde4 in extracts prepared from cycling cells (Gregan *et al.*, 2007). However, it is not known whether Pcs1 interacts with Mde4 during metaphase, when they are required to ensure proper attachment of kinetochores to microtubules. We used a tandem affinity purification (TAP) protocol (Rigaut *et al.*, 1999) to isolate Mde4-TAP together with associated proteins from both cycling and *nda3*-arrested cells. Mass-spectrometry analysis revealed that Mde4 associated with Pcs1 as well as with other proteins such as Net1/Cfi1-related protein Dnt1 (Jin *et al.*, 2007) and TRiC/CCT chaperonin complex (Broadley & Hartl, 2009) (Figure S1A). In addition, Mde4 co-purified with Pcs1-TAP isolated from *nda3*-arrested cells (Figure S1A). While only four residues were

phosphorylated on Mde4 purified from cycling cells, Mde4 was phosphorylated on 16 residues and Pcs1 was phosphorylated on serine 47 when purified from *nda3*-arrested cells (Figure S1B). Five of the Mde4 phosphorylation sites have been identified during the course of this work (Wilson-Grady *et al.*, 2008; Beltrao *et al.*, 2009; Choi *et al.*, 2009). Western-blot analysis revealed slower-migrating forms of Mde4-GFP in *nda3*-arrested cells, which likely represent hyper-phosphorylated Mde4-GFP (Figure 3A). Upon release from the *nda3*-arrest, when cells underwent anaphase, slower-migrating forms of Mde4 quickly decreased. We conclude that both Pcs1 and Mde4 are phosphorylated and interact with each other in metaphase-like cells.

**Cdc2 activity prevents precocious localization of Mde4 to the metaphase spindle.**

Interestingly, eleven of the 16 residues phosphorylated on Mde4 are potential Cdk1 phosphorylation sites (SP or TP). To test whether Mde4 phosphorylation depends on the Cdk1 activity, we constructed conditional analog-sensitive allele of the fission yeast cyclin-dependent kinase Cdc2 (Gregan *et al.*, 2007; Dischinger *et al.*, 2008) (Figure S2). Slower-migrating forms of Mde4 rapidly disappeared upon inactivation of the Cdc2-as in *nda3*-arrested cells, suggesting that Cdc2 activity is necessary for Mde4 phosphorylation during mitosis (Figure 3B).

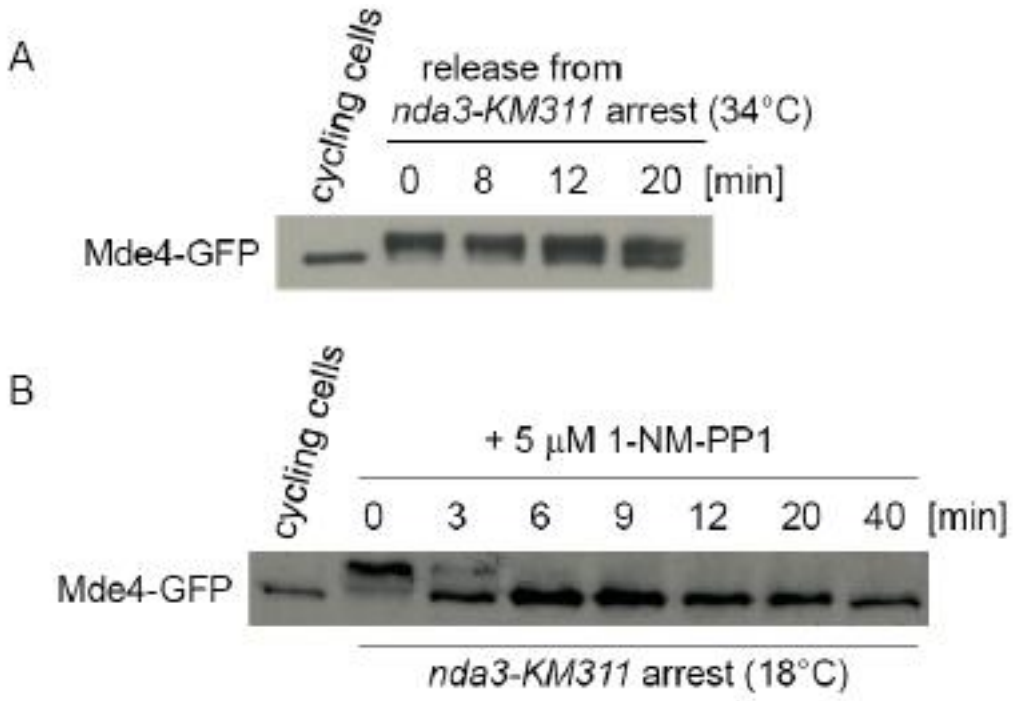


**Figure 3.** Inactivation of Cdc2 inhibits phosphorylation of Mde4. (A) *nda3-KM311* cells expressing Mde4-GFP (JG15033) were grown at permissive temperature of 34°C (cycling cells) or arrested at non-permissive temperature of 18°C for 8 hours and subsequently released to permissive temperature. Samples were taken at the indicated time-points after the release and Mde4-GFP was analyzed by Western blot analysis. (B) *nda3-KM311 cdc2-as* cells expressing Mde4-GFP (JG15356) were grown at permissive temperature of 34°C (cycling cells) or arrested at non-permissive temperature of 18°C for 8 hours. Cdc2-as was subsequently inactivated by adding 5μM 1-NM-PP1. Samples were taken at the indicated time-points after adding 5μM 1-NM-PP1 and Mde4-GFP was analyzed by Western blot analysis.

Previous observation that Cdc2 was able to phosphorylate bacterially expressed 6His-Mde4 suggests that Cdc2 may directly phosphorylate Mde4 (Choi *et al.*, 2009). Interestingly, Lrs4 (Rabitsch *et al.*, 2003; Huang *et al.*, 2006; Gregan *et al.*, 2007), which is the budding yeast ortholog of Mde4, was also found to be phosphorylated by Cdk1 (Ubersax *et al.*, 2003).

In wild-type cells, Mde4 is hyper-phosphorylated during metaphase, whereas inactivation of the Cdc2-as in *nda3*-arrested cells creates a situation where Mde4 is hypo-phosphorylated in metaphase-like cells. We shifted *nda3*-arrested cells with inactivated Cdc2-as to permissive temperature to allow formation of spindles and analyzed localization of Mde4-GFP (Figure 4A). While in wild-type cells, Mde4 localizes

to spindles only in late anaphase (Choi *et al.*, 2009; Khmelinskii & Schiebel, 2009), hypo-phosphorylated Mde4 localized to short metaphase spindles in 83% of cells (Figure 4B). A similar phenomenon has been described in budding yeast where preventing phosphorylation of Cdk substrates such as Fin1 and Sli15/INCENP also leads to precocious localization to metaphase spindle (Pereira & Schiebel, 2003; Woodbury & Morgan, 2007). Chromatin immunoprecipitation showed that both hyper-phosphorylated and hypo-phosphorylated Mde4 were enriched at the central centromeric region (Figure 4C). During the course of this work, Choi et al. showed that mutant allele of Mde4 with 12 predicted phosphorylation sites mutated to alanine (Mde4-12A) failed to localize to kinetochores and localized to metaphase spindles (Choi *et al.*, 2009). While the premature localization of Mde4-12A to spindles is consistent with our observations (Figure 4B), further experiments are needed to establish the role of Mde4 phosphorylation in its kinetochore localization. Thus, we conclude that Cdc2 activity prevents precocious localization of Mde4 to the metaphase spindle.

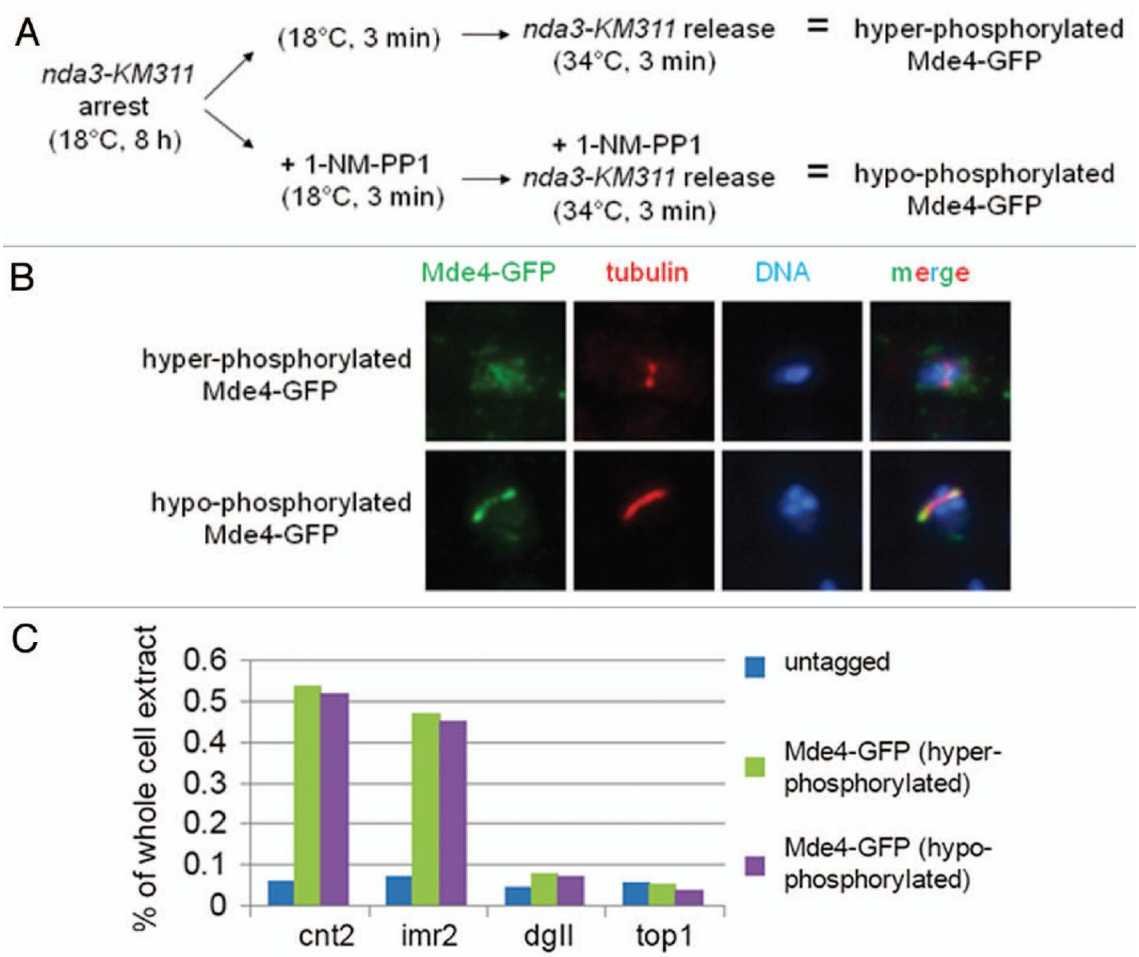


**Figure 4.** Hypo-phosphorylated Mde4 localizes to metaphase spindles and kinetochores. (A) A scheme of experimental procedure. Hypo-phosphorylated Mde4 and hyper-phosphorylated Mde4 indicate experimental stages, which are referred to in other parts of the figure. (B) *nda3-KM311 cdc2-as* cells expressing Mde4-GFP (JG15356) were harvested at stages indicated in the Figure 4A, fixed and stained with antibodies against tubulin and GFP. Nuclei were visualized by Hoechst staining. (C) Untagged *nda3-KM311* cells (JG12013) or *nda3-KM311 cdc2-as* cells expressing Mde4-GFP (JG15356) were harvested at stages indicated in the Figure 4A. Chromatin binding of the Mde4-GFP was analyzed by chromatin immunoprecipitation followed by quantitative PCR using oligonucleotide primers specific for the centromeric central region (cnt2), innermost centromeric repeats (imr2), outer centromere (dgII) and chromosome arm (top1).

**Pcs1/Mde4 complex shares similar features with Spc24/Spc25 complex.**

As in fission yeast, kinetochores in higher eukaryotes bind multiple microtubules and therefore must prevent merotelic attachment. However, it is not clear if higher eukaryotes employ a complex similar to Pcs1/Mde4 to prevent merotelic attachment, since Pcs1 and Mde4 orthologs have only been identified in yeast species (Gregan *et*

*al.*, 2007). Interestingly, we noticed that PfamA Spindle_Spc25 domain was found in Pcs1 proteins (Pfam release 23.0., see Material and Methods for detailed analysis) (Finn *et al.*, 2008). Spindle_Spc25 domain is characteristic for Spc25 proteins, a conserved family of eukaryotic kinetochore proteins. Spc25 forms a dimer with Spc24 and interacts with Ndc80/Nuf2 dimer to form a four-subunit Ndc80 complex (Janke *et al.*, 2001; Wigge & Kilmartin, 2001; McCleland *et al.*, 2004; Asakawa *et al.*, 2005; Cheeseman *et al.*, 2006; DeLuca *et al.*, 2006; Ciferri *et al.*, 2008). A closer analysis revealed several lines of evidence suggesting that Pcs1/Mde4 complex is similar to Spc24/Spc25 complex. All four proteins share similar architecture, namely N-terminal coiled-coil domain followed by a globular domain. The globular part of Pcs1 and Spc25 proteins is combined in the Spindle_Spc25 domain, which aligns conserved secondary structure elements of Pcs1 to structural elements of Spc25 determined by X-ray crystallography and NMR (Wei *et al.*, 2006) (Figure 5A). Mutating the three most conserved residues in the Spindle_Spc25 domain (L140A, F154A, F154D, F212A, F212D) diminished the Pcs1 function (Figure 5B).

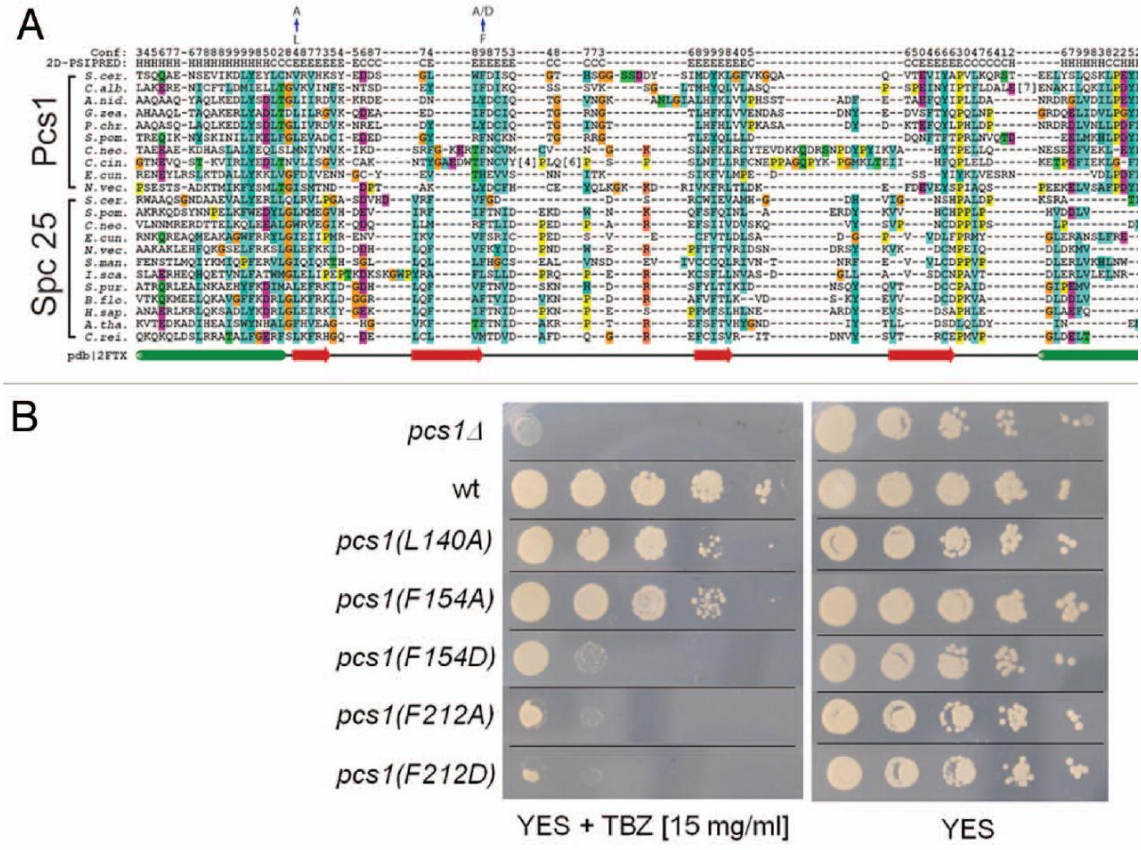


**Figure 5**. Pcs1 and Spc25 proteins share sequence similarity. (A) Sequence alignment of the Spindle_Spc25 domain of Pcs1 and Spc25 proteins. Mutated Pcs1 residues described in the Figure 5B are indicated. A selected set of Pcs1 and Spc25 protein family sequences, covering a coiled coil region (the first helix shown) and the globular sequence domain, were aligned with T-coffee (default parameters, (Notredame *et al.*, 2000)). The sequences used are derived from the NCBI RefSeq database (Pruitt *et al.*, 2007) with the following accessions for the Pcs1 sequence family: *S.cer.* (*Saccharomyces cerevisiae*, NP_010009.1), *C.alb.* (*Candida albicans*, XP_719422.1), *A.nid.* (*Aspergillus nidulans*, XP_664652.1), *G.zea.* (*Gibberella zeae*, XP_382110.1), *P.chr.* (*Penicillium chrysogenum*, XP_002559041.1), *S.pom.* (*Schizosaccharomyces pombe*, NP_001018298.1), *C.neo.* (*Cryptococcus neoformans*, XP_571257.1), *C.cin.* (*Coprinopsis cinerea*, XP_001833086.1), *E.cun.* (*Encephalitozoon cuniculi*, NP_585824.1), *N.vec.* (*Nematostella vectensis*, XP_001632729.1); the Spc25 protein family was represented by *S.cer.* (NP_010934.1), *S.pom.* (NP_588208.1), *C.neo.* (XP_570912.1), *E.cun.* (NP_586429.1), *N.vec.* (XP_001635865.1), *S.man.* (*Schistosoma mansoni*, XP_002580161.1), *I.sca.* (*Ixodes scapularis*, XP_002434782.1), *S.pur.* (*Strongylocentrotus purpuratus*, XP_797276.1), *B.flo.* (*Branchiostoma floridae*, XP_002592012.1), *H.sap.* (*Homo sapiens,* NP_065726.1), *A.tha.* (*Arabidopsis thaliana*, NP_566900.1), *C.rei.* (*Chlamydomonas reinhardtii*, XP_001697914.1). Stretches of unaligned sequence have been removed and the number of deleted residues is indicated in brackets. The top two lines of the alignment indicate predicted secondary structure and corresponding confidence values of *S. pombe* Pcs1 calculated with PSIPRED (Bryson *et al.*, 2005). The bottom line shows the secondary structure of *S. cerevisiae* Spc25 derived from X-ray crystallography (2FTX) as depicted from Wei et al.

2006 (Wei *et al.*, 2006). (B) Serial dilutions of wild-type cells (wt) (JG15414), *pcs1Δ* mutant cells (JG14821) and cells carrying the indicated *pcs1* mutations (*pcs1*(L140A) (JG15549), (*pcs1*(F154A) (JG15550), (*pcs1*(F154D) (JG15551), (*pcs1*(F212A) (JG15552), (*pcs1*(F212D) (JG15553) were spotted on YES medium or YES medium containing 15 mg/ml of thiabendazole (TBZ) and grown for 2 days at 32°C.

Similarly as Spc24, which binds tightly to Spc25, Mde4 binds tightly to Pcs1 (Gregan *et al.*, 2007; Ciferri *et al.*, 2008). All four proteins localize to kinetochores, their kinetochore binding is interdependent (Figure S3 and ref. (McCleland *et al.*, 2004; Choi *et al.*, 2009)) and CENP-C is required for kinetochore binding of both Pcs1 and components of the Ndc80 complex (Liu *et al.*, 2006; Kwon *et al.*, 2007; Tanaka *et al.*, 2009). In addition, both Mde4 and Spc24 are phosphorylated during mitosis (Figure S1B and (Nousiainen *et al.*, 2006)).

