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The Characterization of the Roles of Ipl1 and Cdc5 in Spindle Pole Body Duplication in Yeast Meiosis

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THE CHARACTERIZATION OF THE ROLES OF IPL1 AND CDC5 IN SPINDLE POLE BODY DUPLICATION IN YEAST MEIOSIS

By

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I dedicate this to Ayn Rand
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LIST OF ABBREVIATIONS AND SYMBOLS

SPB: Spindle Pole Body
MTOC: Microtubule Organizing Center
MT: Microtubules
WT: Wild-Type
MI: Meiosis I
MII: Meiosis II
GFP: Green Fluorescence Protein
ABSTRACT

The centrosome is the microtubule organizing center (MTOC) in higher eukaryotes. During meiosis, proper duplication and separation of the centrosomes are necessary for accurate chromosome segregation and leads to the production of gametes containing half of the parental genome. During meiotic interphase I, centrosomes are duplicated when chromosomes replicate. A pair of centrosomes establish a bipolar microtubule spindle that facilitates the segregation of homologs during meiosis I. Centrosomes duplicate once more at interphase II, when DNA duplication is absent, and form two independent spindles for sister chromatid separation during meiosis II. The centrosome in yeast is called the spindle pole body (SPB). Here we show that the Aurora Kinase Ipl1, which protects sister chromatid cohesion, is also required for the maintenance of a tight association between duplicated sister SPBs, referred to here as SPB cohesion. Premature loss of cohesion leads to SPB over-duplication and the formation of multipolar spindles during meiosis II. The Polo-like kinase Cdc5 is a licensing factor for SPB duplication at interphase II and promotes SPB separation during meiosis II. Our data suggests Ipl1 and Cdc5 interact antagonistically at the SPB to maintain proper duplication and separation of SPBs during meiosis.
CHAPTER ONE

INTRODUCTION

1.1. Meiosis and Spindle Pole Body Duplication

The centrosome is the microtubule-organizing center (MTOC) in higher eukaryotes, which nucleates microtubules to form a bi-polar spindle during meiosis, except in oogenesis. During meiosis, the proper duplication and separation of centrosomes is necessary for accurate chromosome segregation, and leads to the production of gametes containing half of the parental genome. As DNA begins to replicate, the parental centrosome duplicates into two sister centrosomes during interphase I. The sister centrosomes begin to separate during meiosis I (MI) by establishing a bi-oriented microtubule spindle which facilitates the segregation of homologs to the opposite poles. Sister centrosomes duplicate once more before meiosis II (MII), during interphase II, which occurs in the absence of DNA replication. Two independent spindles are established to facilitate the separation of centrosomes and segregation of sister chromatids during MII.

In yeast, the MTOC is referred to as the spindle pole body (SPB); it shares structural and functional components with the vertebrate centrosome (Adams and Kilmartin, 2000). Structurally and conceptually, the yeast SPB is best known from vegetative cells (Adams and Kilmartin, 1999; Jaspersen and Winey, 2004). It is embedded in the nuclear envelope as a structure comprised of an inner, core, and outer plaque-like layers (Moens and Rapport, 1971; Byers and Goetsch, 1975).

Cytological observations suggest that duplication of SPBs begin with the elongation of a protenacious membrane structure, called the half bridge. The half bridge is known to consist of the calmodulin-like centrin homolog Cdc31 and Sfi1 (Kilmartin, 2003), and is tethered to the SPB core through its interaction between integral membrane proteins Mps3 and Mlp2. A satellite SPB forms de novo and is comprised of a few SPB components, including Spc42, a core plaque component and the satellite SPB is then deposited onto the distal end of the half bridge which doubles in length. The satellite then matures to form into a duplication plaque and is subsequently inserted into the nuclear membrane. It has been suggested the half bridge retracts away from the nuclear membrane so the newly formed duplication plaque can interact with the nucleoplasm, assemble nuclear SPB components, and assemble into a mature SPB (Adams and
Kilmartin, 1999). Sfi1 associates with Cdc31 through a series of conserved coiled-coil domains, regulating the elasticity and retactability of the half bridge (Li et al., 2006). It has also been suggested that Kar1, another known component found at the cytoplasmic face of the SPB half bridge, (Spang et al., 1995) and along with Mps3, a nuclear envelope protein, have a role in the assembly of the physical half bridge structure. Together these bridge components; Sfi1, Cdc31, Mps3, and Kar1 may play important roles in regulation of SPB duplication and SPB maturation.

After half-bridge formation and SPB duplication, sister SPBs begin to separate as the bridge is severed, which occurs upon the formation of a bipolar spindle (Adams and Kilmartin, 2003). Studying SPB dynamics during meiosis allows a novel approach to study SPB cohesion, in addition to SPB duplication and separation.

### 1.2 The Aurora Kinase Ipl1

Ipl1 is the founding member of the Aurora kinase family. It was first identified during a genetic screen looking for mutations that led to increased ploidy levels, and it is a chromosomal passenger protein (Chan and Botstein, 1993). In mammals, there are three Aurora kinases that have been identified; Aurora A, B, and C, each having different functions in the cell (Nigg, 2001). Aurora A has been shown to be predominately located at the poles of the cell in vertebrates, where it localizes to the centrosomes immediately after duplication occurs thus mediating centriole cohesion and maintaining the bipolarity of the spindle (Barr and Gergely, 2007; Lukasiewicz and Lingle, 2009). Aurora B has been found to regulate mitotic and meiotic chromosomal segregation, associating with the microtubules, and in yeast, Ipl1 has been shown to be analogous to Aurora B. During yeast meiosis, Ipl1 activity has been previously characterized and was shown to have a role in chromosome movement and segregation by promoting accurate segregation of homologs in meiosis I, in addition to preventing the precocious separation of sister chromatids during meiosis I through its role in maintaining PP2A at the kinetochore. (Monje-Casas et al. 2007; Yu and Koshland 2007).