Collectively, this provides evidence that Pcs1/Mde4 complex shares similar features with the Spc24/Spc25 complex. Although a phylogenetic relationship could not be detected with sequence statistical methods, it is possible that Pcs1 and Mde4 originated by a gene duplication event in the yeast lineage, but not in higher eukaryotes. This may explain why we failed to identify orthologs of Pcs1 and Mde4 in higher eukaryotes. Alternatively, Pcs1 and Mde4 orthologs in higher eukaryotes diverged to such extend that we are not able to identify them by sequence homology. Importantly, the common features shared by Pcs1/Mde4 and Spc24/Spc25 complexes suggest that the molecular mechanism how these complexes function and their regulation may be similar.

## Materials and Methods

### Laser scanning confocal microscopy and laser ablation

The strains JG15161 and JG15351 were cultured in liquid EMM with appropriate supplements at 25°C. The expression of mCherry was partially repressed by the addition of 2 µM thiamine. A cover slip was coated with 2 mg/ml Lectin and glued to the round opening in the bottom of a microwell dish. Cells were mounted on the lectin spot and covered with EMM plus supplements. The microwell dish was placed in a Bachhoffer chamber to keep a constant temperature of 25°C.

Imagining of the cells was performed on an Olympus FV-1000 laser scanning confocal system. GFP and mCherry were excited at 488 nm and 561 nm, respectively, with a multi-line Argon laser (Melles Griot Bensheim, Germany). A dichroic mirror DM405/488/561/633 and a UPLSAPO 60x/1.35 oil LSM objective (Olympus) were used. Emission was detected at 500-600 nm for GFP and 600-700 nm for mCherry. During laser ablation single-plane images were taken at a free-run mode with 2 µs/pixel scanning speed. After ablation of the mitotic spindle single-plane pictures were taken using the free-run mode and Kalman filter line 2. All images have a *xy*-pixel size of 40 nm.

The laser setup for ablation consists of a PDL 800-B picoseconds pulsed diode laser with a LDH-P-C.405B laser head (PicoQuant, Berlin, Germany) emitting 70 picosecond pulses at 40 MHz. The laser is coupled to the bleaching port (SIM scanner) of the Olympus FV-1000 laser scanning microscope via an optical fibre. The light path of the cutting laser is different from the path of the imaging lasers. The cutting laser light is reflected onto the objective by a long pass dichroic mirror LP450 (cut on wavelength 450 nm). The laser exposure time was 4 seconds. The system was driven by Fluoview Application Software version 1.6a (Olympus) (Raabe *et al.*, 2009).

**Chromatin immunoprecipitation**

*nda3-311* mutant cells were grown in YPD medium to an OD600 of 0.2-0.4 at 30°C and subsequently shifted to 18°C for 10 hours. Chromatin immunoprecipiation was performed essentially as described before (Riedel *et al.*, 2006; Gregan *et al.*, 2007; Rumpf *et al.*, 2010). $2.5x10^8$ cells were fixed with 3% Paraformaldehyde and treated with 0.4mg/ml Zymolyase T100. DNA was sonicated to fragments of 400 bp average size. Immunoprecipitation was performed using an anti-GFP antibody (Roche) in conjunction with ProteinA Dynabeads (Invitrogen).

Real-time PCR was performed using the IQ SYBR Green Mix (BIO-RAD) and an IQ5 Cycler (BIO-RAD). The following primers were used for qRT-PCR of S. pombe chromosome 2 loci:

Cnt2-fw AGCGCTAACTCGTTTAAGTGAA, Cnt2-rev GGCGTGGAAAGTCATCTGTA, Imr2-fw CTTCGGCGACGTGATATAAG, Imr2-revTTTGCAACGATTACCGGTTT, DgII-fw

TGCTCTGACTTGGCTTGTCT, DgII-rev TTGCACTCGGTTTCAGCTAT, top1-fw AGGGTTATTTCGTGGTCGAG, top1-rev TGCCAACCAGGTCACTGTAT.

**Strains, media and growth conditions**

Media and growth conditions were as described in (Rumpf *et al.*; Rabitsch *et al.*, 2003; Gregan *et al.*, 2006).

**Tandem affinity purification**

Tandem affinity purifications and mass spectrometry were performed as previously described (Riedel *et al.*, 2006; Gregan *et al.*, 2007; Cipak *et al.*, 2009).

**Sequence analysis**

Iterative PSI-BLAST searches with the conserved protein sequence domain of Pcs1 family members could identify homologues in various phyla of the fungi kingdom, and one animal sequence (sea anemone *Nematostella vectensis*) applying significant E-values below 0.01 (Altschul *et al.*, 1997) (see Figure 5A). The same approach was performed to collect the Spc25 sequence family. No significant sequence homology between Pcs1 and the Spc25 protein families could be detected and no phylogenetic relationship can be infered. However, sequence similarity between Pcs1 and Spc25 was reported in the PfamA domain Spindle_Spc25 (PF08234, release 23.0) (Finn *et al.*, 2008), where the globular domains of both protein families were aligned. The incorporation of Pcs1 sequences into the Spindle_Spc25 domain is based on sequence searches that could not be reproduced with the latest databases. In the current Pfam release (24.0) the Pcs1 protein family is represented by the Csm1 domain (PF12539), and the Spindle_Spc25 domain contains solely sequences of the Spc25 protein family.

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**Supplemental Information**

**Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast mutants lacking Pcs1 and Clr4.**

Cornelia Rumpf, Lubos Cipak, Alexander Schleiffer, Karl Mechtler, Alison Pidoux, Iva M. Tolić-Nørrelykke, and Juraj Gregan

**The genotypes of *S. pombe* strains used in this study**

| Strains | Genotype |
|---|---|
| JG15031 | *h+ nda3-311 mis6-HA-leu2 pcs1-GFP-leu2 leu1-32* |
| JG12013 | *h+ nda3-311 leu1-32 ura4-D18 his2* |
| JG14985 | *h+ nda3-311 pcs1-GFP-leu2 leu1-32 ura4-D18 his2* |
| JG12239 | *h- nda3-311 sgo2-GFP-Kan$^R$ ura4* |
| JG15033 | *h+ nda3-311 mis6-HA-leu2 leu1-32 mde4-GFP-Kan$^R$* |
| JG15356 | *h? nda3-311 mis6-HA mde4-GFP cdc2::ClonNat$^R$ cdc2-as-Hph$^R$* |
| JG15161 | *h- mCherry-atb2-hph$^R$ nuf2-GFP-Kan$^R$ pcs1::CloneNat$^R$* |
| JG15351 | *h- clr4::LEU2+ Nuf2-GFP-Kan$^R$ mCherry-atb2-hph$^R$ leu1-32* |
| JG15058 | *h? nda3-311 pcs1-HA-TAP-Kan$^R$* |
| JG15137 | *h+ nda3-311 leu1-32 ura4-D18 his2 mde4-Tap-Kan$^R$* |
| JG15414 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$ pcs1-wt-Hph$^R$* |
| JG14821 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$* |
| JG15549 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$ pcs1-L140A-Hph$^R$* |
| JG15550 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$ pcs1-F154A-Hph$^R$* |
| JG15551 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$ pcs1-F154D-Hph$^R$* |
| JG15552 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$ pcs1-F212A-Hph$^R$* |
| JG15553 | *h+ lys1 his7 leu1 ura4 ade6-210 pcs1::ClonNat$^R$ pcs1-F212D-Hph$^R$* |
| JG15383 | *h+ nda3-311 mis6-HA-leu2 pcs1-GFP-LEU2 leu1-32 mde4::Kan$^R$* |
| JG15366 | *h+ nda3-311 mis6-HA-leu2 mde4-GFP-Kan$^R$ leu1-32 pcs1::CloneNat$^R$* |
| JG15123 | *h? cdc2+* |
| JG15125 | *h- cdc2::ClonNat$^R$ cdc2-as-Hph$^R$* |

Publication: Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast mutants lacking Pcs1 and Clr4.

**A**

List of proteins identified by mass spectrometry co-purifyinhg with **Pcs1-TAP** from *nda3*-arrested *S. pombe* cells.

| | Protein (GeneInfo identifier) | Description | Number of unique peptides |
|---|---|---|---|
| 1 | 68005983 | Chromosome segregation protein Pcs1 | 22 |
| 2 | 19112875 | Sequence orphan Mde4 | 18 |

List of proteins identified by mass spectrometry co-purifyinhg with **Mde4-TAP** from cycling *S. pombe* cells.

| | Protein (GeneInfo identifier) | Description | Number of unique peptides |
|---|---|---|---|
| 1 | 19112875 | Sequence orphan Mde4 | 86 |
| 2 | 19113336 | Nucleolar protein Dnt1 | 84 |
| 3 | 68005983 | Chromosome segregation protein Pcs1 | 51 |
| 17 | 19115145 | Karyopherin Kap95 | 6 |
| 18 | 19114970 | Ribosomal-ubiquitin fusion protein Ubi5 | 5 |
| 24 | 19112476 | Ataxin-2 homolog | 4 |
| 37 | 19115509 | TIM22 inner membrane protein import complex subunit Tim13 | 2 |
| 40 | 19113475 | Human LYHRT homolog | 2 |
| 46 | 19114125 | Nucleoporin Nup124 | 2 |
| 52 | 1136783 | Elongation factor 1 alpha-A | 2 |
| 53 | 63054532 | ATP-dependent DNA ligase Cdc17 | 2 |

List of proteins identified by mass spectrometry co-purifyinhg with **Mde4-TAP** from *nda3*-arrested *S. pombe* cells.

| Protein (GeneInfo identifier) | | Description | Number of unique peptides |
|---|---|---|---|
| 1 | 19112875 | Sequence orphan Mde4 | 25 |
| 2 | 19113336 | Nucleolar protein Dnt1 | 23 |
| 4 | 19075845 | RNA polymerase I upstream activation factor complex subunit Spp27 | 11 |
| 10 | 19112198 | Chaperonin-containing T-complex theta subunit Cct8 | 9 |
| 11 | 19112741 | Chaperonin-containing T-complex alpha subunit Cct1 | 9 |
| 12 | 68005983 | Chromosome segregation protein Pcs1 | 8 |
| 17 | 19112476 | Ataxin-2 homolog | 7 |
| 19 | 19111947 | Chaperonin-containing T-complex delta subunit Cct4 | 7 |
| 22 | 19114645 | Mitotic spindle checkpoint protein Dma1 | 6 |
| 23 | 19112602 | Chaperonin-containing T-complex gamma subunit Cct3 | 6 |
| 33 | 19113147 | Chaperonin-containing T-complex eta subunit Cct7 | 5 |
| 48 | 19115145 | Karyopherin Kap95 | 4 |
| 49 | 19112010 | Vid27 family protein | 4 |
| 52 | 19114189 | Chaperonin-containing T-complex epsilon subunit Cct5 | 3 |
| 65 | 19076049 | Acetyl-CoA-ligase | 3 |
| 70 | 19113371 | Sequence orphan | 3 |
| 73 | 19114554 | Serine/threonine protein kinase Cka1 | 2 |
| 75 | 19115509 | TIM22 inner membrane protein import complex subunit Tim13 | 2 |
| 76 | 19114018 | U3 snoRNP protein Nop58 | 2 |
| 80 | 19112161 | Chaperonin-containing T-complex zeta subunit Cct6 | 2 |
| 82 | 19113929 | Chaperonin-containing T-complex beta subunit Cct2 | 2 |
| 85 | 1136783 | Elongation factor 1 alpha-A | 2 |
| 87 | 19076061 | Ribosome biogenesis protein Nsa2 | 2 |
| 88 | 12000355 | Single-stranded TG1-3 binding protein | 2 |
| 90 | 19112160 | U3 snoRNP protein Nop56 | 2 |
| 91 | 19112358 | Histone H4 h4.2 | 2 |

**B**

## Mde4 (cycling cells)

```
  1 MSTISTSTDS KLDNLGLSVT SRRNQILFYL SKALNLAHLL RSDSLQKSFL
 51 DALKQSATDS ELLHKNLDEI KFLQNEKLNN EKLLEQEQNE ANDYRLKVER
101 LEHKISDYVQ EINSLNSQLQ IQKSNPEKHE DAVSQNRLRG SLDTVSSPSK
151 THKANKDEKA TRLHLIIANL KKALKEKDAE VLNLQSHVSS KESELDRFKI
201 KLETEESNWK VRLQVLESKL ATQDRKLRMQ KKSTERKSLL VSPRVSSPKL
251 FSPSKQAIMG TRQPNATSGS PLSVTPFLQK TSTSIGLSSS PPQSSPSAQS
301 SQPFSRDKYP HSMTVSPSNA RYLKKHLDDT IPSNVSDINH NDHLKIPQSP
351 SSLSPSKIPI RKKRKLKDTV SNCEFTEEDS ESSFLLETIQ PTKSTLRRSI
401 SPLKKRNDEI NELKKGFTMK K
```

## Pcs1 (cycling cells)

```
  1 MHSTQDFVNN EDQDAYSVRE NENELHINNS GMSELNKKLQ LPNVELSTLS
 51 HTQEQEFNEL NKLIRKINEL QEFYLLEDLA KPVTNAGADA DEDTIVKDLK
101 KELENEKKAN HSLKNELLKT REQIKNYSKI NILIKELFGL EVADCIEDED
151 GYRFNCKNTG RRGTLEYQLL LDDQNFTFTP RLNVQTDEEL MKHLPDYLLE
201 EIIFTKEQGK LFSARLMKAL QD
```

Mde4 (*nda3*-arrest)

```
  1 MSTISTSTDS KLDNLGLSVT SRRNQILFYL SKALNLAHLL RSDSLQKSFL
 51 DALKQSATDS ELLHKNLDEI KFLQNEKLNN EKLLEQEQNE ANDYRLKVER
101 LEHKISDYVQ EINSLNSQLQ IQKSNPEKHE DAVSQNRLRG SLDTVSSPSK
151 THKANKDEKA TRLHLIIANL KKALKEKDAE VLNLQSHVSS KESELDRFKI
201 KLETEESNWK VRLQVLESKL ATQDRKLRMQ KKSTERKSLL VSPRVSSPKL
251 FSPSKQAIMG TRQPNATSGS PLSVTPFLQK TSTSIGLSSS PPQSSPSAQS
301 SQPFSRDKYP HSMTVSPSNA RYLKKHLDDT IPSNVSDINH NDHLKIPQSP
351 SSLSPSKIPI RKKRKLKDTV SNCEFTEEDS ESSFLLETIQ PTKSTLRRSI
401 SPLKKRNDEI NELKKGFTMK K
```

Pcs1 (*nda3*-arrest)

```
  1 MHSTQDFVNN EDQDAYSVRE NENELHINNS GMSELNKKLQ LPNVELSTLS
 51 HTQEQEFNEL NKLIRKINEL QEFYLLEDLA KPVTNAGADA DEDTIVKDLK
101 KELENEKKAN HSLKNELLKT REQIKNYSKI NILIKELFGL EVADCIEDED
151 GYRFNCKNTG RRGTLEYQLL LDDQNFTFTP RLNVQTDEEL MKHLPDYLLE
201 EIIFTKEQGK LFSARLMKAL QD
```

**Figure S1**. Mde4 and Pcs1 are phosphorylated during mitosis. (A) List of proteins identified by mass spectrometry co-purifying with *S. pombe* Pcs1-TAP and Mde4-TAP isolated from *nda3-KM311*-arrested cells (JG 15058 and JG15137, respectively) or cycling cells (JG15137). Only proteins identified with more than two peptides are included. Proteins found in other unrelated purifications are omitted from this table. (B) Mde4 and Pcs1 phosphorylation sites identified by mass spectrometry. The identified phosphorylated residues are indicated in red. Sequence coverage is highlighted in yellow.

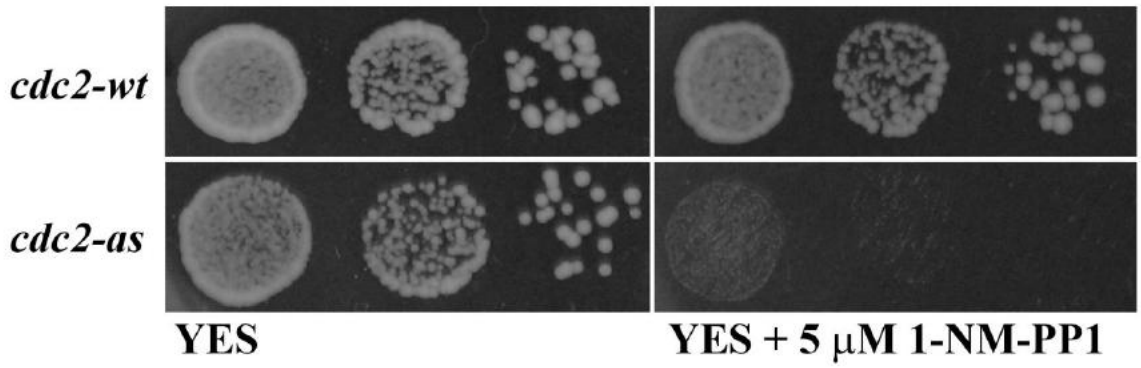


**Figure S2**. Sensitivity of cells expressing Cdc2-as to 1-NM-PP1. Serial dilutions of wild type cells (JG15123) or *cdc2Δ* cells expressing the analog-sensitive Cdc2-as (Cdc2-

F84G) (JG15125) were spotted on YES plates containing or lacking 5 μM 1-NM-PP1 and grown for 2 days at 32 °C.

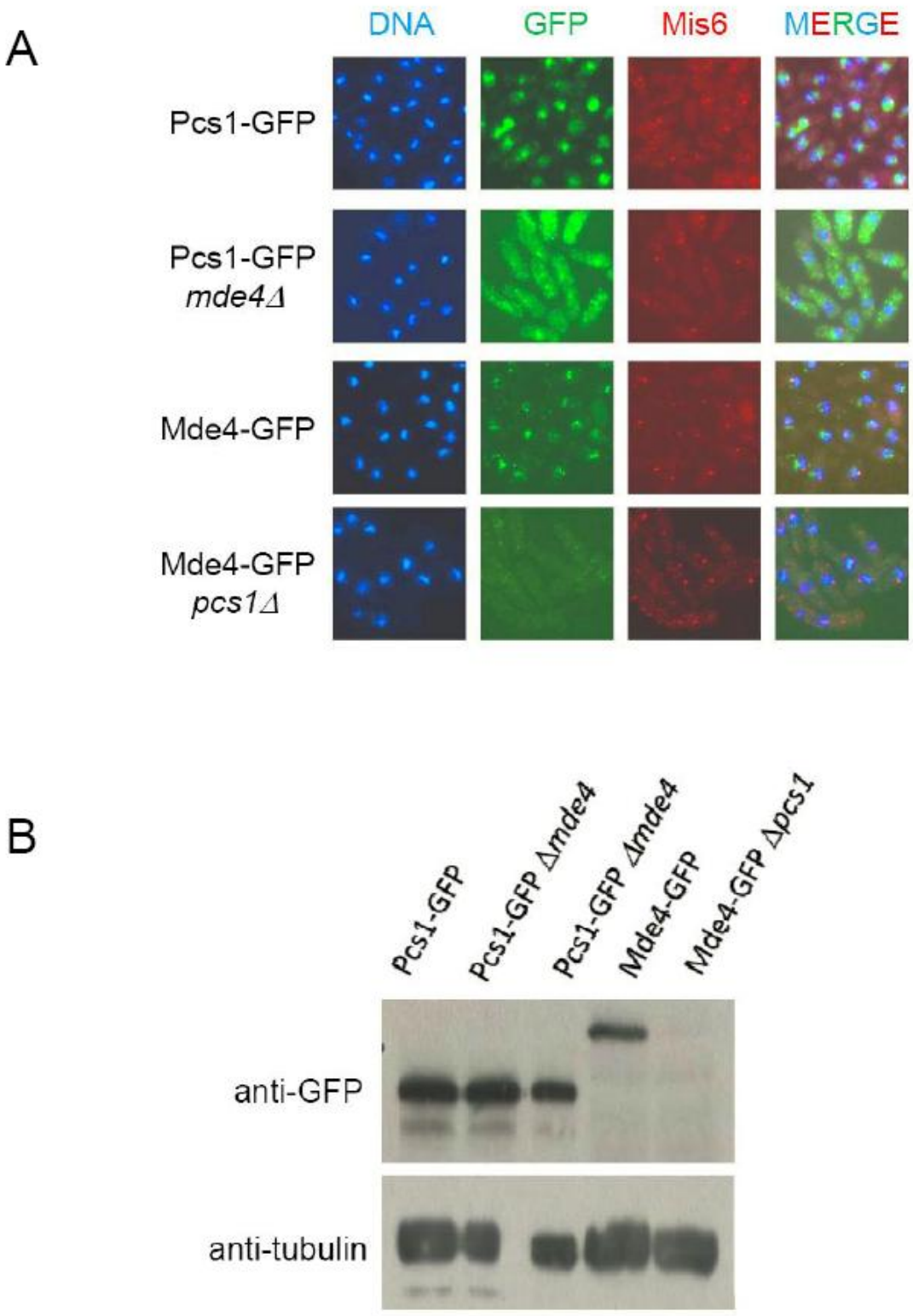


**Figure S3**. Kinetochore localization of Pcs1 and Mde4 is interdependent. (A) Wild-type cells expressing Pcs1-GFP and Mis6-HA (JG15031), or Mde4-GFP and Mis6-HA

(JG15033), *mde4Δ* mutant cells expressing Pcs1-GFP and Mis6-HA (JG15383), and *pcs1Δ* mutant cells expressing Mde4-GFP and Mis6-HA (JG15366) were fixed and stained with antibodies against HA and GFP. Nuclei were visualized by Hoechst staining.(B) Cells as described in Figure S3A were analyzed by Western blot analysis using anti-tubulin and anti-GFP antibodies.