The analogous roles of Aurora B and Ipl1 in mediating chromosome segregation, has led to our interest in other roles Ipl1 may have during meiosis, due to the fact it is the only known Aurora kinase in yeast. These previous studies led us to hypothesize that Ipl1 may play a role at the SPB, regulating SPB cohesion and duplication, that is similar to role of Aurora A at the centrosome.
1.3 Polo-Like Kinase Cdc5

The polo-like protein kinases are a conserved subfamily of serine/threonine protein kinases, and is named Cdc5 in budding yeast. The polo box is a distinct region of homology in the C-terminal noncatalytic domain. It is known that there are two Cdc5 PBDs, and it has been shown that it is critical for Cdc5 localization to the SPB during mitosis (Song et al., 2000). For SPB separation to occur, the bi-polar spindle forms after the SPB half bride structure is severed, and this action has been shown to require the activity of the mitotic cyclin-Cdk and Cdc5 (Kilmartin, 2003; Jaspersen et al., 2004; Crasta et al., 2008). There have been many studies in other model systems showing that the Polo-like kinases have a role in centrosomal regulation. In vertebrates the Polo-like kinase, Plk4, has been shown to promote the dissolution centriole cohesion, which facilitates the separation of centrosomes (Tsou et al., 2009). Due to the roles of Polo-like kinases in other systems, in addition to the role of Cdc5 during mitosis, we investigated the role of Cdc5 in SPB dynamics during meiosis.

1.4 The Centrosome and Human Disease

Centrosomes play an important role in spindle organization and chromosome segregation, in addition to other cellular processes during mitosis and meiosis. Abnormalities in the number, structure and function of human centrosomes have been associated with genomic instability and have been linked to various cancers. For example, studies have suggested centrosome amplification occurs in most types of solid tumors (Fukasawa, 2005). These structural abnormalities observed in supernumerary centrosomes can contribute to improper microtubule organization and nucleation, resulting in multipolar spindle formation and ultimately leading to chromosome missegregation (Salisbury et al., 1999; Zhou et al., 1998). Therefore, the strict regulation of centrosome number and function is essential for maintaining chromosomal stability and is achieved through accurate duplication and separation of the centrosome. Defects in both Aurora Kinases and Polo-like kinases have been found to cause centrosomal abnormalities, such as amplification of centrosomes and centrioles, and are found often in cancerous cells (Sakakura et al., 2001). Better understanding the roles of Ipl1 and Cdc5 during yeast meiosis, will provide more insight on the molecular mechanism of SPB duplication and separation, and may help to shed light on the involvement of these important kinases when studying cancer and other human diseases.
1.5. *Saccharomyces cerevisiae*, the Budding Yeast as a Model System in this Study

For the purposes of the study reported here, the budding yeast, *Saccharomyces cerevisiae*, was used as a model system to characterize the molecular mechanism of SPB duplication during meiosis because it is a very powerful genetic tool due to its short regeneration period and its ability to survive in the haploid and diploid stages. In addition, meiosis can be easily induced in this model system, and through the manipulation of the yeast genome, we were able to effectively create genetic mutations in order to molecularly characterize SPB cohesion and duplication. We were able to visualize mutant phenotypes, by developing a live cell microscopy method where, after meiotic induction, cells expressing fluorescently labeled proteins of interest were observed in real time.

All of these factors taken together, and through the incorporation of cytological, biochemical, and genetic approaches, *S. cerevisiae* provided a good system to study the molecular mechanism of SPB duplication during yeast meiosis, through the characterization of Ipl1 and Cdc5. We hope that the information and knowledge gained from this study will help to further investigate the role of these protein kinases in centrosomal defects that cause human diseases.
CHAPTER TWO

RESULTS

2.1 Ipl1 is required for SPB Cohesion during Meiosis I

To characterize the separation dynamics of duplicated sister SPBs in MI, we developed a live cell culture method of maintaining meiotic induction in budding yeast, on a glass slide, and observed Spc42-GFP-marked SPBs using fluorescence microscopy (Fig. 1A). In wild-type cells, sister SPBs were tightly associated after duplication, primarily forming a single Spc42-GFP focus (Fig. 1A). Immediately before their separation in MI, sister SPBs were resolved by light microscopy as two distinguishable foci (MI, Fig. 1 A, t = 0), which we have referred to as a doublet SPB configuration. For our live cell microscopy, we have defined time zero as the point immediately before SPB separation in MI (~ 4 h after induction of meiosis, see below), which permitted us to objectively examine and compare SPB separation and meiotic progression between wild type (WT) and mutant cells. Ipl1-depletion mutants ($P_{\text{CLB2}}IPL1$) created using PCR based promoter replacement, replacing the endogenous promoter of IPL1 with the promoter of mitosis specific CLB2 to degrade Ipl1 during meiosis (Yu and Koshland, 2007). In contrast to sister SPBs observed in the wild type, most sister SPBs in the Ipl1-depleted ($P_{\text{CLB2}}IPL1$) cells were associated to each other at a greater distance, forming the doublet configuration well before their complete MI separation (Fig. 1B and C), suggesting that the sister SPBs were less cohesive in the absence of Ipl1. To examine the meiotic SPB at a higher resolution, Thomas Giddings, at The University of Colorado at Boulder, serially sectioned resin embedded cells and examined the sections by transmission electron microscopy (Figs. 1D–1F). Consistent with our light-microscopy observations, duplicated SPBs were linked by the bridge and were tightly associated for an extended period of time in MI (Figs. 1C-F1), a situation we termed SPB cohesion. In contrast, sister SPBs were positioned significantly farther from each other with a collapsed bridge before their complete separation in Ipl1-depleted cells (Fig. 1D, 118 nm in the wild type, 162 nm in the mutant). In addition, we observed that the SPB-associated nuclear envelope often became invaginated and bundling of the aster microtubules resulted, in Ipl1-depleted cells (Figs. 1 F2–F6). Even in these cells, the layered structure of SPBs resembled those of the wild-type (Fig. 1F). The average widths of SPB layers from wild-type and Ipl1-depleted cells did not differ significantly, but mutant cells showed a greater variation in SPB size (Fig. 1E). Together,
these results showed that the SPB structure appeared normal in Ipl1-depleted meiotic cells, but sister SPBs were positioned farther apart, suggesting that Ipl1 is required for maintenance of SPB cohesion before their separation.

### 2.2 Ipl1 Localizes to the Spindle Pole

We hypothesized that Ipl1 would localize to the SPB to regulate SPB dynamics during meiosis. We performed time-lapse microscopy to observe Ipl1 localization using Ipl1-GFP (Fig. 2A). Ipl1 was predominantly localized to the astral microtubules at prophase I; it then was enriched at the spindle poles ~12 min before sister-SPB separation (Fig. 2A). In contrast, the kinetochore marker, Mtw1, was not concentrated at the spindle poles during sister-SPB separation in MI (Fig. 2B). These data suggest that Ipl1 localization to the spindle pole is independent of clustered centromeres. After sister-SPB separation, Ipl1 became dispersed inside the nucleus. When the spindle started to elongate at anaphase I, Ipl1 relocalized to the spindle and then recessed to the spindle pole about 40 min after MI SPB separation, at which point spindle microtubules started to disassemble (Fig. 2A). The timing of Ipl1s’ reassociation with the spindle pole corresponds to that of SPB reduplication at interphase II. Because centromeres are clustered at the poles at interphase II and Ipl1 is also associated with the centromeres, our live-cell microscopy observation did not distinguish centromere-associated Ipl1 from SPB-associated Ipl1 at interphase II.

### 2.3 Ipl1 is required for Accurate SPB Duplication at Interphase II

In order to observe SPB dynamics throughout the meiotic cycle, live cell microscopy was utilized. After SPBs separate in MI, they undergo second round of duplication, which is independent of DNA replication. In wild-type cells, four SPBs, revealed by Spc42-GFP and Tub4 (gamma tubulin)-mApple, were formed after completion of MII, (Fig. 3A and B). In contrast, more than 30% of Ipl1-depleted meiotic cells formed five or more SPBs 6 h after induction, a time that corresponded roughly to the completion of MI (Fig. 3A and B), as seen in wild-type cells. These supernumerary SPBs contained the meiotic plaque component Mpc54 (Fig. 4B and 4C), which is characteristic of MII SPBs (Fig. 5A) (Knop and Strasser, 2000). A further investigation of the role of the kinase activity of Ipl1 on SPB dynamics, an experiment was preformed by Hui Jin, in the Yu lab at The Florida State University, where the Ipl1 kinase activity was inactivated through the manipulation of the analog-sensitive (ipl1-as5) allele (Pinsky et al., 2006), using the drug 1-NM-PP1. Ipl1 kinase inactivation led to the formation of
extra Spc42 foci (Fig. 3C). Together, these results suggested that sister SPBs prematurely lose cohesion and subsequently over-duplicate in the absence of Ipl1 activity during meiosis.

To determine when Ipl1 is required for SPB duplication and whether SPBs become fragmented in Ipl1-depleted cells, we arrested the cells at prophase I by eliminating the production of Ndt80, which is necessary for cell cycle progression beyond Prophase I. To do this, we constructed a yeast strain containing the GAL-NDT80 allele and incorporated a GAL4.ER plasmid, to induce the GAL promoter during meiosis, (Carlile and Amon, 2008). SPB separation with and without Ipl1 kinase activity was then observed in cells before and after Prophase I (Fig. 3D). Sister SPBs failed to separate in cells arrested at prophase I, but after inactivation of Ipl1 kinase activity, led to MI SPB separation in ~50% of these cells. Thus supporting the idea that Ipl1 is required for maintaining SPB cohesion. Importantly, these separated SPBs did not commit to another round of duplication unless Ndt80 was reintroduced, releasing Prophase I arrest, using β-estradiol and allowing cells to proceed through meiosis (Fig. 3D). These data suggest that supernumerary SPB formation in Ipl1-depleted cells is less likely to be due to SPB fragmentation. Therefore, we conclude that the Aurora kinase Ipl1 is required for SPB cohesion and prevents SPB over-duplication at interphase II. Together, these results suggest that sister SPBs prematurely lose cohesion and subsequently over-duplicate in the absence of Ipl1 activity during meiosis.