## 2.1 Contributions

| | |
|---|---|
| **Figure 1** | Cornelia Rumpf |
| **Figure 2** | Cornelia Rumpf |
| **Figure 3** | Lubos Cipak |
| **Figure 4** | Cornelia Rumpf |
| **Figure 5** | Alexander Schleiffer, Cornelia Rumpf |
| **Figure S1** | Lubos Cipak, Karl Mechtler |
| **Figure S2** | Lubos Cipak |
| **Figure S3** | Cornelia Rumpf |

# 3. Publication: Casein kinase 1 is required for efficient removal of Rec8 during meiosis I

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**Abstract**

Segregation of chromosomes during meiosis depends on separase cleavage of Rec8, the meiosis-specific alpha-kleisin subunit of cohesin. We mapped Rec8 phosphorylation sites by mass spectrometry and show that, in fission yeast, Rec8 phosphorylation is required for proper chromosome disjunction during meiosis. We further show that the fission yeast casein kinase 1 (CK1) delta/epsilon isoforms Hhp1 and Hhp2 are required for full levels of Rec8 phosphorylation and for efficient removal of Rec8 at the onset of anaphase I. Our data are consistent with the model that Hhp1/Hhp2-dependent phosphorylation of Rec8 is required for separase-mediated cleavage of Rec8 during meiosis I.

**Key Words:** meiosis, chromosome segregation, cohesin, casein kinase, fission yeast

**Introduction**

Sexual reproduction depends on the generation of haploid gametes from diploid cells in a process called meiosis. The reduction of chromosome number during meiosis is achieved by two successive nuclear divisions after one round of DNA replication. The formation of chiasmata, mono-orientation of sister kinetochores and a stepwise loss of sister chromatid cohesion ensure proper segregation of chromosomes during meiosis (Petronczki *et al.*, 2003; Hauf & Watanabe, 2004). In meiosis I, a protease called separase cleaves cohesin along chromosome arms, thus allowing segregation of homologs. Cohesin at centromeres is protected from separase cleavage during meiosis I and is only cleaved at the onset of anaphase II, hence allowing segregation of sister centromeres. The protection of centromeric cohesion requires the conserved Sgo1/MEI-S332 proteins that recruit the protein phosphatase 2A (PP2A) to centromeres (Kerrebrock *et al.*, 1995; Kitajima *et al.*, 2004; Marston *et al.*, 2004; Rabitsch *et al.*, 2004; Gregan *et al.*, 2005; Kitajima *et al.*, 2006; Riedel *et al.*, 2006; Gregan *et al.*, 2008; Xu *et al.*, 2009). The Sgo1/PP2A complex then protects

centromeric cohesin from separase cleavage, possibly by dephosphorylating Rec8, the meiosis-specific alpha-kleisin subunit of cohesin (Brar *et al.*, 2006; Gregan *et al.*, 2008; Kudo *et al.*, 2009; Sakuno & Watanabe, 2009). To understand the role of Rec8 phosphorylation, we mapped Rec8 phosphorylation sites and analyzed chromosome segregation in fission yeast cells expressing a nonphosphorylatable or phosphomimetic version of Rec8. Moreover, we provide evidence that fission yeast casein kinase 1 delta/epsilon activity is required for full levels of Rec8 phosphorylation and for efficient removal of Rec8 at the onset of anaphase I.

## Results

### Mapping Rec8 phosphorylation sites

We purified Rec8-HA3 from *S. pombe* cells harvested around metaphase I (4 hours after induction of synchronous meiosis) and mapped the phosphorylation sites of Rec8 by mass spectrometry. Rec8 was phosphorylated on twelve residues (Figure 1A, Figure S1). To investigate the importance of Rec8 phosphorylation, we mutated the 12 serine and threonine residues found to be phosphorylated to alanine, which can no longer be phosphorylated (Rec8-12A), or to aspartate, which mimics phosphorylation (Rec8-12D). However, cells expressing Rec8-12A or Rec8-12D underwent both meiotic divisions with no apparent defect in chromosome segregation (data not shown and Figure 2).

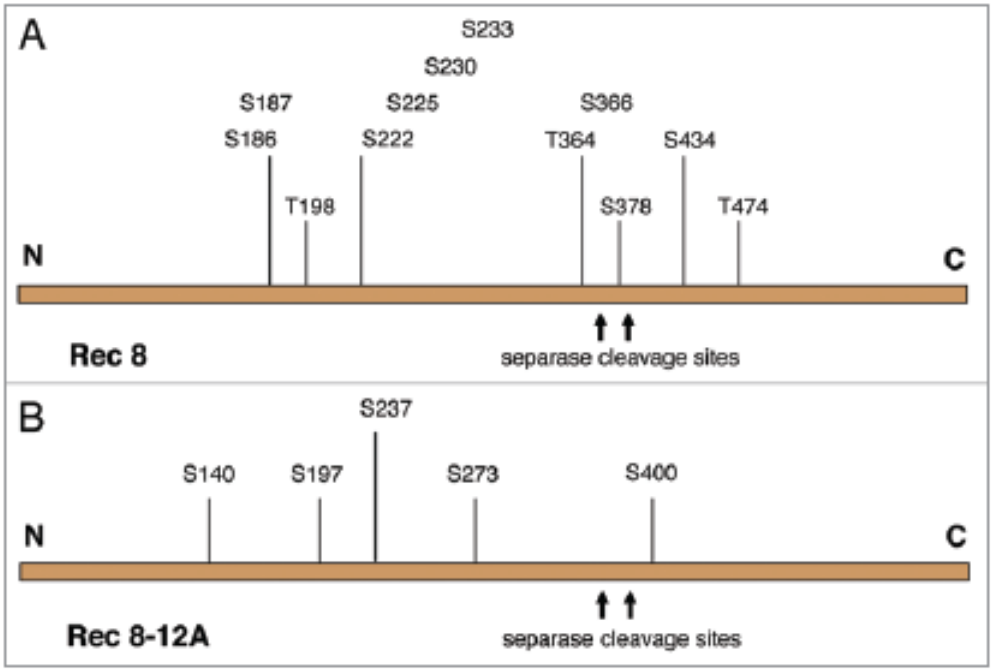



**Figure 1**. Rec8 phosphorylation sites identified by mass-spectrometry. *pat1-114* homozygous diploid cells expressing Rec8-HA3 (JG13460) (A) or Rec8-12A-HA3 (JG15152) (B) were arrested by nitrogen starvation and released into meiosis at 34°C by inactivation of Pat1. The cells were harvested 4 hours after meiosis induction and Rec8-HA3 was isolated using an HA-affinity matrix. The eluate was analyzed by mass-spectrometry and mass spectra were searched for phosphopeptides using sequest software. Rec8-HA3 was fully functional as assessed by spore viability (data not shown).

Therefore, either Rec8 phosphorylation is not important for meiotic chromosome segregation, or there are additional residues phosphorylated on Rec8. Indeed, mass spectrometry analysis of Rec8-12A-HA3 purified from cells harvested around metaphase I revealed five additional phosphorylated residues (Figure 1B, Figure S1).

**Rec8 phosphorylation is required for proper chromosome disjunction during meiosis.**

We next mutated all 17 Rec8 residues found to be phosphorylated to alanine (Rec8-17A) and analyzed chromosome segregation in a strain in which both copies of chromosome I contained *lys1* sequences marked with GFP (*lys1*-GFP) (Nabeshima *et al.*, 1998). Wild-type cells produced four nuclei each containing one *lys1*-GFP dot, whereas cells expressing Rec8-17A showed a mild nondisjunction phenotype. In contrast, when we analyzed chromosome segregation in cells expressing a mutant version of Rec8-17A in which one of the two separase cleavage sites was mutated (Rec8-17A-RD1), meiotic nuclear divisions were greatly inhibited and most of the cells

showed all of the *lys1*-GFP dots in one nucleus. A similar phenotype has been observed when both Rec8 cleavage sites were mutated (Rec8-RDRD) (Figure 2) (Kitajima *et al.*, 2003). This indicates that meiotic chromosome segregation was impaired in cells expressing Rec8-17A-RD1. Proteolytic cleavage at either of the two cleavage sites is sufficient for Rec8 degradation and normal progression of meiotic divisions (Kitajima *et al.*, 2003), suggesting that Rec8 phosphorylation is important for proper chromosome disjunction during meiosis.

| | *lys1*-GFP dots in three or four nuclei | *lys1*-GFP dots in two nuclei | *lys1*-GFP dots in one nucleus |
|---|---|---|---|
| wild-type | 100% | - | - |
| *rec8Δ* | 87% | 13% | - |
| Rec8-12A | 99% | 1% | - |
| Rec8-12D | 99% | 1% | - |
| Rec8-17A | 96% | 2% | 2% |
| Rec8-RD1 | 97% | 3% | - |
| Rec8-RDRD | - | 26% | 74% |
| Rec8-17A-RD1 | 2% | 29% | 69% |

**Figure 2.** Rec8 phosphorylation is required for proper chromosome disjunction during meiosis. Meiotic segregation of chromosome I was scored in $h^{90}$ *lys1*-GFP cells with deleted *rec8* (JG14208), or carrying either wild-type Rec8 (JG11248) or mutant Rec8 (Rec8-12A (JG14541), Rec8-12D (JG14987), Rec8-17A (JG15557), Rec8-RD1 (JG15595), Rec8-RDRD (JG12069), Rec8-17A-RD1 (JG15594)). Cells were sporulated on PMG-N plates for 16 hours, stained with Hoechst and examined under the fluorescence microscope. Chromosome segregation was scored in at least 100 asci. See Figure S5 for schematic presentation of Rec8 mutations.

Segregation of chromosomes during meiosis I is triggered by separase cleavage of Rec8 along chromosome arms, while centromeric cohesin is protected from separase cleavage during meiosis I by Sgo1/MEI-S332 proteins in complex with protein phosphatase 2A (Kitajima *et al.*, 2006; Riedel *et al.*, 2006). The fact that Rec8 phosphorylation promotes its cleavage by separase suggests that the protection of centromeric cohesion might be conferred by dephosphorylation of Rec8 by the Sgo1/PP2A complex. We therefore speculated that a Rec8 phosphomimetic mutant might be cleaved at the centromeres during meiosis I, resulting in precocious separation of sister centromeres and nondisjunction during meiosis II. However, cells expressing Rec8 with phosphomimetic mutations (Rec8-16D) showed only mild nondisjunction during meiosis II (data not shown). This may be due to the fact that aspartate is not able to fully substitute phosphorylated residues of Rec8 or we failed to identify all of the Rec8 phosphorylation sites. Alternatively, the protection of centromeric cohesion might not be conferred by dephosphorylation of Rec8.

**Hhp1 and Hhp2 are required for full levels of Rec8 phosphorylation.**

Interestingly, four of the residues phosphorylated on Rec8 match the CK1 consensus phosphorylation site S/T(P)-$X_{1-2}$-S/T (Hoekstra *et al.*, 1994; Knippschild *et al.*, 2005). We have recently shown that the fission yeast CK1 delta/epsilon isoforms Hhp1 and Hhp2 are required for proper segregation of chromosomes during meiosis I (Petronczki *et al.*, 2006). Moreover, TAP-tagged Hhp1 protein purified from cells harvested around metaphase I was able to phosphorylate a bacterially-expressed Rec8 fragment (Rec8(323-562)) on serine 366, as revealed by mass-spectrometry. This phosphorylation was likely to be due to Hhp1 or Hhp2 activity, because mass-spectrometry confirmed the presence of Hhp1 and Hhp2 (data not shown) and no phosphorylation of Rec8 was observed when the CK1 inhibitor D4476 (Rena *et al.*, 2004) was added (Figure 3).

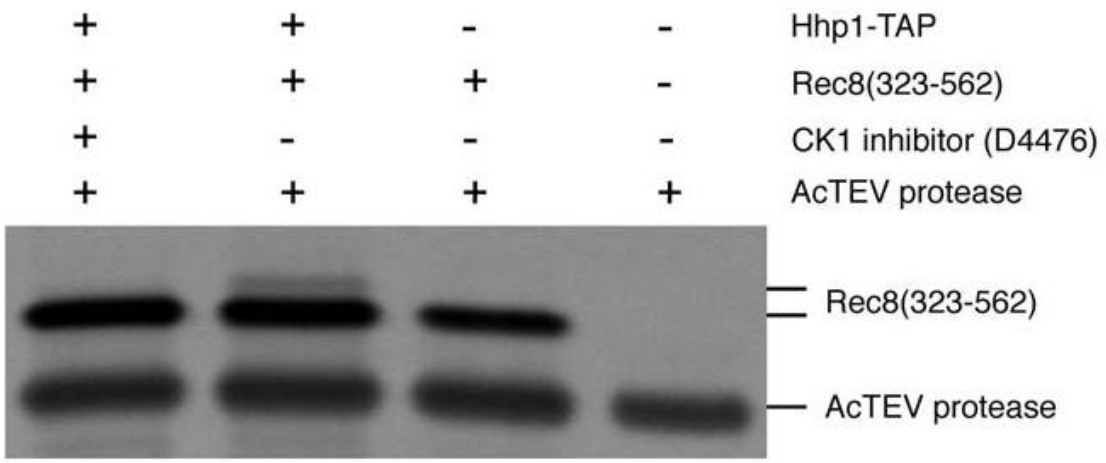



**Figure 3.** The Hhp1 complex can phosphorylate bacterially expressed Rec8 fragment. The MBP-Rec8(323-562) fusion protein purified from *E. coli* was mixed with the Hhp1 complex purified by tandem affinity purification from yeast cells harvested around metaphase I and incubated in the presence of 200 µM ATP at 30°C for 2 hours. The Rec8(323-562) fragment was removed from the MBP by adding AcTEV protease. In the control reaction, 5 µM D4476 was added to inhibit CK1 activity. The proteins were visualized by SDS-PAGE and silver staining and the Rec8(323-562) fragment was analyzed by mass-spectrometry. Mass spectra were searched for phosphopeptides using sequest software. Phosphorylation of serine 366 was detected in the sample containing the Hhp1 complex and lacking the inhibitor D4476.

We therefore investigated whether Rec8 phosphorylation depends on Hhp1 and Hhp2. Whereas Rec8 became phosphorylated in wild-type cells approaching anaphase I, phosphorylation of Rec8 was largely abolished in *hhp1-as hhp2Δ* mutant cells (Figure 4).

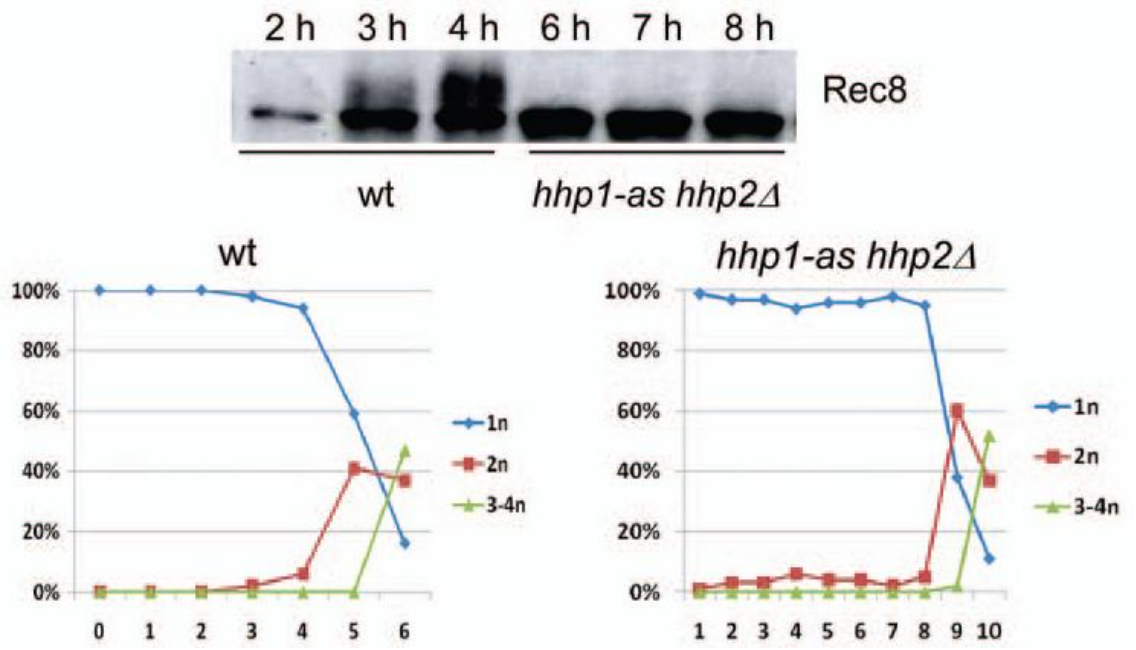
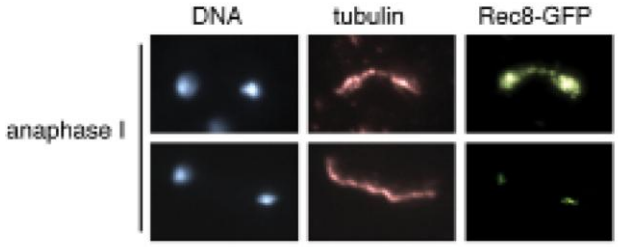



**Figure 4.** Inhibition of CK1 delta/epsilon limits the extent of Rec8 phosphorylation. *pat1-114* cells carrying wild-type alleles of *hhp1* and *hhp2* (JG12015) or *hhp1-as hhp2Δ* (JG15600) were arrested by nitrogen starvation and released into meiosis at 34°C by inactivation of Pat1 in the presence of 1NM-PP1. Cells were harvested at the indicated time points (hours) after meiosis induction, stained with DAPI and nuclei were counted in 100 cells per time point. The fraction of cells that contained one nucleus (1n), two nuclei (2n) or more than two nuclei (3-4n) at the indicated time points are shown. Rec8 protein was detected by western blotting using anti-Rec8 polyclonal antibodies.

Phosphorylation of cohesin's alpha-kleisin subunit is known to promote its cleavage by separase (Alexandru *et al.*, 2001; Hornig & Uhlmann, 2004; Brar *et al.*, 2006). Consistent with this, we observed high levels of Rec8-GFP in *hhp1-as hhp2Δ* mutant cells during anaphase I, whereas in wild-type cells, most of the Rec8-GFP disappeared at the onset of anaphase I (Figure 5). We conclude that Hhp1 and Hhp2 are required for full levels of Rec8 phosphorylation and for efficient removal of Rec8 at the onset of anaphase I.




| Rec8-GFP in anaphase cells | wild-type | *hhp1-as hhp2Δ* |
|---|---|---|
| general nuclear flourescence | 2% | 21% |
| distinct nuclear foci | 98% | 79% |

**Figure 5.** Hhp1 and Hhp2 are required for Rec8 removal at the onset of anaphase I. Wild-type (JG11218 x JG11219) or *hhp1-as hhp2Δ* mutant cells expressing Rec8-GFP (JG15722 x JG15723) were sporulated in the presence of 1NM-PP1, fixed and stained with antibodies against tubulin and GFP. Nuclei were visualized by Hoechst staining. The Rec8-GFP signal was scored in 100 anaphase I cells.

**Hhp1 and Hhp2 colocalize with Rec8.**

To analyze subcellular localization, we tagged Hhp1 and Hhp2 with GFP at their carboxyl termini. These constructs contained endogenous 3' untranslated regions (3' UTR) to ensure physiological expression levels. In most meiotic cells, GFP-tagged Hhp1 was evenly distributed throughout the cell, while Hhp2-GFP was enriched in nuclei (Figure 6A). To determine whether Hhp1 and Hhp2 associate with chromatin, we arrested cells by nitrogen-starvation, induced meiosis by inactivation of Pat1 kinase (Yamamoto, 1996) and harvested cells 2.5 and 4 hours after induction of synchronous meiosis. These fractions were enriched for prophase and metaphase I cells, respectively (Figure S2). Chromatin immunoprecipitation (ChIP) followed by real-time PCR showed that both proteins were enriched at several Rec8 binding sites (Figure 6B) (Riedel *et al.*, 2006). These results are consistent with the notion that Hhp1 and Hhp2 may phosphorylate Rec8 on chromatin.

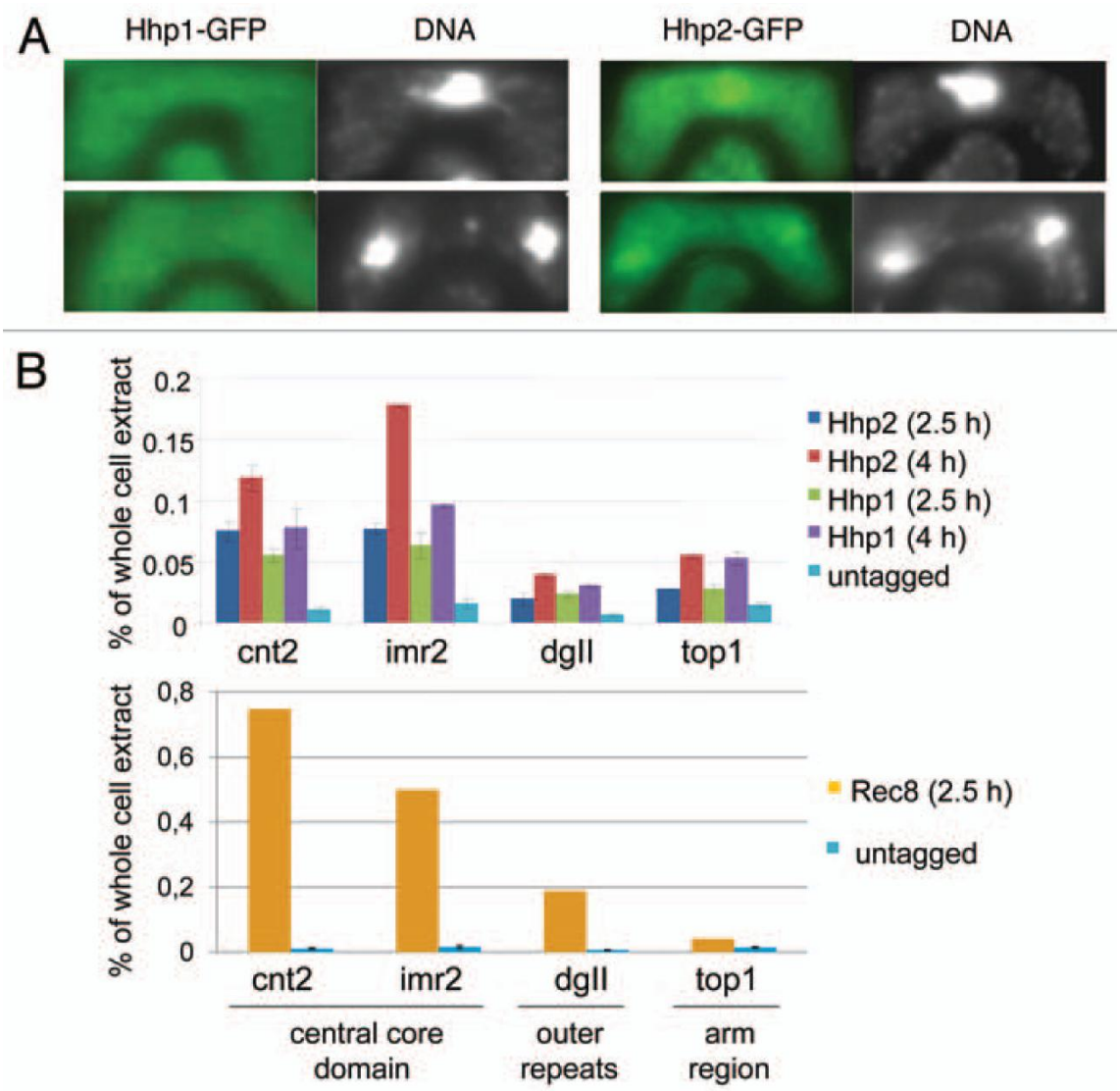



**Figure 6.** Subcellular localization of Hhp1 and Hhp2 during meiosis. (A) $h^{90}$ strains expressing Hhp1-GFP (JG14824) or Hhp2-GFP (JG14825) were sporulated, stained with Hoechst and analyzed by fluorescence microscopy. Both Hhp1-GFP and Hhp2-GFP were functional (see Figure S3). (B) *pat1-114* homozygous diploid cells either wild-type (JG12209), or expressing Hhp1-GFP (Hhp1) (JG14846), Hhp2-GFP (Hhp2) (JG14847), or Rec8-GFP (Rec8) (JG12094) were arrested by nitrogen starvation and released into meiosis at 34°C by inactivation of Pat1. Cells were harvested at the indicated time points (2.5 and 4 hours) after meiosis induction (see Figure S2) and chromatin binding was analyzed by chromatin immunoprecipitation (ChIP) followed by real-time PCR using oligonucleotide primers specific for the centromeric central region (cnt2), innermost centromeric repeats (imr2), outer centromere (dgII) and chromosome arm (top1).

**Discussion**

Previous studies established that Rec8 becomes phosphorylated as cells proceed through meiosis (Parisi *et al.*, 1999; Watanabe & Nurse, 1999; Clyne *et al.*, 2003; Lee & Amon, 2003; Brar *et al.*, 2006). Our analyses reveal that, in fission yeast, Rec8 phosphorylation is required for proper chromosome disjunction during meiosis. It is likely that the nondisjunction observed in cells expressing Rec8-17A-RD1 is caused by defective cleavage of Rec8 protein. During the course of this work, two studies showed that, indeed, Rec8 phosphorylation promotes its cleavage by separase in both budding and fission yeast (Ishiguro *et al.*, 2010; Katis *et al.*, 2010). Similarly, phosphorylation of the mitotic kleisin Rad21/Scc1 by Polo-like kinase is known to enhance its cleavage by separase (Alexandru *et al.*, 2001; Hornig & Uhlmann, 2004; Hauf *et al.*, 2005).
In budding yeast, CK1 delta/epsilon is a subunit of the monopolin complex required for the mono-orientation of sister kinetochores during meiosis I. Our observation that the fission yeast CK1 delta/epsilon isoforms Hhp1 and Hhp2 are required for full levels of Rec8 phosphorylation and for efficient removal of Rec8 at the onset of anaphase I reveal a novel function of this evolutionarily conserved kinase. However, it is possible that other protein kinases act redundantly in the regulation of Rec8 cleavage. Previous studies showed that Rec8 phosphorylation depends on Polo-like kinase and Dbf4-dependent Cdc7 kinase (Clyne *et al.*, 2003; Brar *et al.*, 2006; Kudo *et al.*, 2009; Katis *et al.*, 2010).

Segregation of chromosomes during meiosis I is triggered by separase cleavage of Rec8 along chromosome arms, while centromeric cohesin is protected from separase cleavage during meiosis I by the Sgo1/PP2A complex. Centromeric cohesin is only cleaved during meiosis II, hence allowing segregation of sister chromatids. Our finding that Rec8 phosphorylation promotes its removal at the onset of anaphase raises the possibility that the Sgo1/PP2A complex protects centromeric cohesin by dephosphorylating Rec8. Although we failed to identify a phosphomimetic Rec8 mutant that would be cleaved at the centromeres during meiosis I, two very recent studies showed that *S. cerevisiae* Rec8 with phosphomimetic mutations is no longer protected from separase at centromeres during meiosis I (Katis *et al.*, 2010) and forced

localization of excess CK1 delta/epsilon at the pericentromeric region abrogates the ability of Sgo1/PP2A to protect centromeric Rec8 (Ishiguro *et al.*, 2010). These results suggest that Sgo1/PP2A protects centromeric cohesin from separase cleavage by opposing CK1-dependent phosphorylation of Rec8. This mechanism may be evolutionarily conserved, because similar observations were made in budding and fission yeast, and both Rec8 and CK1 proteins are present in most eukaryotes.

## Material and Methods

### Tandem affinity purification

Tandem affinity purifications and mass spectrometry were performed as previously described (Riedel *et al.*, 2006; Gregan *et al.*, 2007).

### Chromatin immunoprecipitation

Chromatin immunoprecipiation was performed essentially as described before (Pidoux *et al.* 2004). $2.5x10^8$ cells were fixed with 3% Paraformaldehyde and treated with 0.4mg/ml Zymolyase T100. DNA was sonicated to fragments of 400 bp average size. Immunoprecipitation was performed using an anti-GFP antibody (Roche) in conjunction with ProteinA Dynabeads (Invitrogen).

Real-time PCR was performed using the IQ SYBR Green Mix (BIO-RAD) and an IQ5 Cycler (BIO-RAD). The following primers were used for qRT-PCR of S. pombe chromosome 2 loci:

Cnt2-fw AGCGCTAACTCGTTTAAGTGAA, Cnt2-rev GGCGTGGAAAGTCATCTGTA, Imr2-fw CTTCGGCGACGTGATATAAG, Imr2-revTTTGCAACGATTACCGGTTT, DgII-fw TGCTCTGACTTGGCTTGTCT, DgII-rev TTGCACTCGGTTTCAGCTAT, top1-fw AGGGTTATTTCGTGGTCGAG, top1-rev TGCCAACCAGGTCACTGTAT.

### Preparation of Rec8 fragment from *Escherichia coli*

*S. pombe rec8* fragment (970-1686) was PCR amplified with primers rec8fw (5'-ATATATGAATTCTATGTTCGTCGTAGAGGAGCTTCTTCGGCAAAGAAG-3' (EcoRI site is underlined)) and rec8rev (5'-ATATCTGCAGTCAAATGGCATCGGTGCTTTTTAGG-3' (PstI site is underlined)), digested with *EcoRI* and *PstI*, and cloned into pMAL-TEV/6xHIS

vector creating in-frame fusion to the carboxyl terminus of maltose binding protein (MBP). The MBP-Rec8(323-562) fusion protein was expressed in *E. coli*, extracted, bound to maltose-binding beads, and eluted with maltose according to the manufacturer's instructions (New England Biolabs). The eluted fusion protein was concentrated and washed with kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT) by centrifugation using the Centricon-30 system and stored at -80°C.

***In vitro* kinase assay**

The MBP-Rec8(323-562) fusion protein (~ 500 ng) was mixed with Hhp1 complex (20 µl) purified by TAP from cells harvested around metaphase I and incubated in 80 µl of kinase buffer containing 200 µM ATP at 30°C for 2 hours, followed by cleavage with TEV protease at 16°C for 1 hour to remove MBP from the Rec8(323-562) fragment. The reaction was mixed with 2xSDS-PAGE loading buffer and analyzed by SDS-PAGE. The proteins were visualized by silver staining and the Rec8(323-562) fragment was analyzed by mass-spectrometry.

**Microscopy**

The Immunofluorescence and microscopy techniques were as described in (Rabitsch *et al.*, 2003).

**Strains, media and growth conditions**

Media and growth conditions were as described in (Rabitsch *et al.*, 2003). The genotypes of *S. pombe* strains used in this study are listed in the Figure S4.

**Mutagenesis and Trasformation**

Plasmid pY137 (Y. Watanabe) carrying $rec8^+$ and $ura3^+$ selection marker was used for *rec8* mutagenesis. STRATAGENE QuikChange® Lightning Site-Directed Mutagenesis Kit was used to introduce *rec8* mutations and all constructs were verified by sequencing. Plasmids carrying mutated *rec8* were linearised with Bsp68I (Fermentas) and transformed into *S. pombe* cells. Proper integration of the *rec8* construct was verified in Ura+ colonies by colony PCR.

**Acknowledgements**

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**Supplementary Material**

**Casein kinase 1 is required for efficient removal of Rec8 during meiosis I.**

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**Rec8-HA**

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  1 MFYNQDVLTK EKGGMGVIWL AATLGSKHSL RKLHKKDIMS VDIDEACDFV
 51 AFSPEPLALR LSSNLMIGVT RVWAHQYSFF HSQVSTLHLR VRKELDHFTS
101 KPFKNIDIQN EQTNPKQLLL AEDPAFIPEV SLYDAFNLPS VDLHVDMSSF
151 TQPKENPNIS VLETLPDSTS YLINTSQNYS LRNNVSSFVY EDSRAFSTEE
201 PLDFEFDENG DIQELTKGTI NSDPSLQAAS QHSNLGSVQR EYNSEEQESR
251 IHMFEIDEDV LPLPVPLQSV MDSEHNENEP RALKRRKVQK LLEPDENIEL
301 STRTLSQWRK NYVERMIALE ATKYVRRRGA SSAKKKELNK FFDWESFHPL
351 LKPWIEKLKP SNNTPSEIDD VLRNIDTSEV EVGRDVQGEL GLNIPWNTSS
401 RSNSAINSKS HSQTGSEHST PLLDTKYRKR LPHSPSMPSR VEFLPALESS
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451 QFHETLNSEL SLQLSDDFVL YKNTQEENAH LMLSMEKECA NFYEYAKTAI
501 YENNGRITFS SLLPNDLKRP VVAQAFSHLL SLATKSAFLV KQDKPYSEIS
551 VSLNLKSTDA I
```

**Rec8-12A-HA**

```
  1 MFYNQDVLTK EKGGMGVIWL AATLGSKHSL RKLHKKDIMS VDIDEACDFV
 51 AFSPEPLALR LSSNLMIGVT RVWAHQYSFF HSQVSTLHLR VRKELDHFTS
101 KPFKNIDIQN EQTNPKQLLL AEDPAFIPEV SLYDAFNLPS VDLHVDMSSF
151 TQPKENPNIS VLETLPDSTS YLINTSQNYS LRNNVAAFVY EDSRAFSAEE
201 PLDFEFDENG DIQELTKGTI NADPALQAAA QHANLGSVQR EYNSEEQESR
251 IHMFEIDEDV LPLPVPLQSV MDSEHNENEP RALKRRKVQK LLEPDENIEL
301 STRTLSQWRK NYVERMIALE ATKYVRRRGA SSAKKKELNK FFDWESFHPL
351 LKPWIEKLKP SNNAPAEIDD VLRNIDTAEV EVGRDVQGEL GLNIPWNTSS
401 RSNSAINSKS HSQTGSEHST PLLDTKYRKR LPHAPSMPSR VEFLPALESS
451 QFHETLNSEL SLQLSDDFVL YKNAQEENAH LMLSMEKECA NFYEYAKTAI
501 YENNGRITFS SLLPNDLKRP VVAQAFSHLL SLATKSAFLV KQDKPYSEIS
551 VSLNLKSTDA I
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**Figure S1**. Rec8 peptides identified by mass-spectrometry.
The matched peptides are indicated in red.

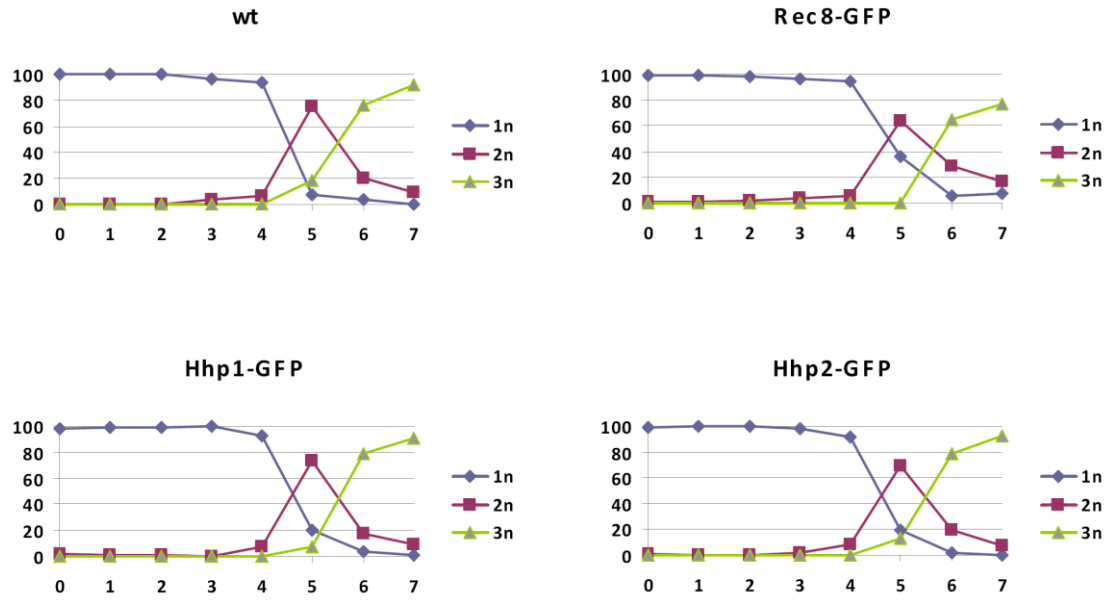


**Figure S2.** Progression of *pat1*-induced meiosis. *pat1-114* homozygous diploid cells expressing Hhp1-GFP (JG14846), Hhp2-GFP (JG14847) or Rec8-GFP (JG12094) were harvested as described in Figure 6B, stained with DAPI and nuclei were counted in 100 cells per time point. The fraction of cells that contained one nucleus (1n), two nuclei (2n) or more than two nuclei (3n) at the indicated time points are shown.

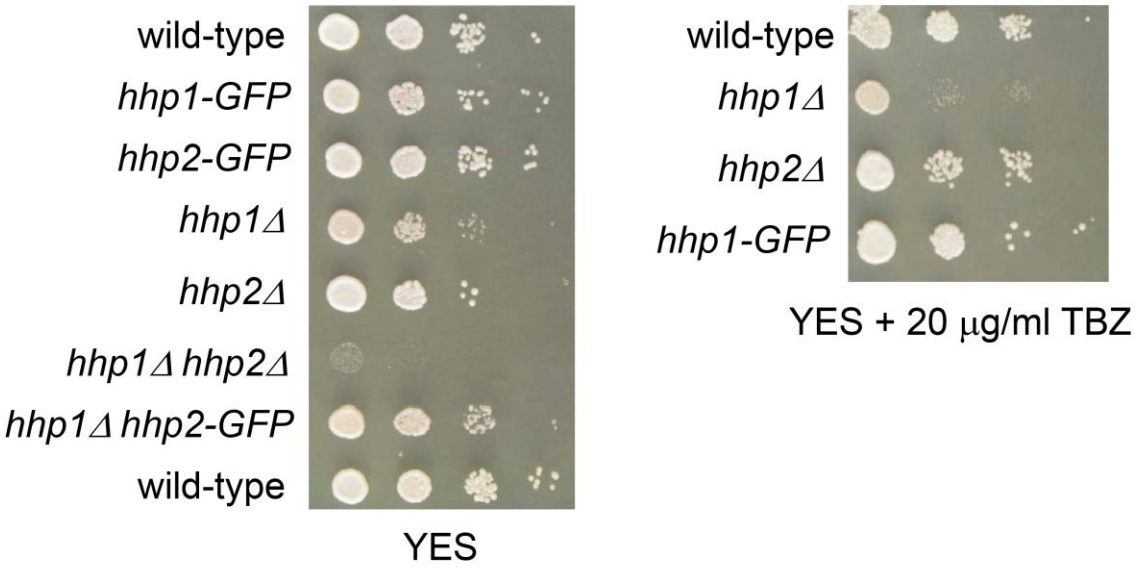


**Figure S3.** Testing the functionality of Hhp1-GFP and Hhp2-GFP fusions. Serial dilutions of the indicated strains were spotted on YES plates containing or lacking 20μg/ml thiabendazole (TBZ) and grown for two days at 32 °C.