2.4 Ipl1 Prevents Multipolar Spindle Formation during Meiosis II

To determine whether over-duplicated SPBs in Ipl1-depleted cells function to nucleate microtubules, we performed time-lapse microscopy to observe SPB dynamics and spindle formation using Spc42-GFP and mApple-tagged α-tubulin (called Tub1 in yeast; Fig. 5A and B). In wild-type cells, sister SPBs remained tightly associated after duplication, in general forming a single Spc42-GFP focus with a diameter less than 0.5 μm (Fig. 5A). Sister SPBs were separated to form a 2- to 3- μm -long spindle (Metaphase I, Fig. 5 A and C). About 30 min after SPB separation, the spindle elongated to reach a length of ~6 μm. Then pole-to-pole distance decreased slightly during an ~8-min window before SPBs were separated again in MII (Fig. 5 C). On average, the interval from the beginning of MI SPB separation to that of MII was 40 (±3, n = 14) min. Duplicated SPBs were kept together before the formation of the MII spindle (Fig. 5A, from t = 40 min to t = 46 min), as sister SPBs were in MI. In Ipl1-depleted meiotic cells, long before MI separation, sister SPBs formed the doublet configuration (~1 μm in size)
but were associated with the aster microtubules (Fig. 5B, t = –24 min and see below). Notably, mutant cells lacked a clear spindle-elongation phase; instead, 30 (±8, n = 12) min after MI SPB separation, a third Spc42-GFP focus appeared, apparently originating from an existing SPB; then additional Spc42-GFP foci were observed to form (Fig. 5B and C). The newly formed SPBs were initially present at a very low fluorescence intensity of Spc42-GFP (Fig. 5B, t = 39 min); they grew to intensity similar to that of the old MI SPBs in about 20–30 min (Fig. 5B). All Spc42-GFP foci formed in Ipl1-depleted cells were able to nucleate microtubules, often forming multipolar spindles (Fig. 5B and 5E), demonstrating that these SPBs are functional in microtubule organization. The SPB morphology appears normal in Ipl1-depleted cells (Figs. 1 and Fig. 5D) suggesting that these SPBs are fully formed. Together, these data further support the idea that Ipl1 is required for faithful duplication of existing SPBs at interphase II to ensure proper MII spindle formation.

2.5 Cdc5 Promotes SPB Separation and Licenses SPB Duplication

We hypothesized that the completion of sister-SPB separation depends on the outward force between them, resulting from the formation of a bipolar spindle. The Polo-like kinase, Cdc5 in yeast, is implicated in centriole separation in vertebrate cells and SPB separation in vegetative yeast cells (Crasta et al., 2008; Tsou et al., 2009). Cdc5, C-terminally tagged with GFP, appeared in the nucleus ~20 min before MI SPB separation in wild-type cells, forming foci that were not associated with SPBs as observed by live cell microscopy (Fig. 6A, from t = –18 to t = –4 min, arrows). Cdc5 concentrated on the SPB during MI sister-SPB separation (Fig 6A, t = 0 min, arrows), but diminished from SPBs afterwards (Fig. 6A, t = 2 and 8 min). Before spindle elongation, Cdc5 reformed foci that colocalized with SPBs (Fig. 6A, t = 20 min). In contrast, in Ipl1-depleted cells, the separation of sister SPBs in MI started at the appearance of Cdc5-GFP detected by fluorescence microscopy (Fig. 6B and C), indicating that, upon Cdc5 production, cells immediately initiated sister-SPB separation. As in wild type cells, Cdc5 was concentrated on the SPB during spindle elongation (Fig. 6B, t = 26 min) ~10 min before the appearance of a third SPB (Fig. 6B, T = 36 min). Also in the Ipl1-depleted mutant, five Tub4-mApple foci formed 66 min after sister-SPB separation and all Tub4 foci were enriched with Cdc5-GFP (Fig. 6B). Together, our data suggests that sister-SPB cohesion, maintained by Ipl1, prevents the premature separation of sister SPBs, by the separating force, which is regulated by Cdc5.
To determine whether Cdc5 is required for SPB separation and duplication during meiosis, we depleted meiotic Cdc5 by means of replacing the endogenous CDC5 promoter with CLB2, a mitosis specific gene, using the $P_{CLB2}CDC5$ allele (Clyne et al., 2003; Lee and Amon, 2003). In Cdc5-depleted cells, after MI, SPB failed to separate (Fig. 6 E); supporting the idea that Cdc5 is necessary for MII SPB separation. We reasoned that overproduction of meiotic Cdc5 would promote SPB separation. Indeed, over-production of Cdc5 using the promoter driven Cdc5 expression allele ($P_{CUP1}CDC5$) led to sister-SPB separation, and dramatically increased the number of meiotic cells observed forming supernumerary SPBs (Fig. 6 D and E).

In cells depleted of both Ipl1 and Cdc5 ($P_{CLB2}IPL1 P_{CLB2}CDC5$), ~40% sister SPBs were able to separate and duplicated to form three or four but not five SPBs (Fig. 6 E), suggesting that other factors, in addition to Cdc5, can also promote SPB duplication. Alternatively, residual Cdc5 activity might have remained in the mutant. Together, these data support the idea that Ipl1 protects sister-SPB cohesion during interphase I and prevents SPB over-duplication during interphase II, whereas Cdc5 promotes SPB separation and duplication at interphase II.
CHAPTER THREE

DISCUSSION

3.1 Ipl1 is required for Spindle Pole Body Cohesion, Accurate Spindle Pole Body Duplication and Proper Spindle formation during Yeast Meiosis

3.1.1 Ipl1 Mediated Spindle Pole Body Cohesion

In yeast, Ipl1 is the only Aurora kinase that functions both on the spindle and at the spindle poles, whereas in metazoans Aurora A is differentiated to be the one predominately concentrated at the centrosomes, protecting centriole cohesion, and also mediating spindle bipolarity (Barr and Gergely, 2007; Lukasiewicz and Lingle, 2009). In this study, through the use of the meiotic specific Ipl1 allele ($P_{CLB2}IPL1$) we report that Ipl1’s activity during meiosis is involved in mediating SPB cohesion and preventing SPB over-duplication and multi-polar spindle formation, which resembles that of Aurora A kinase in higher eukaryotes.

Our results suggest Ipl1 is required to maintain cohesion between duplicated SPBs during interphase I, before MI separation. In WT cells, duplicated MI SPBs maintain cohesion, a tight association to each other, until before the onset of MI separation, where the SPBs loose their tight association and gain distance between each other and form a doublet configuration (Fig 1). Structurally, the side-by-side configuration, of these sister SPBs, is maintained via the half bridge, which acts as a physical link maintaining their tight association. Sister SPBs embed into the nuclear membrane at their central plaque, and nucleate aster microtubules into the nucleus, parallel to each other (Fig. 1D). MI separation immediately follows doublet SPB formation, and is facilitated by bipolar spindle orientation and elongation (Fig 2). In contrast, our results show that in Ipl1-depleted mutants, cohesion is prematurely lost much before the onset of MI separation, resulting in a significant increase in the association between sister SPBs when visualized using light microscopy (Fig. 1). When investigating the dynamics between Ipl1 and SPBs during Meiosis, we used light microscopy. Our results show, Ipl1 concentrates at the spindle poles before meiosis I separation (Fig. 2A), which coincides with the period when SPBs begin duplicating, this result may suggest Ipl1 is acting at the spindle pole to maintain SPB cohesion. It is known that Ipl1 also localizes to the centromeres; however, we were able to visualize the concentration of Ipl1 to the spindle pole before centromere clustering at the poles.
near the end of interphase I, but we were unable to distinguish Ipl1 localization between centromeres and SPBs, during later meiosis (Fig. 2). Further analysis of SPBs using the serial sectioned electron microscopy preformed by Thomas Gidding, we were able to visualize the structural morphology of these mutant SPBs. Consistent with our live cell microscopy data, we observed a significant increase in the distance between sister SPBs in the Ipl1-depleted cells. In these mutants, we observed invagination of the nuclear envelope, and what appeared to be, a collapsed half bridge between sister SPBs. Unlike the parallel nuclear MT formation in WT SPBs, the nuclear MTs in the Ipl1- depletion mutants were positioned toward each other, forming misoriented and bundled MT formations (Figs. 1D and 1F).

In this context, the loss of cohesion observed in our Ipl1-depletion mutants, implies an association between two duplicated SPBs that is greater in distance then in WT cells. The EM data presented here may suggest that cohesion loss may occur upon disruption of the half bridge. Bridge collapse may be due to the misorientation of nuclear MTs between two sister SPBs, which creates a pulling force between them, thus compromising the integrity of the bridge that acts to link them together. If cohesion is maintained physically through the half bridge, why doesn’t SPB separation immediately follow cohesion loss? A possible explanation may be that SPBs remain embedded into the invaginated nuclear envelope and it is the invaginated nuclear membrane that creates an increase in the distance between sister SPBs. It is also feasible the sister SPBs remain side by side until separating forces are able to act on the SPBs which signal spindle elongation. The timing of separation in Ipl1-depleted mutant cells are similar to WT, which further supports this explanation. Another hypothesis may be the invagination, resulting from half bridge collapse, is due to improper assembly of the bridge during interphase I, when SPBs duplicate, in the absence of Ipl1. This leads to the question, how exactly does Ipl1 regulate SPB cohesion? Are there Ipl1 substrates at the meiotic SPB? If so, what are those substrates? We hypothesize Ipl1 has substrates at the SPB and also at the half bridge. Those Ipl1 substrates act to maintain the integrity of the half bridge during SPB duplication, during interphase I. These results would suggest cohesion loss, in addition to resulting in an increase in the distance between sister SPBs, is due to the loss of the physical integrity of the half bridge. These results would also be consistent with the explanation that premature MI separation does not occur until factors promoting SPB separation and bi-polar spindle formation are signaled due to the association of the sister SPBs to the nuclear envelope.
Our lab is continuing to collaborate with Dr. Mark Winey’s lab at the University of Colorado at Boulder in order to better understand SPB regulation, and we are currently performing phosphoproteome analysis of the meiotic SPB in order to locate Ipl1 phosphorylation sites. We predict there will be phosphorylation sites at the meiotic SPB half bridge, and that Ipl1 has a role in the regulation of SPB cohesion and duplication at those sites. It will be interesting to explore the direct substrates of Ipl1 to see exactly how cohesion is maintained between sister SPBs during Interphase I.