| Strains | Genotype |
|---|---|
| JG13460 | *h⁻/h⁻ pat1-114/pat1-114 ade6-210/ade6-216 rec8-HA-Kan$^R$/rec8-HA-Kan$^R$* |
| JG15152 | *h⁻/h⁻ pat1-114/pat1-114 ade6-210/ade6-216 rec8::Nat$^R$/rec8::Nat$^R$ rec8 ST12A-HA-Kan$^R$/rec8 ST12A-HA-Kan$^R$* |
| JG14208 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+ rec8::Nat$^R$* |
| JG11248 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+* |
| JG14541 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+ rec8::Nat$^R$ rec8 ST12A-GFP-Kan$^R$-ura4+* |
| JG14987 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+ rec8::Nat$^R$ rec8 ST12D-ura4+* |
| JG15557 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+ rec8::Nat$^R$ rec8 ST17A-ura4+* |
| JG15595 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+ rec8::Nat$^R$ rec8 RD1-ura4+* |
| JG12069 | *h$^{90}$ rec8::Kan$^R$<<ura4+<<Prec8-rec8-RDRD-FLAG<<Kan$^R$ lys1+<<lacO his7+<<Pdis1-GFP-lacI-NLS ade6-M216 leu1 ura4-D18* |
| JG15594 | *h$^{90}$ leu1 ura4 LacO-lys1+ GFP-LacI-his7+ rec8::Nat$^R$ rec8 ST17A RD1-ura4+* |
| JG12015 | *h⁺ pat1-114 ade6-216* |
| JG15600 | *h⁻ pat1-114 ade6-216 hhp1::Nat$^R$ hhp1-as::Hyg$^R$ hhp2::Ble$^R$* |
| JG11218 | *h⁺ rec8-GFP::Kan$^R$ ade6-210* |
| JG11219 | *h⁻ rec8-GFP::Kan$^R$ ade6-216* |
| JG15722 | *h⁻ hhp1::Nat$^R$ hhp2::Kan$^R$hhp1-M84G::Hyg$^R$ rec8-GFP::Kan$^R$* |
| JG15723 | *h⁺ hhp1::Nat$^R$ hhp2::Kan$^R$ hhp1-M84G::Hyg$^R$ rec8-GFP::Kan$^R$* |
| JG14824 | *h$^{90}$ leu1-32 ura4-D18 hhp1-GFP-UTR::Kan$^R$* |
| JG14825 | *h$^{90}$ leu1-32 ura4-D18 hhp2-GFP-UTR:::Kan$^R$* |
| JG14846 | *h⁻/h⁻ pat1-114/pat1-114 ade6-210/ade6-216 hhp1-GFP-UTR::Kan$^R$/hhp1-GFP-UTR::Kan$^R$* |
| JG14847 | *h⁻/h⁻ pat1-114/pat1-114 ade6-210/ade6-216 hhp2-GFP-UTR::Kan$^R$/hhp2-GFP-UTR::Kan$^R$* |
| JG12094 | *h⁻/h⁻ pat1-114/pat1-114 ade6-210/ade6-216 rec8-GFP/rec8-GFP* |
| JG12209 | *h⁻/h⁻ pat1-114/pat1-114 ade6-210/ade6-216* |

**Figure S4.** Strain list.

| | S140 | S186 | S187 | S197 | T198 | S222 | S225 | S230 | S233 | S237 | S273 | T364 | S366 | S378 | E379 | E381 | R384 | S400 | S434 | T474 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rec8-12A | | A | A | | A | A | A | A | A | | | A | A | A | | | | | A | A |
| Rec8-12D | | D | D | | D | D | D | D | D | | | D | D | D | | | | | D | D |
| Rec8-16D | D | D | D | | D | D | D | D | D | D | D | D | D | D | | | | D | D | D |
| Rec8-17A | A | A | A | A | A | A | A | A | A | A | A | A | A | A | | | | A | A | A |
| Rec8-17A-RD1 | A | A | A | A | A | A | A | A | A | A | A | A | A | A | R | R | D | A | A | A |
| Rec8-RD1 | | | | | | | | | | | | | | | R | R | D | | | |

**Figure S5.** Schematic presentation of Rec8 mutations used in this study.

## 3.1 Contributions

| | |
|---|---|
| **Figure 1** | Cornelia Rumpf, Andrej Dudas, Lubos Cipak, Christian G. Riedel |
| **Figure 2** | Cornelia Rumpf, Andrej Dudas |
| **Figure 3** | Lubos Cipak, Miroslava Pozgajova, Gustav Ammerer |
| **Figure 4** | Cornelia Rumpf |
| **Figure 5** | Zsigmond Benko, Juraj Gregan |
| **Figure S1** | Lubos Cipak, Karl Mechtler |
| **Figure S2** | Cornelia Rumpf |
| **Figure S3** | Cornelia Rumpf |

# 4. Publication: High-throughput knockout screen in *Schizosaccharomyces pombe* identifies a novel gene required to prevent meiosis I non-disjunction.

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key words: fission yeast, meiosis, chromosome segregation, dynein, nuclear movement

running title: Dil1 protein is required to prevent meiosis I non-disjunction

**Abstract**

Meiosis is the process which produces haploid gametes from diploid precursor cells. This reduction of chromosome number is achieved by two successive divisions. Whereas homologs segregate during meiosis I, sister chromatids segregate during meiosis II. To identify novel proteins required for proper segregation of chromosomes during meiosis, we applied a high-throughput knockout technique to delete 87 *S. pombe* genes whose expression is upregulated during meiosis and analyzed the mutant phenotypes. Using this approach, we identified a new protein, Dil1, which is required to prevent meiosis I homolog non-disjunction. We show that Dil1 acts in the dynein pathway to promote oscillatory nuclear movement during meiosis.

**Introduction**

The reduction of chromosome number during meiosis is achieved by a single round of DNA replication followed by two rounds of chromosome segregation, called meiosis I and meiosis II. While the second meiotic division is similar to mitosis in that sister centromeres segregate to opposite poles, the first meiotic division is fundamentally different and ensures segregation of homologous centromeres. Paliulis and Nicklas demonstrated that chromosome segregation during meiosis is determined by special properties of the meiotic chromosomes rather than by spindle components or other cytosolic factors (Paliulis & Nicklas, 2000). There are three major features of meiotic chromosomes and their associated proteins responsible for their unique meiosis I segregation (Page & Hawley, 2003; Petronczki *et al.*, 2003; Gregan *et al.*, 2008). The first is recombination in which homologous chromosomes cross over to form chiasmata and to designate bivalents for disjunction. The second meiosis-specific feature is mono-orientation of sister kinetochores. The third meiosis-specific feature is the protection of centromeric cohesion. Disturbing any of these processes may lead to missegregation of chromosomes and aneuploidy, which is the major cause of miscarriages and mental retardation in humans.

The fission yeast *Schizosaccharomyces pombe* is an excellent model organism for the study of chromosome segregation, as it is amenable to both genetic and cell biology

techniques. Moreover, fission yeast chromosomes have large complex centromere structure, similar to those of higher eukaryotes (Pidoux & Allshire, 2005). We have been studying the consequences on meiotic chromosome segregation of deleting *S. pombe* genes whose mRNAs were upregulated during meiosis, arguing that meiotic regulators governing the aforementioned processes would be preferentially expressed during meiosis. We previously deleted 180 genes and identified new regulators of meiotic chromosome segregation, including the protector of centromeric cohesion Sgo1 and a new protein required for the initiation of meiotic recombination Mde2 (Rabitsch *et al.*, 2004; Gregan *et al.*, 2005). Martin-Castellanos *et al.* used a similar approach to delete 160 genes and identified seven novel genes required for critical meiotic events (Martin-Castellanos *et al.*, 2005). Here we report deletion and phenotypical characterization of additional 87 meiotically upregulated genes.

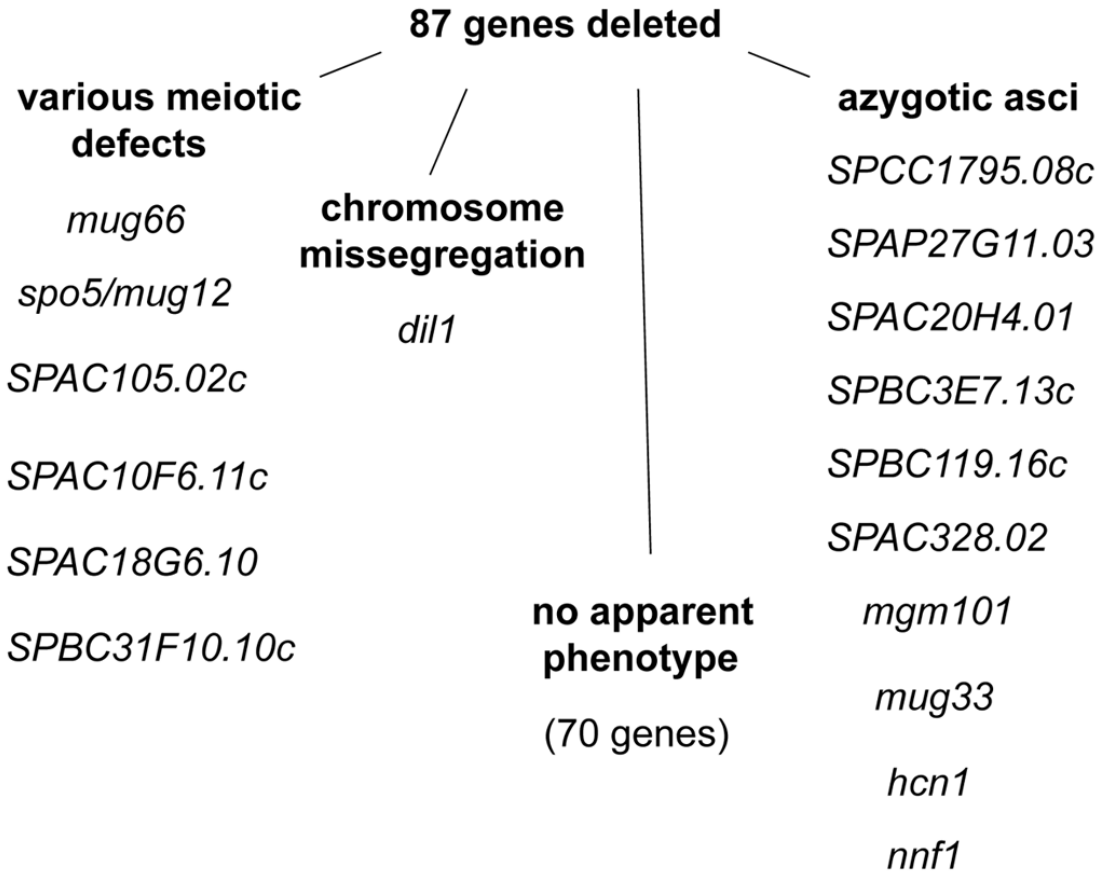


**Figure 1.** Summary of the screen. Chromosome segregation in deletion mutants generated by a large-scale knockout approach was analyzed by scoring *lys1*-GFP in meiotic cells. The genes were sorted into four categories according to their phenotype. See Table S1 for the full list of analyzed genes.

## Results

### A screen for genes required for meiotic chromosome segregation

We have designed a high-throughput strategy to knock-out genes in the fission yeast *S. pombe* on a large scale (Gregan *et al.*, 2005; Gregan *et al.*, 2006). In addition to its high knockout efficiency, our strategy has the advantage that a library of knockout plasmids is created. The plasmids are freely available and can be used to knockout genes in strains with various genetic backgrounds. Here we applied this technique to delete selected meiotically upregulated genes (Mata *et al.*, 2002). We were able to delete 87 genes out of 92 selected (Table S1). The genes which resisted deletion may be essential genes or genes refractory to homologous recombination (Krawchuk & Wahls, 1999). Two of these genes have been shown to be essential for cell growth during the course of this work (Ma *et al.*, 1999; Martin-Castellanos *et al.*, 2005).

In order to analyze chromosome segregation, we knocked-out the genes in a haploid homothallic $h^{90}$ strain where chromosome I was marked with GFP (*lys1*-GFP) (Nabeshima *et al.*, 1998). In this strain, GFP tagged LacI molecules bind to *lacO* repeats inserted within the *lys1* locus located near the centromere. This strain generates cells of both mating types and undergoes mating and meiosis on sporulation medium. We sporulated mutant cells, stained nuclei with Hoechst dye and scored segregation of *lys1*-GFP dots in asci. 70 mutants had no apparent phenotype and faithfully segregated their chromosomes. Ten mutants formed azygotic asci, which may indicate that the deletion caused defects in mitotic growth, as we have previously observed (Fig. 1) (Gregan *et al.*, 2005). Consistent with this hypothesis, $hcn1^{+}$ and $nnf1^{+}$ genes have been shown to be required for normal mitotic growth (Yoon *et al.*, 2006; Kobayashi *et al.*, 2007). One mutant (*SPAC458.04c*) missegregated chromosomes during meiosis and six other mutants displayed various meiotic defects such as fragmented nuclei and abnormal number of spores (Fig. 1). Abnormal spore morphology in *spo5*/*mug12* and *mug66* mutants have been previously described (Martin-Castellanos *et al.*, 2005; Kasama *et al.*, 2006).

**Dil1 is required for proper segregation of homologs during meiosis I**

We further analyzed the *SPAC458.04cΔ* mutant that missegregated chromosomes during meiosis. The open reading frame of *SPAC458.04c* is predicted to encode a 38 kDa protein with no obvious orthologs in other organisms (Hertz-Fowler *et al.*, 2004). In the current *S. pombe* genome database (Hertz-Fowler *et al.*, 2004) SPAC458.04c is listed as an orphan sequence with no known sequence domains and a distinctive up-regulation during the meiotic cell cycle and in stressed cells (Mata *et al.*, 2002; Chen *et al.*, 2003). We performed a more detailed computational analysis of SPAC458.04c which revealed a similarity to dynein light intermediate chain proteins (DLIC) (Fig. 2, Fig. S1 and http://mendel.imp.ac.at/SEQUENCES/DLIC/).

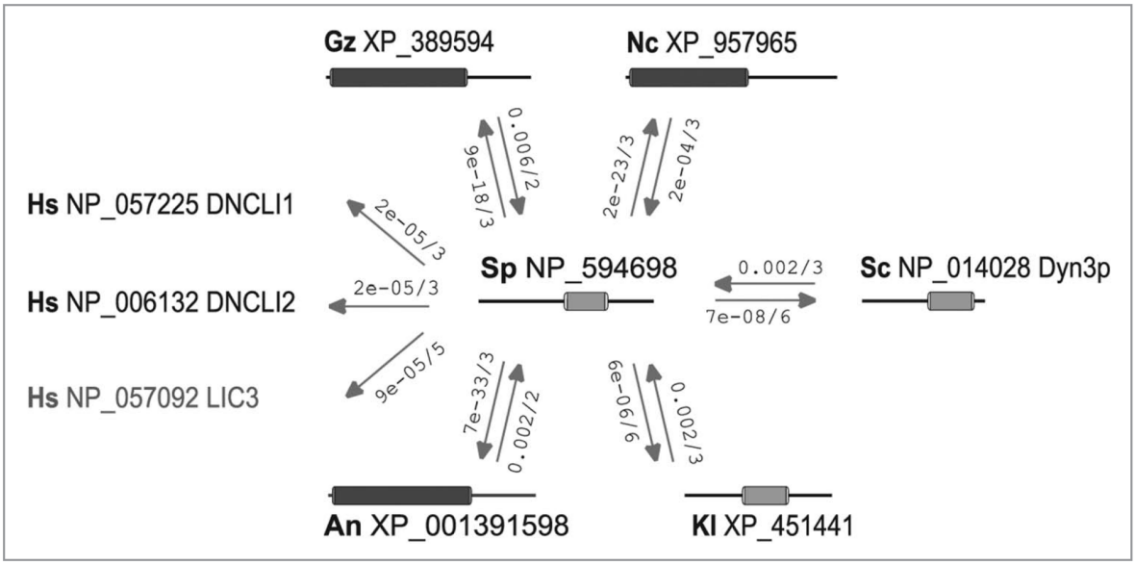


**Figure 2.** Dil1/SPAC458.04c shares similarity with dynein light intermediate chain proteins (DLIC). Schematic representation of bidirectional best PSI-BLAST similarities which were used to establish the homology of SPAC458.04c to fungal DLIC family members. The query database used is the NCBI non-redundant protein sequence database (Dec 2007) supplemented by the *Schizosaccharomyces octosporus* SPAC458.04c sequence (Wheeler *et al.*, 2000). Directed arrows mark significant PSI-BLAST links, with E-value and iteration round of the hit indicated alongside. Protein sequences are listed using a 2 letter species code followed by the NCBI accession number. Protein domain architecture corresponding to the concise result view of NCBI-CD-search is indicated for each protein. Dark grey boxes indicate DLIC/PF05783.2 domain hits found with highly significant E-values (E<1e-20) in fungal proteins which are known members of the DLIC family and are reciprocal best DNCLI1/2 proteome hits (more distant LIC3 shown in grey). Light grey boxes indicate borderline DLIC/PF05783.2 domain similarity with E-values between 0.005 and 0.10. Links of SPAC458.04c to non-fungal DLIC family members are exemplified for human. The observed transitive reciprocal best links from *S. pombe* over fungal DLIC to non-fungal DLIC are not shown. Species code: An *Aspergillus niger*, Gz *Gibberella zeae*, Kl *Kluyveromyces lactis*, Nc

*Neurospora crassa*, Hs *Homo sapiens*, Sc *Saccharomyces cerevisiae*, Sp *Schizosaccharomyces pombe*.

Members of the DLIC family are associated with cytoplasmic dynein which plays a central role in chromosome segregation during both mitosis and meiosis (Yoder & Han, 2001; Davis & Smith, 2005; Hook & Vallee, 2006; Chikashige *et al.*, 2007; Wickstead & Gull, 2007). Structural and sequence similarity analyses suggest that DLIC proteins are closely related to the structural family of G proteins, and might adopt a P-loop containing nucleoside triphosphate hydrolases-like fold (see the Materials and Methods and http://mendel.imp.ac.at/SEQUENCES/DLIC/). Given the similarity between the SPAC458.04c and DLIC proteins, we decided to call *SPAC458.04c* gene *dil1* (*d*ynein *i*ntermediate light chain - *l*ike *1*). Sequence alignments suggested that the N-terminus of the Dil1 may be longer than predicted (see Materials and Methods and Fig. S1). Consistent with this notion, our mass-spectrometry analysis of TAP-tagged Dil1 purified from cycling cells identified N-terminally acetylated peptide (MDELLEK) mapping to this region.

In synchronous *pat1*-induced meiosis, *dil1Δ* mutant cells underwent both meiotic divisions with kinetics similar to wild type (Fig. S2). Analysis of *lys1*-GFP dots in mature asci of strains carrying homozygous *lys1*-GFP indicated meiosis I homolog non-disjunction in *dil1Δ* mutant cells (Fig. 3A).

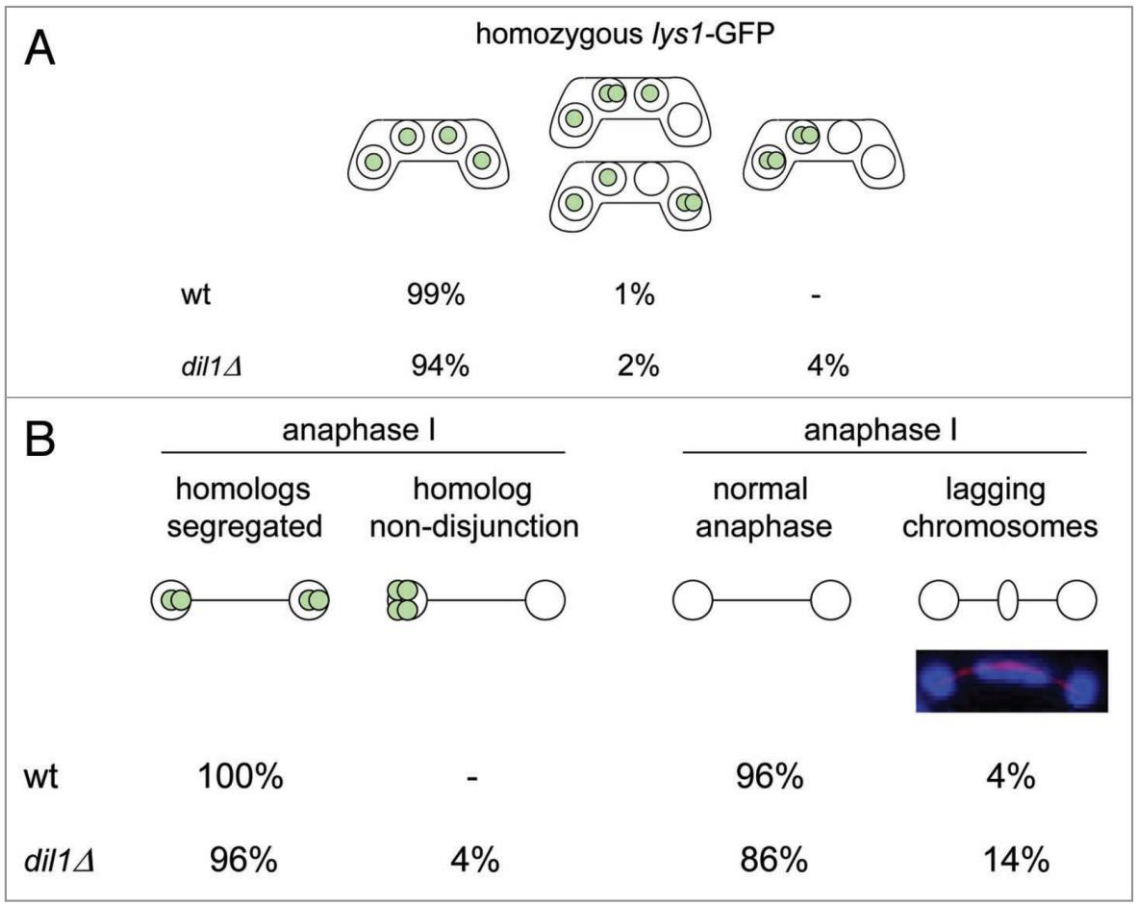


**Figure 3.** Dil1 is required for proper segregation of chromosomes during meiosis. (A) Meiotic segregation of chromosome I was scored in a wild-type $h^{90}$ *lys1*-GFP strain (K11248) and $h^{90}$ *lys1*-GFP strains carrying the knockout allele of *dil1* (*dil1Δ*) (JG14997). Unfixed cells were stained with Hoechst and examined under the fluorescence microscope. Chromosome segregation was scored in at least 100 asci. (B) Strains as described in (A) were fixed and immunostained for tubulin and GFP. DNA was visualized by Hoechst staining. 100 anaphase I cells were examined under the fluorescence microscope and segregation of chromosome I marked by *lys1*-GFP was scored.