3.1.2 Ipl1 Regulates Spindle Pole Body Duplication and Proper Bipolar Spindle Formation

In vertebrates, it has been suggested that the disengagement of centrioles is a licensing factor for centrosome duplication, and occurs one cell cycle before the duplication event. (Tsou and Stearns, 2006). In yeast, the SPBs do not contain centrioles like vertebrate centrosomes; however, the half bridge is known to consist of the calmodulin-like centrin homolog Cdc31 and Sfi1 (Kilmartin, 2003). It is possible that these components may function similarly in regulating SPB cohesion in a manner similar to centriole engagement. In this study, our results support the notion that cohesion loss between sister SPBs licenses SPBs to undergo duplication during the second round of the meiotic cell cycle at interphase II when SPBs are competent for a second round of duplication.

Here we have shown evidence that suggests Ipl1 maintains the accurate duplication of SPBs and proper bipolar spindle formation during interphase II due to premature loss of cohesion (Fig. 1) that licenses the cell to undergo extra round of duplication during the next cell cycle, resulting in over duplication of SPBs and multi-polar spindle formation during MII (Fig. 5).

After observing supernumerary SPBs in the Ipl1-depleted cells, we investigated how the extra SPBs were formed. Centrosome amplification in higher eukaryotes has been shown to occur as a result of additional rounds of duplication, fragmentation of the centrosome, or occur de novo (Tsou and Stearns, 2006). Our data shows that kinase activity is required for proper SPB duplication, and loss of kinase activity results in supernumerary SPBs as seen in Ipl1-depleted cells (Fig. 3C). In addition, loss of Ipl1 kinase activity before Prophase I results in only two SPBs which is expected during MI, while supernumerary SPBs did not result until Ipl1 kinase activity was shut off after Prophase I arrest was released, allowing cells to continue through the cell cycle (Fig. 3D). These results suggest that it is less likely the supernumerary
SPBs, observed in Ipl1-depletion mutants, are a result of fragmentation, due to cell cycle arrest, or occur de novo. We suggest that SPB over duplicate and that duplication event is restricted to the time after MI separation, at Interphase II. In addition, over duplicated SPBs contained meiotic specific SPB plaque component Mpc54 (Fig 4B), as in WT cells, suggesting these SPBs are able to assemble and mature similarly to WT SPBs (Figs. 4 and 5D-F).

The Aurora A in vertebrates is also implicated in causing centrosome amplification and multi-polar spindles, and results when Aurora A is over-expressed (Sakakura et al., 2001). Unlike vertebrates, the supernumerary SPBs observed in this study are a result of Ipl1 depletion; however, because yeast is a much less complex system, which only has one Aurora kinase and does not contain centrioles, we might expect results that differ from higher eukaryotes. Here we report that over duplicated SPBs are functional and are able to nucleate microtubules, which results in multi-polar spindle formation (Fig. 5B and 5D).

3.2 Cdc5 Promotes Spindle Pole Body Separation during Meiosis II and is a Licensing Factor for Spindle Pole Body Duplication at Interphase II

In addition to elucidating the role of Ipl1 in SPB of cohesion and duplication, we have further investigated the process of separation of SPBs by studying the Polo-like kinase Cdc5. Our results suggest Cdc5 is required for licensing SPB duplication at interphase II presumably by promoting the dissolution of SPB cohesion. Antagonistic to the Aurora kinase, Polo-like kinases play a role in promoting the separation of vertebrate centrioles, facilitating the duplication of centrosomes in the next cell cycle (Tsou et al., 2009). Here in this study, we have shown Cdc5 is required for SPB separation during meiosis II (Fig. 6E). Promoter-driven over-expression of meiotic Cdc5 results in supernumerary SPBs that contain gamma tubulin (Fig. 6D and 6E), which is similar to the phenotype observed in Ipl1-depleted mutants, suggesting it may have an antagonistic relationship with Ipl1. Cdc5 is most likely not the only factor that is regulating SPB separation due to the appearance of more than two SPBs in the Cdc5 and Ipl1 double depletion mutant. However the depletion of Cdc5 inhibits the formation of supernumerary centrosomes that are normally observed in Ipl1 depleted cells, which confirms Cdc5s role in SPB duplication. The Polo-like kinases have been repeatedly shown to have a role in promoting the separation of centrosomes in higher eukaryotes; PLK4 in mammalian cells, and SAK in drosophila melanogaster. The up-regulation of these kinases has shown amplification of centrosomes, which is consistent with our observations in this study, during meiosis in
Saccharomyces cerevisiae. It will be interesting to determine exactly how Cdc5 interacts antagonistically with Ipl1 at the SPB. It is possible that there are other factors that mediate Cdc5 localization to the meiotic SPB at Ipl1 phosphorylation sites, and would potentially help to answer the question of how Cdc5 antagonizes Ipl1 function at the meiotic SPB duplication and separation.

We have shown that meiotic SPB separation and duplication requires the antagonistic interaction between two important cell-cycle regulators, Ipl1 and Cdc5. These two important cell cycle regulators are known to be involved in regulating the chromosome and the spindle microtubules. The opposing roles of Ipl1 and Cdc5 at the meiotic SPB are also reminiscent of their roles in regulation of sister-chromatid cohesion during meiosis I, when Ipl1 protects centromeric cohesion (Monje-Casas et al., 2007; Yu and Koshland, 2007), whereas Cdc5 promotes meiotic cohesin removal from the chromosome to disassociate sister chromatids (Lee and Amon, 2003; Brar et al., 2006). The SPB duplication process at interphase II provides a unique experimental system for further elucidation of the molecular mechanisms of SPB duplication during yeast cell proliferation.
### Materials and Methods

#### 4.1 Yeast strains

Table 1. Yeast Strains used in this study

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<th>Genotype</th>
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<tr>
<td>HY1423C</td>
<td><em>ura3, leu2, his3D200, SPC42-GFP::HIS5/ura3, leu2, his3D200, SPC42-GFP::HIS5</em></td>
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Table 2. Primers used in this study

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<td>CDC5R1</td>
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<td>IPL1F1</td>
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<td>5’—AAAATTTGGCGGCGGTGTAATAT GCCTGAAACAGGAAGGCCATATA—3’</td>
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4.2 Construction of Yeast Strains

All strains used in the study reported here were diploid SK1 derivatives. To create the PCLB2-IPL1 (referred to here as P_{CLB2IPL1}), we used a PCR-based approach to replace IPL1 endogenous promoter with CLB2 (Yu and Koshland, 2007). This method was used for the P_{CLB2CDC5} (Lee and Amon, 2003) strain as well. For the strain, ipl1-as5 (Pinsky et al., 2006) for the strain, GAL4.ER and GAL-NDT80 (Carlile and Amon, 2008), a plasmid containing GAL4.ER was transformed into the strain, GAL-NDT80. We used the same method to create PCUP1-CDC5 (referred to here as P_{CUP1CDC5}). A 1 kb DNA sequence upstream of the CUP1 open reading frame was used to replace the endogenous CDC5 promoter (Jin et al., 2009). A similar PCR-based method was used to construct strains expressing fluorescent fusion labels, all
genes under expression of their endogenous promoter; *SPC42-GFP*, *CDC5-GFP*, *MTW1-GFP*, *IPL1-GFP*, *MPC54-GFP*, *TUB1-mApple*, and *TUB4-mApple*.

### 4.3 Yeast Culture methods

Yeast cells were grown at 30°C with standard culture methods. Before yeast cells were induced to enter meiosis in 2% KOAC, they were grown in Yeast Protein Extract, Peptone, and 1% KOAC (YPA) medium with vigorous shaking for about 12 h, to an optical density *(l = 600)* of 1.5. To induce *P_CUP1/CDC5* expression during meiosis, we added 60 mM (final concentration) of CuSO₄ to the sporulation medium after induction of meiosis. Inactivation of *ipl1-as5* (Pinsky *et al.*, 2006) was induced by addition of 100 μM (final concentration) of 1-NA-PP1 to the sporulation medium after induction of meiosis. To induce *GAL-NDT80* expression, we added 100 μM β-estradiol (final concentration) 6 h after induction of meiosis (Fig. 2 D). For monitoring of SPB formation, aliquots of cells were withdrawn at the indicated times, fixed with 1% formaldehyde for 1 h at room temperature, washed twice with 1´ PBS, and visualized under a fluorescence microscope.

For live-cell microscopy, we used a concave glass slide as a culture chamber, which was filled with 100mL of 2% agarose dissolved in 2% KOAC. The agarose pad was allowed to solidify for 5 min at room temperature before use. About 1.5 ml yeast culture was laid on top of the agarose pad then sealed with a glass coverslip. The glass coverslip was sealed with 1:1:1 Lanolin, Vaseline, Paraffin, and applied around the edges with a toothpick. The slide was temperature balanced for 15 min at 30°C before microscopy.

### 4.4 Fluorescence microscopy

Live-cell microscopy was carried out on a DeltaVision imaging system equipped with an environmental chamber (Applied Precision, Inc.). All live-cell images were acquired at 30°C with a 60´ (NA = 1.41) objective lens on an Olympus ix71 microscope. Seven or eight z-stacks were collected at each time point. Each optical section was 1 μm thick. Exposure time for each optical section was set between 60 and 100 ms. To minimize phototoxicity, we blocked either 68% or 90% of output light from the GFP emission channel and 68% from the mCherry emission channel, which was used to acquire images from mApple-tagged strains. The time-lapse interval was set between 1 and 3 min. Each time-lapse experiment lasted for ~4 h.
Fixed cells were visualized under an epifluorescence microscope (AxioImager, Zeiss) with a 100′ objective lens (NA = 1.40). At least 200 cells were counted at each time point for evaluation of Spc42-GFP and Tub4-GFP focus formation.

4.5 Electron microscopy

Five-ml yeast aliquots were collected 4 h after induction of meiosis. Cell blocks were prepared by a high-pressure freezing and freeze-substitution method described previously (Winey et al., 2005). Serial sections of embedded cells were obtained and visualized under a transmission electron microscope (Philips, CM10).