To investigate chromosome segregation directly in anaphase I cells, we fixed cells and stained with antibodies against tubulin and GFP. During anaphase I, homologous centromeres in wild-type cells segregate to opposite poles. However, we frequently observed lagging chromosomes and homolog non-disjunction in *dil1Δ* anaphase I cells (Fig. 3B). Missegregation of homologs during meiosis I could be caused either by a defective recombination leading to a failure to produce chiasmata or by defective sister-chromatid cohesion along chromosome arms. To analyze sister chromatid cohesion, we used a strain in which both copies of chromosome II contained *cut3* sequences marked with GFP (*cut3*-GFP) (Nabeshima *et al.*, 2001). The strain expressed

a mutant version of cohesin's α-kleisin subunit (*rec8-RDRD*) that is resistant to separase cleavage and blocks meiotic divisions (Kitajima *et al.*, 2003). The strain also contained a *mes1-B44* mutation which prevents reaccumulation of the Cdc13 cyclin and any attempt to undergo meiosis II (Izawa *et al.*, 2005). This strain produces uninucleate meiotic cells with sister chromatids cohesed as indicated by one or two *cut3*-GFP dots. Defects in sister-chromatid cohesion along chromosome arms are expected to result in cells with more than two *cut3*-GFP dots. Indeed, almost 60% of cells contained more than two *cut3*-GFP dots in a strain deleted for *rec11*, which is known to negatively affect sister-chromatid cohesion along chromosome arms (Kitajima *et al.*, 2003). This indicates that the system is efficient in detecting defective cohesion. Consistent with previous reports, sister chromatid cohesion was intact in *rec12Δ* mutant cells that do not initiate meiotic recombination (Kitajima *et al.*, 2003; Gregan *et al.*, 2005). Deletion of *dil1* did not increase the appearance of cells with more than two *cut3*-GFP dots, suggesting that sister chromatid cohesion along chromosome arms is intact in these mutants (Fig. S3). Moreover, deletion of *dil1* permitted a small fraction of *mes1-B44* mutant cells to undergo nuclear division, which is consistent with the observed homolog non-disjunction phenotype (data not shown). We next compared meiotic recombination in wild-type and *dil1Δ* mutant cells.

**Table 1.** Meiotic recombination is reduced in the absence of *dil1+*

| **Intergenic recombination** | |
|---|---|
| Prototrophic recombinants/100 viable spores | *his4-lys4* |
| *dil1*$^+$ (JG14960 x JG14961) | 5 ± 0.4[a] |
| *dil1*Δ (JG15605 x JG15606) | 2.2 ± 0.4[a] |
| **Intragenic recombination** | |
| Prototrophic recombinants/100,000 viable spores | *ade6-M375* x *ade6-52* |
| *dil1*$^+$ (JG14960 x JG14961) | 4.5 ± 0.8[a] |
| *dil1*Δ (JG15605 x JG15606) | 1.6 ± 0.4[a] |
| **Spore viability** | |
| *dil1*$^+$ (JG14960 x JG14961) | 88% |
| *dil1*Δ (JG15605 x JG15606) | 65% |

[a]Standard error of the mean was calculated from three independent experiments.

Both intergenic and intragenic recombination was moderately reduced in *dil1Δ* mutant cells (Table 1). These data suggest that homolog non-disjunction observed in *dil1Δ* mutant cells is due to a defect in recombination but not sister-chromatid cohesion.

**Dil1 acts in the dynein pathway to promote oscillatory nuclear movement and efficient pairing of homologous centromeres during meiotic prophase**

Dynein-driven meiotic oscillatory nuclear movement is known to play a central role in recombination and pairing of homologs (Yamamoto *et al.*, 1999; Miki *et al.*, 2002; Ding *et al.*, 2004; Chikashige *et al.*, 2007; Vogel *et al.*, 2009). The nuclear movement during meiotic prophase in fission yeast results in a typical horsetail shape of nuclei.

**Table 2.** Nuclear morphology and number of *lys1*-GFP dots scored in uninuclear zygotes

| | Horsetail shape of the nucleus | Regular shape of the nucleus | 1 *lys1*-GFP dot | *2 lys1*-GFP dots |
|---|---|---|---|---|
| **wt** (K11248) | 74 % | 26 % | 54 % | 46 % |
| ***dil1*Δ** (JG14997) | 54 % | 46 % | 27 % | 73 % |

We observed a reduced number of horsetail nuclei as well as defect in pairing of homologous centromeres in *dil1Δ* mutant cells (Table 2) suggesting that Dil1 may be required for the horsetail nuclear movement during meiotic prophase. Live cell imaging showed that indeed, horsetail movement was impaired in 22 out of 24 *dil1Δ* mutant cells (Fig. 4).

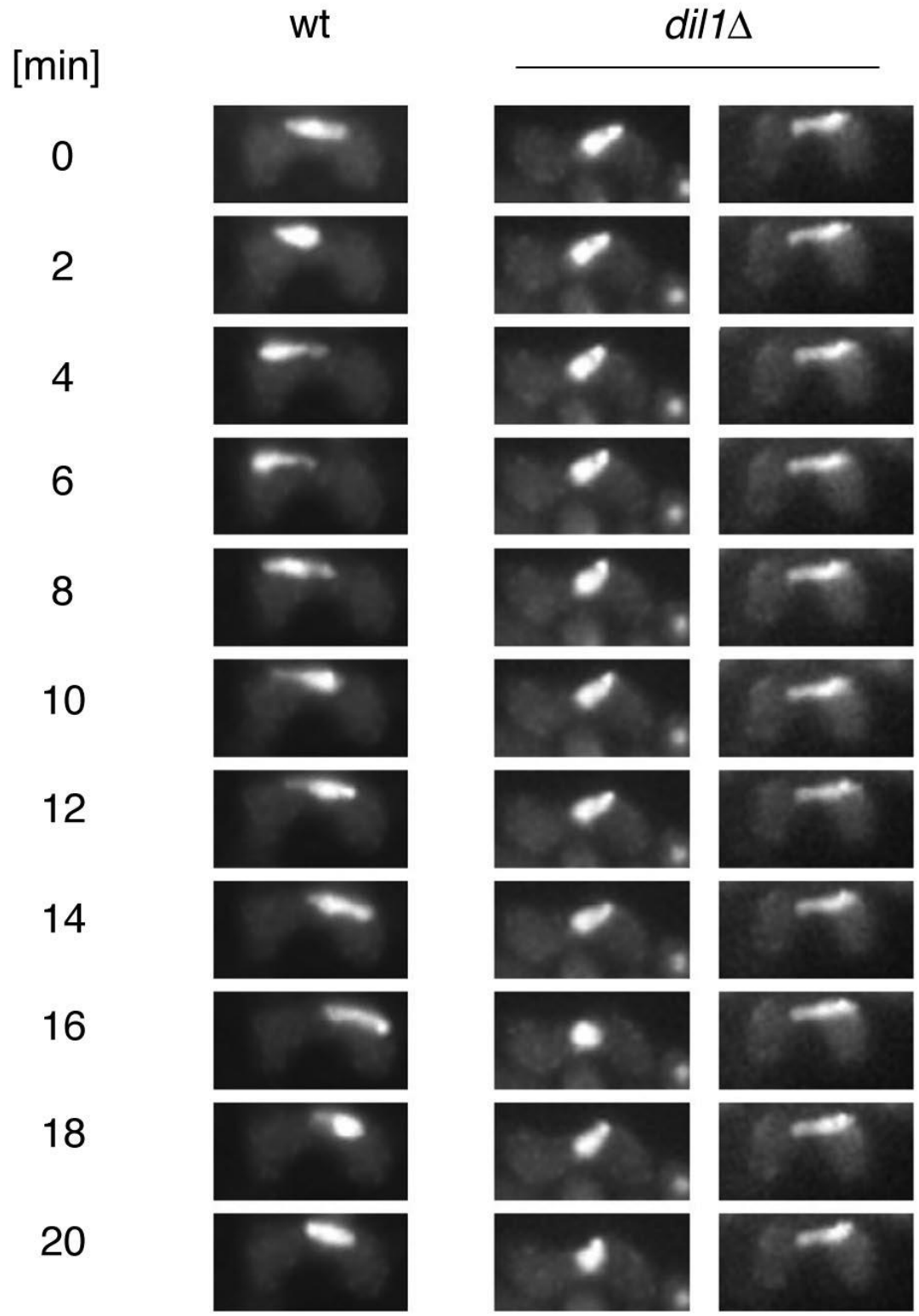


**Figure 4.** Dil1 is required for meiotic oscillatory nuclear movement. The dynamics of meiotic horsetail nuclei in the wild-type (K11248) and the *dil1Δ* cells (JG14997) (two examples are shown) was followed by time-lapse observation. The numbers indicate time in minutes. We observed impairment of the oscillatory nuclear movement n *dil1Δ* mutant cells, although some nuclei assumed the horsetail shape.

Cytoplasmic dynein is a microtubule motor that drives meiotic nuclear movement (Yamamoto *et al.*, 1999; Miki *et al.*, 2002; Moore *et al.*, 2009; Vogel *et al.*, 2009). The heavy chains of cytoplasmic dynein contain both ATPase and microtubule binding activities. We further asked whether Dil1 acts in dynein pathway by analyzing genetic interaction of the *dil1* with the *dhc1*, encoding dynein heavy chain (Yamamoto *et al.*, 1999; Davis & Smith, 2005). Homolog non-disjunction phenotype and nuclear morphology of the double mutant *dil1Δ dhc1Δ* resembled that of single mutants suggesting that the Dil1 acts in the same pathway as Dhc1 (Fig. 5).

| | anaphase I | | nuclear morphology | |
|---|---|---|---|---|
| | homologs segregated | homolog non-disjunction | horsetail shape | regular shape |
| |  |  |  |  |
| wt (K11338xK11321) | 99% | 1% | 71% | 29% |
| *dhc1Δ* (GP5117xGP5118) | 95% | 5% | 41% | 59% |
| *dil1Δ* (JG15071xJG15070) | 95% | 5% | 44% | 56% |
| *dhc1Δ dil1Δ* (JG15072xJG15069) | 95% | 5% | 51% | 49% |

**Figure 5.** Analysis of chromosome segregation in *dil1Δ dhc1Δ* double mutant. The indicated $h^-$ *lys1*-GFP strains were crossed to a corresponding $h^+$ *lys1*-GFP strains. Cells were fixed and stained with antibodies against tubulin and GFP. DNA was visualized by Hoechst staining. 100 anaphase I cells were examined under the fluorescence microscope and segregation of chromosome I marked by *lys1*-GFP was scored.

Similarly as the dynein complex, Dil1 protein localizes to the cytoplasm in cycling cells (Matsuyama *et al.*, 2006). We observed that Dil1-TAP localized to the leading edge of the horsetail nucleus, where the spindle pole body (SPB) is expected, and to the leading microtubules (Fig. S4). In cycling cells, Dil1-TAP formed foci which distributed asymmetrically during cell division (Fig. S4). A similar phenomenon has been described for the budding yeast dynein (Shaw *et al.*, 1997; Grava *et al.*, 2006). Thus, Dil1 and dynein display similar localization pattern, raising the possibility that Dil1 may directly interact with dynein. To analyze if Dil1 is physically associated with the dynein complex, we purifed Dil1-TAP from cycling cells. Mass-spectrometry revealed that Dil1-

TAP co-purified with several proteins whose function in nuclear movement has not been analyzed, but not with dynein (Table 3).

**Table 3.** List of proteins identified by mass spectrometry co-purifying with S. pombe Dil1-TAP

| Accession number | Description | Mascot score | Molecular weight [kDa] | Number of peptides assigned |
|---|---|---|---|---|
| 19115610 | Dil1 (sequence orphan) | 180 | 49.1 | 11 |
| 19115897 | multidomain vesicle coat component Sec16 | 138 | 218.3 | 8 |
| 19112952 | conserved fungal protein | 77 | 14.6 | 4 |
| 19115783 | GTPase activating protein Rga3 | 74 | 110.1 | 5 |
| 19112432 | AAA family ATPase Rvb2 | 65 | 51.7 | 2 |
| 19113091 | actin cytoskeletal protein Syp1 | 64 | 90.9 | 2 |
| 19112502 | sterol binding ankyrin repeat protein | 44 | 148.6 | 3 |
| 19115105 | dipeptidyl aminopeptidase | 41 | 46.1 | 2 |
| 19114566 | exocyst complex subunit Sec10 | 34 | 92.8 | 3 |

This suggests that Dil1 either does not bind to dynein or binds only loosely. Further analysis of proteins co-purifying with Dil1 will be important to fully understand the function of Dil1. Identification of proteins interacting with Dil1 during meiosis may shed light on the role of Dil1 in the horsetail nuclear movement. However, our attempts to purify Dil1-TAP from meiotic culture failed.

Taken together, we show that Dil1 acts in the dynein pathway to promote oscillatory nuclear movement which is essential for efficient pairing of homologous centromeres and recombination during meiosis.

**Discussion**

The strategy of knocking-out genes upregulated during meiosis proved to be efficient in identifying key regulators of meiotic chromosome segregation. In fission yeast, such strategy led to the identification of the long sought-after protector of centromeric cohesion Sgo1 and a new protein required for the initiation of meiotic recombination Mde2 (Rabitsch *et al.*, 2004; Gregan *et al.*, 2005). Martin-Castellanos et al. identified three mutants (*rec24*, *rec25*, and *rec27*) with reduced meiosis-specific DNA double-strand breakage, one mutant (*tht2*) deficient in karyogamy, and two mutants (*bqt1* and *bqt2*) deficient in telomere clustering (Martin-Castellanos *et al.*, 2005). A similar approach in budding yeast identified Mam1 component of the monopolin complex, Mnd1 recombination protein and Mnd2 subunit of the anaphase-promoting complex (APC/C) (Toth *et al.*, 2000; Rabitsch *et al.*, 2001; Rabitsch *et al.*, 2003). In our current study we report the identification of a novel gene, *dil1*$^{+}$, required for faithful segregation of chromosomes during meiosis. We establish that Dil1 acts in the dynein pathway to promote oscillatory nuclear movement, which is known to be required for pairing of homologous centromeres and recombination during meiosis. Recombination leads to formation of chiasmata which physically link homologous chromosomes. This linkage is essential for faithful segregation of chromosomes during meiosis I (Petronczki *et al.*, 2003). In many model organisms studied so far, disturbances in the recombination pathway are frequently associated with abnormalities in chromosome segregation at meiosis I (Hassold *et al.*, 2007). Defective recombination is therefore likely to be the cause of homolog non-disjunction observed in *dil1Δ* mutant cells. Our analysis revealed that Dil1 shares sequence similarity with dynein light intermediate chain proteins (DLIC), indicating that Dil1 may be a fission yeast counterpart of the DLIC proteins. Dynein-dependent nuclear movements play important roles in both

yeasts and higher eukaryotes (reviewed in (Tolic-Norrelykke; Yamamoto & Hiraoka, 2003; Gennerich & Vale, 2009; Moore *et al.*, 2009; Vallee *et al.*, 2009)), suggesting that the basic mechanism regulating dynein-dependent nuclear movements may be conserved in evolution. Finally, greater knowledge of the processes that promote proper segregation of chromosomes during meiosis might help us to understand the origins of human meiotic aneuploidy, the leading cause of miscariages and genetic disorders such as Down syndrome (Hassold *et al.*, 2007).

## Materials and Methods

### Strains, Media and Growth Conditions

The genotypes of *S. pombe* strains used in this study are listed in the Table S2. Genes were deleted according to knockout protocol described in (Gregan *et al.*, 2006). *S. pombe* media and growth conditions were as described in (Rabitsch *et al.*, 2004).

Spore viability and recombination analysis was performed as described in (Spirek et al.).

### Microscopy

The immunofluorescence and microscopy techniques used to analyze chromosome segregation were as described in (Rabitsch *et al.*, 2004).

### Live cell imaging

For time-lapse observations of living cells a Zeiss Axio Imager equipped with a digital spot camera (Visitron System) was used. Cells were sporulated on PMG-N plates for 20 hours, resuspended in 1µg/ml Hoechst 33342 and incubated for 15 min. 3µl of stained cells were mixed with 6µl of PMG-N medium containing 1% low melt agarose and mounted on a coverslip. The coverslip was placed on a microscope slide and sealed with hot Vaseline. Each time series of images was obtained with a 0.1-s exposure at 2 min intervals under low fluorescence excitation conditions to limit bleaching (BFP filter).

**Protein purification**

8-liter cultures of strains expressing TAP-tagged proteins were grown to log phase and the tagged proteins were isolated as described previously (Riedel *et al.*, 2006; Cipak *et al.*, 2009).

LC-MS/MS analysis was performed as described previously (Gregan *et al.*, 2007).

**Sequence Analysis and Protein Database Searches**

In the current *S. pombe* genome database (Hertz-Fowler *et al.*, 2004) SPAC458.04c is listed as an orphan sequence with no known sequence domains and a distinctive up-regulation during the meiotic cell cycle. While in the up-to-date NCBI non-redundant protein database (Wheeler *et al.*, 2000) SPAC458.04c indeed lacks clear homologs (with E<0.001), orthologs can be readily identified in the genomic sequence of *Schizosaccharomyces octosporus* and *Schizosaccharomyces sp. OY26* (www.broad.mit.edu). Using these orthologous sequences and a number of sensitive computational tools we can demonstrate the sequence similarity of SPAC458.04c to the family of dynein light intermediate chain (DLIC) proteins.

When applying the S. pombe SPAC458.04c protein sequence as a query to available profile-based similarity methods the following links to the dynein light intermediate chain (DLIC) family are obtained: 1) FFAS03 (Jaroszewski *et al.*, 2005) reports DLIC/PF05783.2 as the top and only profile with significant similarity to SPAC458.04c with a score of -10.7 (scores below -9.5 are considered significant by FFAS03) 2) HHpred (Soding *et al.*, 2005) and CD-search list the similarity to DLIC/PF05783.2 as the top, and by far best hit with E-value of 0.043 and 0.1, respectively. These initial indications of a SPAC458.04c-DLIC similarity are strongly reinforced, when the S. octosporus full-length homolog of SPAC458.04c is applied to FFAS03 and HHpred queries. In these searches DLIC/PF05783.2 is the top and only significant hit obtained with a FFAS03 score of -50 and E-value of 5.6e-44, respectively. Highly significant hits to the DLIC family are also obtained when using S. pombe SPAC458.04c itself in a PSI-BLAST search against the NCBI non-redundant protein sequence database (Dec 2007) supplemented by the S. octosporus SPAC458.04c. The first fungal DLIC homologs are retrieved in round 2 of the PSI-BLAST search, and are followed by various metazoan homologs in round 3. The PSI-BLAST remains in the DLIC family until its convergence in

round 11 and collects homologs from a broad range of taxa including apicomplexans, kinetoplastids, ciliates, green algae, cellular slime molds, trichomonads. Conservation within the family is particularly high in the central part of the proteins. An alignment of representative sequences of the fungal/metazoan DLIC group is shown in Fig. S1.

The de-novo gene prediction of S. OY26 SPAC458.04c extends further N-terminally than the publicly available S. pombe SPAC458.04c (NP_594698.1). The extended N-terminal segment of S. OY26 seems to be confirmed by similarity to other DLIC family members and can be used to extend the publicly available S. pombe SPAC458.04c protein sequence. While all sequence analyses were performed with the publicly available SPAC458.04c variant, the extended variant is shown in Fig. S1.

It has been previously noted that DLIC proteins show similarity to P-loop containing nucleoside triphosphate hydrolases (Grissom *et al.*, 2002). In addition the protein family DLIC/PF05783.4 has been assigned to the Pfam AAA Clan (CL0023) of P-loop containing nucleoside triphosphate hydrolases. For a more precise protein classification HHpred searches are performed with individual DLIC members against the hierarchically structured SCOP and NCBI-CD databases. PSI-BLAST iterations within HHpred are restricted to a minimal number ensuring that a DLIC-specific model is used by the method. HHpred searches against the SCOP database indicate that DLIC proteins belong to the structural family of G proteins (c.37.1.8). Notable among the top hits is the domain model of Spg1/Tem1 GTPases (cd04128) - a protein family that is also encountered in BLAST and PSI-BLAST searches using LIC3 family members. For further details and search results see http://mendel.imp.ac.at/SEQUENCES/DLIC/.

**Acknowledgments**

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**Supplementary Material**

**High-throughput knockout screen in *Schizosaccharomyces pombe* identifies a novel gene required for efficient homolog disjunction during meiosis I.**

Cornelia Rumpf, Lubos Cipak, Maria Novatchkova, Zhang Li, Silvia Polakova, Andrej Dudas, Ines Kovacikova, Eva Miadokova, Gustav Ammerer, and Juraj Gregan

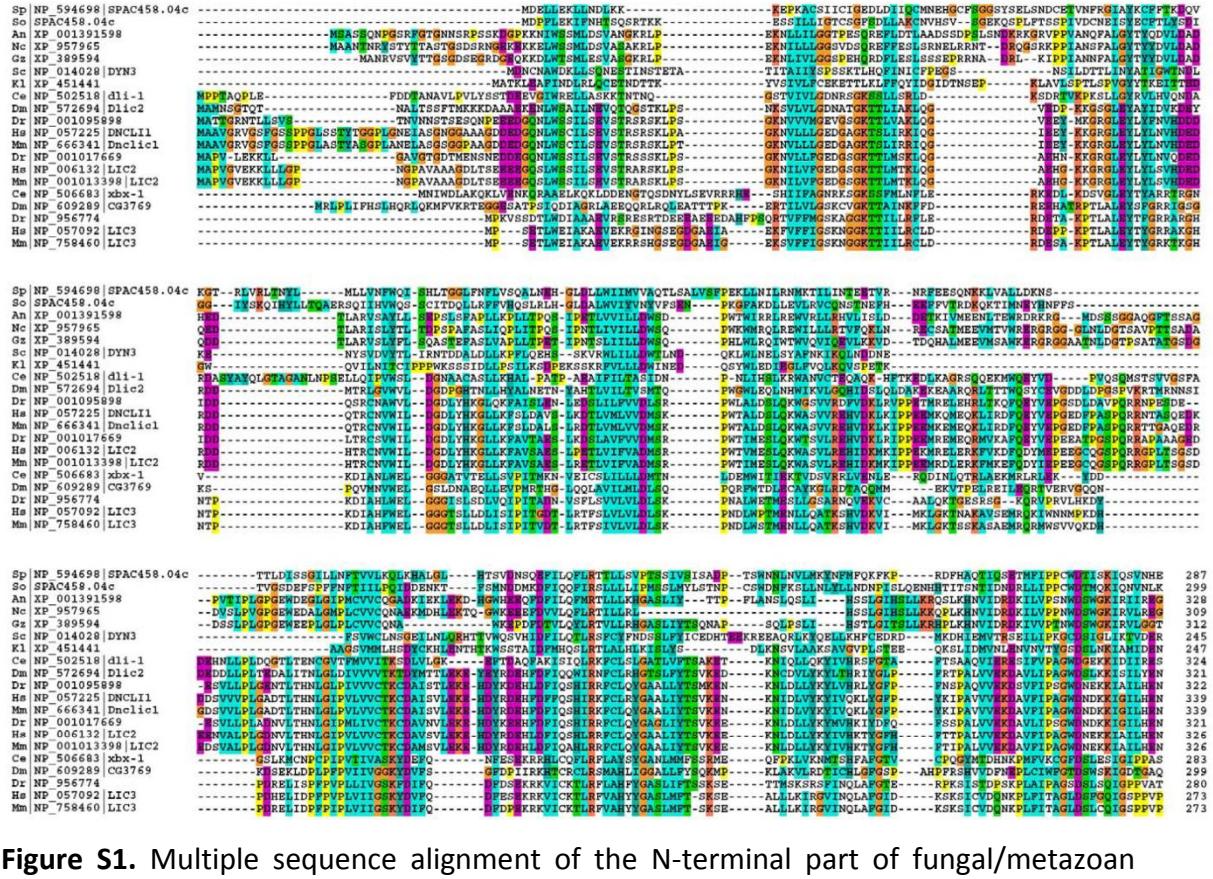

**Figure S1.** Multiple sequence alignment of the N-terminal part of fungal/metazoan DLIC homologs up-to the central region of highest conservation. The aligned protein segments represent the region in DLIC similar to P-loop containing nucleoside triphosphate hydrolases. Protein sequence identifiers are composed of a 2 letter species code, followed by a unique NCBI accession number for all sequences but the *S. octosporus SPAC458.04c*. NCBI gene names are indicated thereafter. Species code as in Fig. 2 and Ce *Caenorhabditis elegans*, Dm *Drosophila melanogaster*, Dr *Danio rerio*, Mm *Mus musculus*, So *Schizosaccharomyces octosporus*.

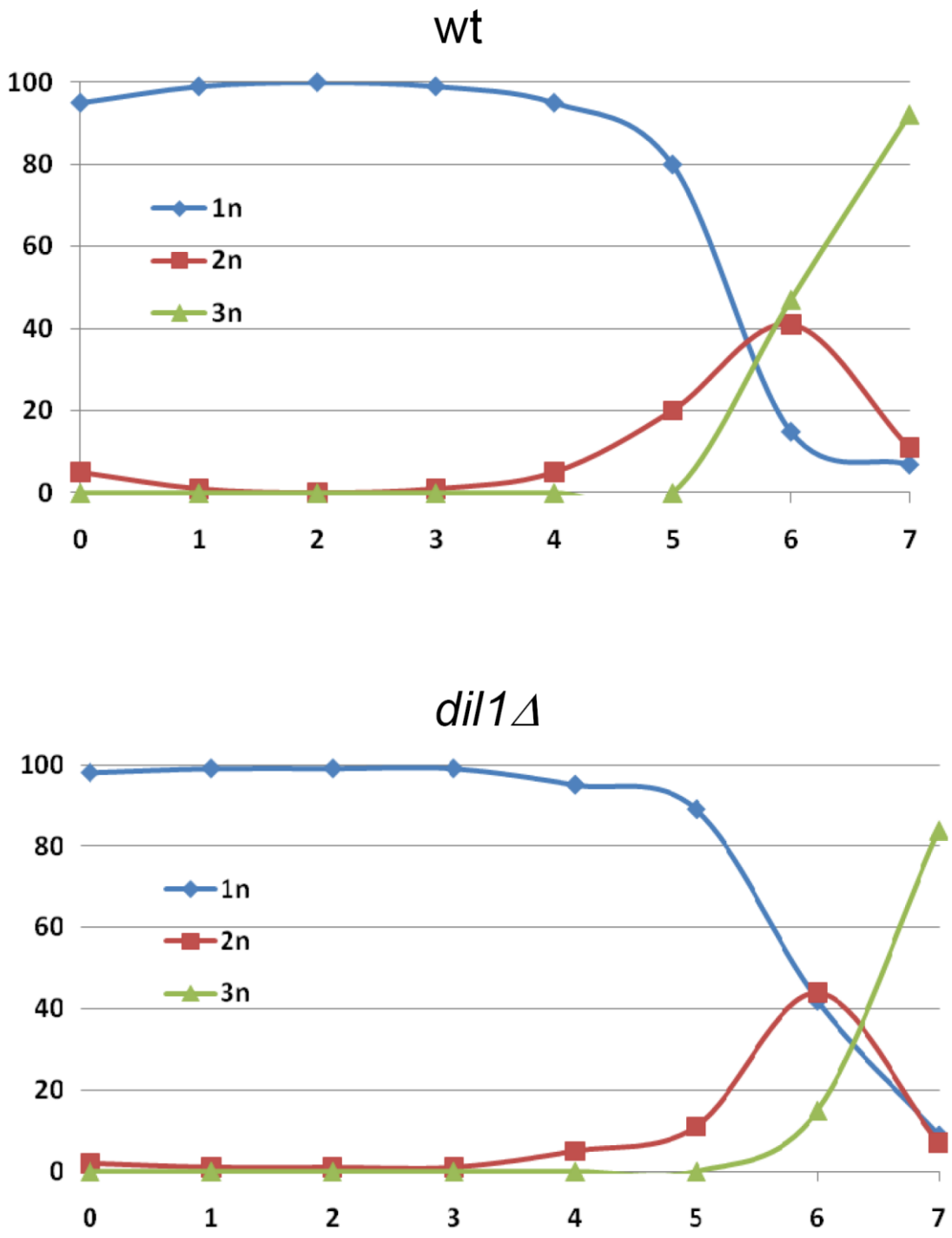


**Figure S2.** Progression of *pat1*-induced meiosis. Wild-type or *dil1Δ* mutant cells carrying *pat1-114* mutation (JG15602 and JG11322, respectively) were arrested by nitrogen starvation and released into meiosis at 34°C by inactivation of Pat1. Cells were harvested at the indicated time points after meiosis induction, stained with DAPI and nuclei were counted in 100 cells per time point. The fraction of cells that contained one nucleus (1n), two nuclei (2n) or more than two nuclei (3n) at the indicated time points are shown.

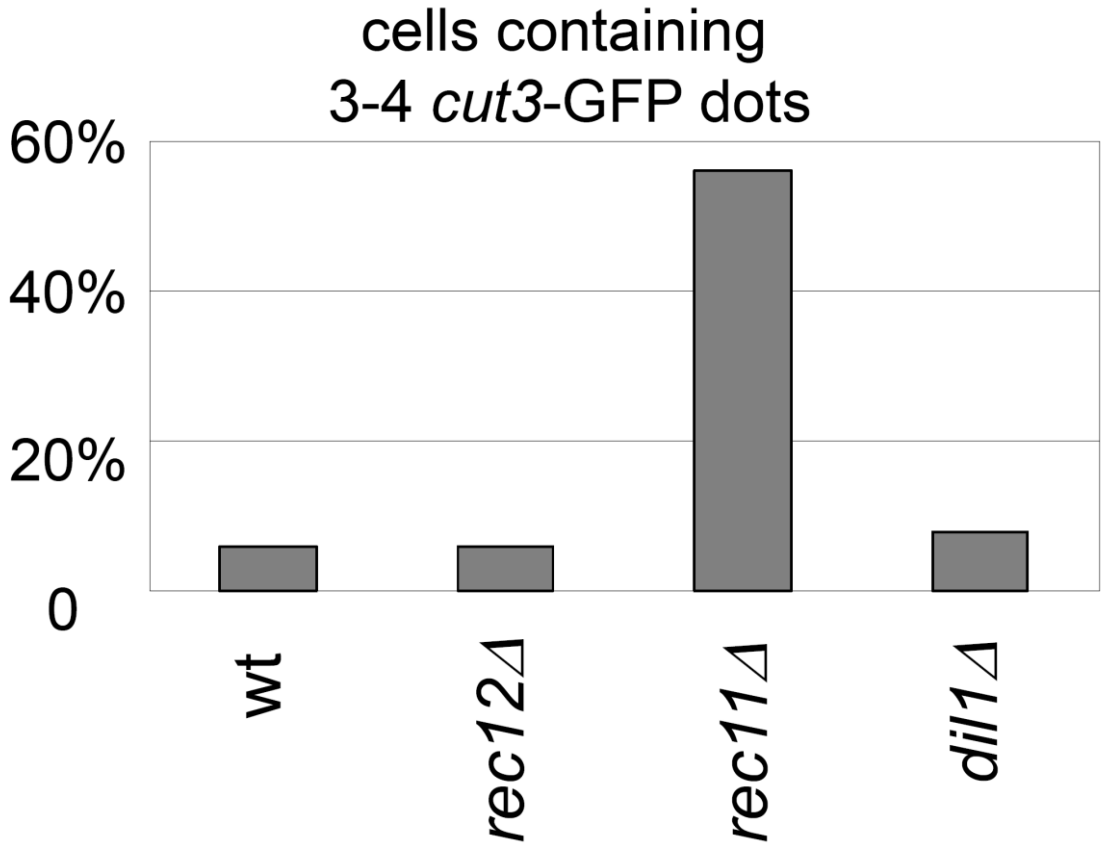


**Figure S3.** Sister-chromatid cohesion along chromosome arms is intact in *dil1Δ* mutant cells. $h^{90}$ *cut3*-GFP *rec8-RDRD mes1-B44* strains were either wild-type (wt) (K12924) or carried a knockout allele of either *rec12* (*rec12Δ*) (K12920), *rec11* (*rec11Δ*) (K12921), or *dil1* (*dil1Δ*) (JG14994). Strains were sporulated and examined under the fluorescence microscope. The percentage of cells containing more than two GFP dots, representing split sister chromatids at the *cut3* locus, was scored in at least 100 cells.

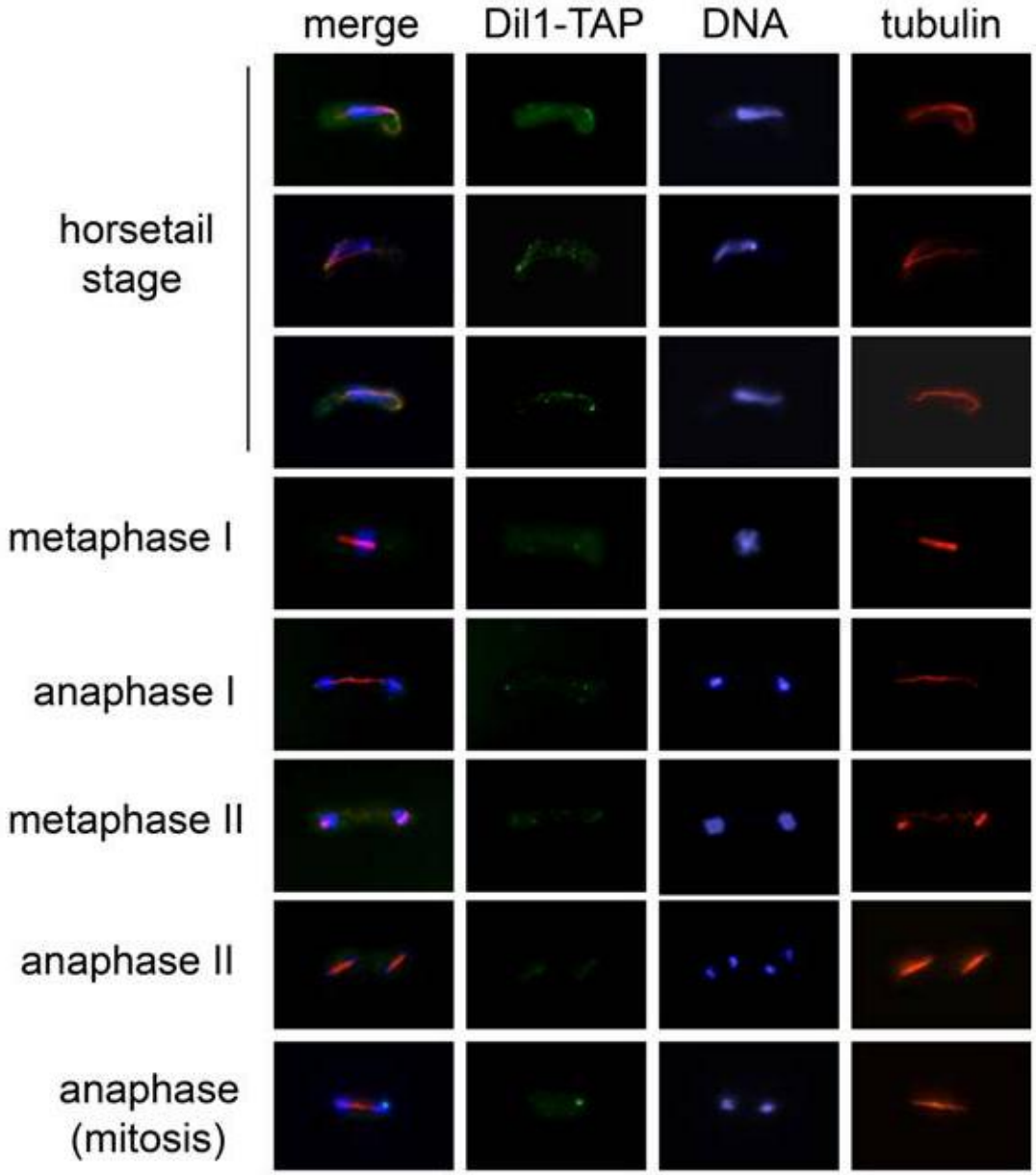


**Figure S4.** Localization of Dil1-TAP during meiosis and mitosis. Meiotic cells expressing Dil1-TAP (JG15110 x JG15608) were fixed and immunostained for tubulin and TAP. DNA was visualized by Hoechst staining. The following antibodies were used: TAT1 mouse monoclonal anti-tubulin (1:200) (K. Gull), rabbit polyclonal anti-TAP (CAB1001 - anti-TAP antibody 50ug (0.5mg/ml)) (1:500), secondary antibodies (anti-mouse-Alexa568 (1:500), anti-rabbit-Alexa488 (1:500)).

**Table S1.** List of selected genes.

| | | | |
|---|---|---|---|
| *SPBC3E7.13c* | *SPBC119.16c* | *SPAC6G10.03c* | *SPBC543.10* |
| *SPCC4G3.12c* | *SPBC36B7.02* | *SPBC2F12.15c* | *SPAC18G6.10* |
| *SPBC12C2.04* | *SPAC458.04c* | *SPAC1142.08* | *SPAC7D4.03c* |
| *SPCC16A11.16c* | *SPAPB1A10.14* | *SPCC16A11.04* | *SPAC8C9.06c* |
| *SPCC622.03c* | *SPCC191.04c* | *SPCC1795.08c* | *SPBC146.06c* |
| *SPBC30D10.09c* | *SPAP27G11.03* | *SPAC29E6.04* | *SPAC1006.04c* |
| *SPAC27E2.11c* | *SPAC6F6.12* | *SPAC30C2.08* | *SPBC29A10.02* |
| *SPCC663.15c* | *SPAC328.02* | *SPBC119.17* | *SPAC22A12.13* |
| *SPCC4F11.03c* | *SPCC1682.12c* | *SPAC20H4.01* | *SPCC1739.10* |
| *SPBC21C3.17c* | *SPCC11E10.05c* | *SPBC1921.05* | *SPAC22H12.01c* |
| *SPAC14C4.10c* | *SPCC1259.12c* | *SPCC4B3.04c* | *SPAC22F8.02c* |
| *SPAC23C11.12* | *SPBC13E7.05* | *SPCC16C4.01* | *SPBC146.10* |
| *SPACUNK4.15* | *SPBC30D10.08* | *SPAC694.03* | *SPAC1296.04* |
| *SPBC27B12.05* | *SPAC222.14c* | *SPBC31F10.10c* | *SPAC25H1.03* |
| *SPBC32H8.09* | *SPAC5D6.04* | *SPCC126.07c* | *SPAC24C9.05c* |
| *SPBC14C8.11c* | *SPAC105.02c* | *SPAC458.06* | *SPAC56E4.05** |
| *SPAC1142.09* | *SPAC1952.13* | *SPAC1782.08c* | *SPBC8D2.05C** |
| *SPCC188.07* | *SPAC23C11.04c* | *SPAC22F8.07c* | *SPAC8F11.06** |
| *SPCC162.06c* | *SPAC3G6.03c* | *SPAC23H3.05c* | *SPAC20H4.02** |
| *SPAC10F6.11c* | *SPAC12B10.01c* | *SPAC3H5.08c* | *SPAC7D4.11C** |
| *SPBC32F12.12c* | *SPBC4.03c* | *SPBC16E9.13* | |
| *SPBC31F10.17c* | *SPBC543.04* | *SPBC36B7.05c* | |
| *SPBP19A11.07c* | *SPAC3F10.07c* | *SPBC19C7.04c* | |

| | | | |
|---|---|---|---|
| *SPAC1782.04* | *SPAC589.06c* | *SPCC338.04* | |

* genes which resisted deletion

**Table S2.** Strain list.

| Strain | Genotype |
|---|---|
| K11248 | *h90 leu1 ura4 LacO-lys1+ GFP-LacI-his7+* |
| JG14997 | *h90 leu1 ura4 LacO-lys1+ GFP-LacI-his7+ SPAC458.04C::natMX* |
| K12924 | *h90 rec8::kanr<<ura4+<<Prec8-rec8-RDRD-FLAG<<kanr cut3+<<lacO his7+<<Pdis1-GFP-lacI-NLS mes1-B44 ade6 leu1 ura4-D18* |
| K12920 | *h90 rec8::kanr<<ura4+<<Prec8-rec8-RDRD-FLAG<<kanr cut3+<<lacO his7+<<Pdis1-GFP-lacI-NLS rec12-152::LEU2+ mes1-B44 ade6 leu1 ura4-D18* |
| K12921 | *h90 rec8::kanr<<ura4+<<Prec8-rec8-RDRD-FLAG<<kanr cut3+<<lacO his7+<<Pdis1-GFP-lacI-NLS rec11-156::LEU2+ mes1-B44 ade6 leu1 ura4-D18* |
| JG14994 | *h90 rec8::kanr<<ura4+<<Prec8-rec8-RDRD-FLAG<<kanr cut3+<<lacO his7+<<Pdis1-GFP-lacI-NLS mes1-B44 ade6 leu1 ura4-D18 SPAC458.04C::NatMX* |
| K11338 | *h- LacO-lys1+ GFP-LacI-his7+ leu1 ura4 ade6-210* |
| K11339 | *h+ lys1 his7 leu1 ura4 ade6-210* |
| K13790 | *h90 rec8::kanr cut3+<<lacO his7+<<Pdis1-GFP-lacI-NLS mei4-P572 ade6 leu1 ura4-D18 rec8+ura3* |
| K13755 | *h90 rec8::kanr cut3+<<lacO his7+<<Pdis1-GFP-lacI-NLS mei4-P572 ade6 leu1 ura4-D18* |
| JG15000 | *h+ ade6-M210 ura4-D18* |
| JG15003 | *h+ ade6-M210 ura4-D18 SPAC458.04c::natMX* |
| JG15004 | *h- ade6-M216 ura4-D18* |
| JG15007 | *h- ade6-M216 ura4-D18 SPAC458.04c::natMX* |
| JG15068 | *h- leu1-32 lacO-lys1 GFP-LacI-his7 ura4D18 dlc1::ura4 SPAC458.04c:: natMX* |

| | |
|---|---|
| JG15069 | *h+ leu1-32 lacO-lys1 GFP-LacI-his7 dhc1::kanMX SPAC458.04c:: natMX* |
| JG15070 | *h+ leu1-32 lacO-lys1 GFP-LacI-his7 SPAC458.04c::natMX* |
| JG15071 | *h- leu1-32 lacO-lys1 GFP-LacI-his7 SPAC458.04c::natMX* |
| JG15072 | *h- leu1-32 lacO-lys1 GFP-LacI-his7 dhc1::kanMX SPAC458.04c::natMX* |
| JG11322 | *h- pat1-114* |
| JG15602 | *h- pat1-114 dil1::natMX* |
| JG14960 | *h+ ade6-M375 his4-239* |
| JG14961 | *h- ade6-52 lys4-94* |
| JG15605 | *h+ ade6-M375 his4-239 dil1::natMX* |
| JG15606 | *h+ ade6-52 lys4-94 dil1::natMX* |
| JG 15110 | *h- dil1-TAP::kanMX, leu1-32 ura4-D18 ade6-M210* |
| JG 15608 | *h+ dil1-TAP::kanMX* |
| K11321 | *h+ leu1-32 ura4-D18 lacO-lys1 GFP-LacI -his7* |
| GP4957 | *h+ leu1-32 lacO-lys1 GFP-LacI-his7* |
| GP4958 | *h- leu1-32 lacO-lys1 GFP-LacI-his7* |
| GP5117 | *h- leu1-32 lacO-lys1 GFP-LacI-his7 dhc1::kanMX* |
| GP5118 | *h+ leu1-32 lacO-lys1 GFP-LacI-his7 dhc1::kanMX* |

## 4.1 Contributions

| | |
|---|---|
| **Figure 1** | Zhang Li, Juraj Gregan |
| **Figure 2** | Maria Novatchkova |
| **Figure 3** | Cornelia Rumpf, Juraj Gregan |
| **Figure 4** | Cornelia Rumpf |
| **Figure 5** | Cornelia Rumpf, Ines Kovacikova |
| **Figure S1** | Maria Novachkova |
| **Figure S2** | Cornelia Rumpf |
| **Figure S3** | Juraj Gregan |
| **Figure S4** | Andrej Dudas |
| **Table 1** | Silvia Polakova |
| **Table 2** | Cornelia Rumpf, Ines Kovacikova |
| **Table 3** | Lubos Cipak |

# 5. Discussion

The major aim of this thesis was to uncover new factors and mechanisms required for a correct segregation of chromosomes. Chromosome segregation is an important process in both mitosis and meiosis. The consequences of incorrect chromosome segregation are cells with unequal amounts of DNA, called aneuploidy. Mitotically dividing aneuploid cells are hallmarks of many cancers. During meiosis, errors in chromosome segregation can lead to infertility, miscarriages and a variety of genetic diseases such as the Down Syndrome.

Due to the extreme importance of this topic, it has been studied extensively in the recent past. Hence, in the last part of my thesis I want to discuss my own results in the context of most recent findings from other laboratories and outline open questions.

## 5.1 Conservation of the monopolin complex

During mitosis in higher eukaryotes, like in *S. pombe*, multiple microtubules are attached to a single kinetochore. Therefore, there must be factors preventing merotelic attachments and promote syntelic kinetochore orientation in higher eukaryotes as well. The factors ensuring syntelic attachments are only poorly characterized so far but it is likely that the basic principle of a molecular clamp holding together microtubule attachment sites is conserved between the fission yeast and higher eukaryotes. Unfortunately we failed to identify clear homologs of Pcs1 and Mde4 in higher eukaryotes so far. The most promising candidates are the proteins Spc24 and Spc25. They are part of the highly conserved Ndc80 complex and required to establish and maintain kinetochore-microtubule attachments (McCleland *et al.*, 2004). Although the sequence identity between Spc24/25 and Pcs1 or Csm1 is quite low (Rumpf *et al.*, 2010) recent structural analysis showed that Spc24 and Spc25 form a heterodimer similar to the Csm1 homodimer (Corbett *et al.*, 2010).

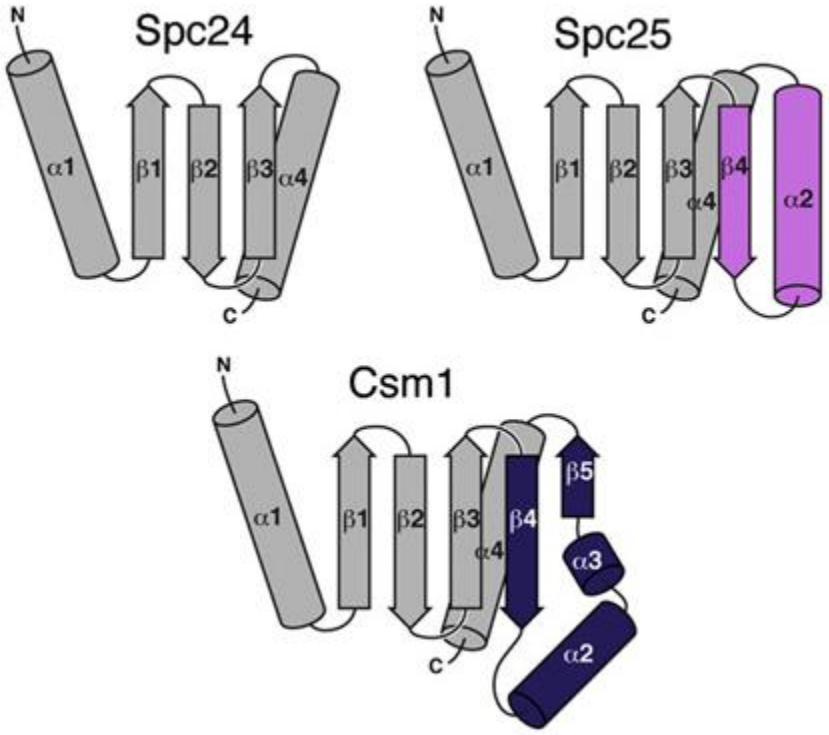


**Figure 5.1** The secondary structure of Spc24, Spc25 and Csm1. Csm1 as well as Pcs1 are structural relatives of Spc24 and Spc25. All four proteins share significant features like an N-terminal coiled coil domain followed by a globular domain. Their common fold is shown in grey. Figure taken from (Corbett *et al.*, 2010)

These recent findings support our hypothesis that the Spc24/25 and the Pcs1 may have a common evolutionary origin (Rumpf *et al.*, 2010).

Although the Csm1/Lrs4 complex in *S. cerevisiae* is absolutely essential for the mono-orientation during meiosis I (Rabitsch *et al.*, 2003), we could clearly show that the Pcs1/Mde4 complex in *S. pombe* is only required during Meiosis II and Mitosis (Gregan J, 2007). During budding yeast mitosis and meiosis II, only a single microtubule is attached to one kinetochore. Thus *S. cerevisiae* does not require a mechanism for clamping together multiple microtubule attachment sites. The only situation when those cells have to handle such multiple attachment sites is during meiosis I, when the two sister kinetochores have to act as one unit (Rabitsch *et al.*, 2003; Gregan J, 2007). Nevertheless, the question how monopolar attachment is established during meiosis I in the fission yeast is still open. Using genetic screens in order to identify new key players for meiosis I will help to solve that puzzle in the near future.

## 5.2 The role of Mde4 phosphorylation and dephosphorylation

We could clearly show that the phosphorylation of Mde4 is important for its function but not for its localization to kinetochores. We suggest that the phosphorylation is required for the self-association of Mde4 in order to build a clamp which holds together microtubule attachment sites (unpublished data). During the course of this work Choi *et al.* published similar results (Choi *et al.*, 2009). They could show as well that Cdk1 is the master kinase responsible for the Mde4 phosphorylation. Mutating the potential Cdk1 sites to alanine and thereby creating a non-phosphorylatable version of Mde4, they clearly showed that the phosphorylation of Mde4 is essential for correct chromosome segregation during mitosis. However, in contrast to our findings, they claim that the phosphorylation of Mde4 is not only important for its function but also for its localization to kinetochores. In order to resolve this discrepancy, further studies will be necessary. We have already tried to obtain artificial Mde4 dimers using Mde4-FKBP in combination with a small molecular dimerizer (ARGENT™ Regulated Homodimerization Kit from Ariad). Showing that the non-phosphorylatable version of Mde4 can prevent merotelic attachments after artificial dimerization, would give us an indirect evidence that the phosphorylation is important for the Mde4 self-association. Unfortunately, we failed to obtain functional Mde4 homo- or oligomeres (data not shown).

During late anaphase Mde4 becomes dephosphorylated and localizes to the spindle. Choi *et al.* showed that Mde4-GFP coimmunoprecipitated with the substrate-trapping mutant, Clp1-C286S-13Myc. Clp1 is a phosphatase which dephosphorylates sites phosphorylated by Cdk1. Moreover, they showed that Clp1 can dephosphorylate Mde4 *in vitro*. Still their data suggests that other phosphatases play a crucial role in dephosphorylation of Mde4 since in the absence of Clp1, Mde4 still becomes dephosphorylated *in vivo* as these cells exit mitosis (Choi *et al.*, 2009). Finding the master phosphatase will be one of the future aims and will help to uncover the regulation of the Pcs1/Mde4 complex.

## 5.3 Casein Kinase 1 is responsible for the phosphorylation of Rec8

We could clearly show that the Casein Kinase 1 (Hhp1/Hhp2 in *S. pombe*) is the kinase which phosphorylates Rec8 (Rumpf *et al.*, 2010). The phosphorylated Rec8 is cleaved

during meiosis I in order to resolve chiasmata and segregate the homologs. The phosphatase PP2A dephosphorylates the centromeric fraction of Rec8 in order to prevent it from cleavage. This protection of centromeric cohesin is important for proper segregation of sisters during meiosis II. The results of our work were confirmed in two recent publications. It was shown that in *S. cerevisiae* the casein kinase CK1δ/ε and the Dbf4-dependent Cdc7 kinase (DDK) are required for the efficient removal of Rec8 from chromosome arms (Katis *et al.*, 2010). In a nice and elegant way Katis *et al.* demonstrated that it is indeed phosphorylation produced by CK1 and DDK rather than by PLK1 which is required for the cleavage of Rec8. They postulate that PP2A protects the centromeric cohesin by the dephosphorylation of Rec8 directly and thereby counteracting CK1 and DDK.

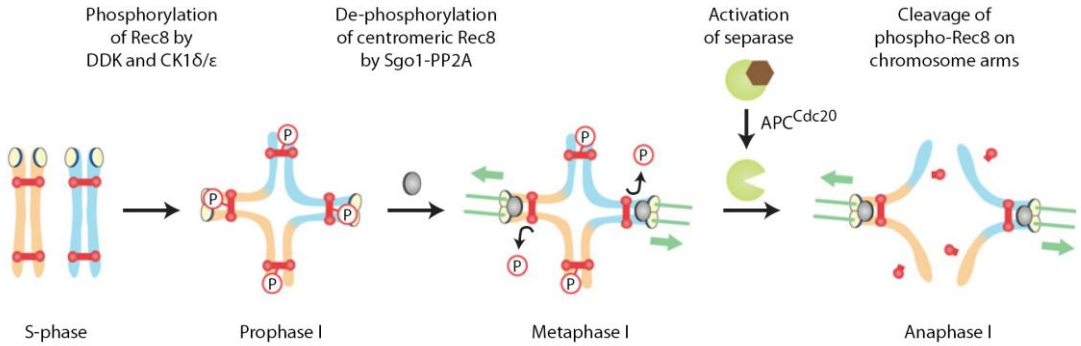


**Figure 5.2** A model – The phosphorylation of Rec8 by CK1 and DDK is counteracted by the dephosphorylation of Rec8 by PP2A at the centromeres. Figure taken from (Katis *et al.*, 2010)

Another recent publication strengthened our findings that in *S. pombe* the two casein kinases Hhp1 and Hhp2 are the key players in the phosphorylation of Rec8 (Ishiguro *et al.*, 2010). The fact that the mechanism of Rec8 phosphorylation is similar in two very distinct yeast species raises the possibility that it might be evolutionary conserved.

Still it could not be shown that Rec8 is a direct target of PP2A. Another possibility is that PP2A inhibits CK1 and thereby prevents the phosphorylation and subsequently the cleavage of centromeric Rec8.

One of the most important questions is how Sgo1 and PP2A lose their ability to protect centromeric cohesin during meiosis II. In *S. pombe* Sgo1 is inhibited by *Sgo1*'s 3'UTR in meiosis II (Rabitsch *et al.*, 2004). However, even after artificial expression of Sgo1 without its 3'UTR cells underwent both meiotic divisions without significant delay

(Gregan *et al.*, 2008). These questions will be the concern of several laboratories in the near future.

## 5.4 Dil1 acts in the dynein pathway

Using a high throughput knock out screen we could identify a novel protein required for efficient homolog disjunction during meiosis I. During the course of this work we showed that the Dil1 shares similarity with dynein light intermediate chain proteins (DLIC) and that it is essential for the oscillatory movement of the nucleus during meiotic prophase I. These observations lead to the assumption that Dil1 acts in the dynein pathway. Although we observed a similar localization pattern for Dil1 as it is described for dynein we were not able to co-purify Dil1 together with subunits of the dynein complex. Recently it was shown that Dil1 is indeed a subunit of the dynein complex (Fujita *et al.*, 2010). Fujita *et al.* were able to co-purify Dil1 together with the dynein heavy chain and therefore showed that the two proteins do not only act in the same pathway but also form a complex. Moreover they suggest that Dil1 is required for the localization of Dhc1 to microtubules. It will be interesting to find out how these two proteins interact. We could show that deletion of both Dhc1 and Dil1 shows only weak chromosome missegregation which is similar to the phenotype of Dil1 and Dhc1 single knock outs. These observations support the model that this two proteins act in the same pathway. Interestingly, in cells lacking both Dil1 and Dlc1, encoding for the dynein light chain, homolog nondisjunction occurs at a significantly higher level (our unpublished data). This observation is consistent with the finding that telomere clustering in *dhc1 dlc1* double mutants is severely impaired while it is only slightly impaired in single mutants and with the suggestion that DLC has some dynein-independent functions (Yamamoto *et al.*, 1999; Miki *et al.*, 2002) reviewed in (Yamamoto & Hiraoka, 2003). Due to the fact that dynein functions are evolutionary conserved, more detailed studies of this dynein sub-complexes will give insights into the molecular functions of these proteins in higher eukaryotes as well.

Taken together, there is a big variety of proteins involved in many different pathways required for proper chromosome segregation. We were able to identify several new

key players which will help to shed some light on the matter and get a bigger picture of how the different pathways are regulated and work together.

Still there are lots of gaps remaining that have to be filled in order to gain greater knowledge of the processes that promote proper segregation of chromosomes during meiosis and mitosis. This is essential to understand the origins of a variety of human diseases.

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# 8. Curriculum Vitae


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***Education***

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| 2011 | Estimated Graduation |
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| 2006 | Graduation |
| 2004 -2006 | Master thesis with Univ. Prof. Dr. Kai-Uwe Fröhlich at the Karl Franzens University, Graz<br>Title: The effect of selective amino acid starvation on apoptosis in respiration competent and deficient yeast cells |
| 2000 -2006 | Study of Microbiology at the Karl Franzens University, Graz |
| 1999 - 2000 | Study of Economy at the Karl Franzens University, Graz |
| 1991 - 1999 | BG/BRG Köflach (Matura: 18.06.1999) |

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| September 2008, January 2009 and May 2009 | Practical training in the Lab of Iva Tolic (Max Planck Institut, Dresden) |
| November 2006 | Practical training in the Lab of Stephen Kearsey (University of Oxford) |

***Other Experience***

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| February 2008, 2009, and 2010 | Teaching practical courses at the University of Vienna |
| 2009 | Co-worker at Dialog Gentechnik |
| August 2007 | Part of the organisational committee of the FEBS conference |

***Scholarships***

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| 2009 | Forschungsstipendium from the University of Vienna |

***Memberships***

ÖGMBT (Austrian Association of Molecular Life Sciences and Biotechnology)

# 9. Publication List

1.) Rumpf C, Cipak L, Schleiffer A, Pidoux A, Mechtler K, Tolić-Nørrelykke I, Gregan J.
Laser microsurgery provides evidence for merotelic kinetochore attachments in fission yeast cells lacking Pcs1 or Clr4. *Cell Cycle,* 2010 Oct3; 9(19)
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13.) Gregan, J. and C. Rumpf,
How might DNA enter the cohesin ring? *Cell Cycle*, 2006. **5**(22): p. 2553

## Posters

1.) Pcs1/Mde4 complex is required to prevent merotelic kinetochore orientation.
Cornelia Rumpf, Alison Pidoux, Robin Allshire, Kim Nasmyth, Iva Tolic-Norrelykke and Juro Gregan
The EMBO Meeting 2009

2.) Pcs1 and Mde4 are required to prevent merotelic kinetochore orientation.
Cornelia Rumpf, Juraj Gregan, Christian G. Riedel, Alison L. Pidoux, Yuki Katou, Alexander Schleiffer, Robin C. Allshire, Stephen E. Kearsey, Katsuhiko Shirahige, Kim Nasmyth
35th Annual Conference on Yeasts 2007

3.) A screen to identify yeast genes involved in apoptosis.
Alexandra Schauer, Christoph Ruckenstuhl, Heide Knauer, Heike Fussi, Elisabeth Hulla, Laura Leitner, Cornelia Rumpf, Julia Spielhofer, Kai-Uwe Fröhlich
XXIIth International Conference on Yeast Genetics and Molecular Biology 2005

## Talks

1.) Casein kinase 1 is required for efficient removal of Rec8 during meiosis I
Cornelia Rumpf, Miroslava Pozgajova, Juraj Gregan
38th Annual Conference on Yeasts 2010

2.) Pcs1/Mde4 complex clamps together microtubule attachment sites
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