Data analysis and image display

Raw data collected from the DeltaVision imaging system were deconvolved with SoftWorx (Applied Precision, Inc.). The three-dimensional pole-to-pole length of the SPB (Fig. 4C) was determined with the measurement tool provided by SoftWorx. Because mApple-tagged \(-\)tubulin strains show a ~5-min delay in MII SPB separation (compare Fig. 2 B and Fig. 4 B), the rate and duration of SPB separation were determined in Spc42-GFP single-tagged strains. Optical sections were projected into two dimensions for display. Images were exported as tiff files and compiled. No additional image adjustment was made. Projected images were used to generate histograms shown in Fig. 1A and Fig. 1B. An area composed of 400 pixels centered on the SPBs is shown. Pixel size is 0.1070 \(\mu\)m. All EM data was done by Thomas Giddings at University of Colorado at Boulder, in Dr. Mark Wineys’ Lab.

4.6 Protein Extraction and Western-blot

1mL yeast aliquots were collected at 2 h intervals after induction of meiosis. Cells were pelleted at 13,000 RPM for 30 seconds and supernatant was removed (Jin, et al., 2009). Cells were resuspended and incubated with 0.1 M NaOH for 5 min at room temperature. Cells were pelleted again and supernatant was removed. Cell pellets were resuspended in 1x SDS-PAGE loading buffer and boiled for 5 min at 100°C. Standard SDS-PAGE and western-blot procedures were followed. The SDS-PAGE gel used was 8%. Cdc5-GFP was detected by a GFP-specific antibody (Cat#632569, Clontech). The level of Tub2 (\(b\)-tubulin) served as a loading control.
CHAPTER FIVE

FIGURES AND FIGURE LEGENDS

5.1 Ipl1 Is Required to Maintain SPB Cohesion during Yeast Meiosis
Figure 1. Ipl1 Is Required to Maintain SPB Cohesion during Yeast Meiosis

(A–B) Fluorescence live-cell microscopy showing the morphology of duplicated sister SPBs in wild-type (WT, HY1423C) and Ipl1-depleted cells (HY1423) in meiosis I (MI). SPBs are marked by Spc42-GFP. Time zero was defined as the point immediately before sister-SPB separation in MI. Time in minutes is shown above each frame. Projected images from seven z-stacks with 1-μm optical sectioning are shown. Exposure time for each optical section was 80 ms. Three-dimensional histograms show Spc42-GFP intensity. Pixel intensity counts are shown to the right. Bar, 0.5 μm. (C) Quantification of SPB doublet formation. Cells were induced to undergo synchronous meiosis, fixed at the indicated times, and visualized under a fluorescence microscope. At least 100 cells were counted for each time points. (D–E) SPB-to-SPB distance and SPB plaque width in MI. Cells were induced to undergo synchronous meiosis, subjected to high-pressure freezing and freeze substitution, serially sectioned, and visualized under EM. Cells with side-by-side SPBs were identified; SPB-to-SPB distance and plaque width were determined. WT, n = 9; mutant, n = 7. Error bars show standard deviation. (F) Representative images showing sister-SPB configuration from WT (F1) and mutant (F2–F6). Five serial sections are shown for the mutant (Thomas Giddings, CU). Note that the SPB-associated nuclear envelope became invaginated in the mutant. NE, nuclear envelope. Bar, 50 μm.
5.2 Ipl1 Localization at the Spindle Pole is Independent of Centromere Clustering

Figure 2. Ipl1 Localization at the Spindle Pole is Independent of Centromere Clustering
(A) Live-cell microscopy was performed for visualization of GFP-tagged Ipl1 (HY1957). SPB is marked by Tub4-mApple. Time in minutes is shown below each frame. Red, Tub4; green, Ipl1. Bar, 2 μm. (B) Mtw1 dynamics during yeast meiosis. Mtw1 was tagged with GFP, and Tub4 with mApple. Projected images from eight z-stacks with 1-μm optical sectioning are shown. Time zero was defined as the point of SPB separation in MI. Exposure time for each optical section was 60 ms.
5.3 Ipl1 is required for Accurate SPB Duplication during Yeast Meiosis
Figure 3. Ipl1 is required for Accurate SPB Duplication during Yeast Meiosis

(A) Fluorescence microscopy showing SPB segregation in WT (HY1675) and \( P_{CLB2}IPL1 \) (HY1886) cells during yeast meiosis. SPBs are marked by Spc42-GFP and Tub4-mApple. Projected images from eight z-stacks with 1- \( \mu \)m optical sectioning are shown. Exposure time for each optical section was 100 ms. Note that Spc42 foci largely colocalize with the Tub4 foci in both cell types. Red, Tub4; green, Spc42. *Duplicated sister-SPBs formed a doublet configuration in \( P_{CLB2}IPL1 \) cells before separation in MI. Bar, 2 \( \mu \)m. 

(B) The number of Spc42 foci in fixed samples from WT (right) and \( P_{CLB2}IPL1 \) (right) cells. Yeast cells were induced to undergo synchronous meiosis and fixed at the indicated times. Spc42-GFP foci were scored by fluorescence microscopy. One and two SPB cells were grouped for MI; three and four were grouped for meiosis II (MII); five or more represented cells with overduplicated SPBs. Averages from two independent experiments are shown.

(C) Spc42-GFP foci formation in the \( ipl1-as5 \) mutant (HY2486) during meiosis. Addition of 1-NM-PP1 inhibits the Ipl1 kinase activity. DMSO treatment (left) serves as a control (Hui Jin). Spc42-GFP foci were determined as in B.

(D) The execution point of Ipl1 on SPB cohesion and duplication. Yeast cells (HY2627) were induced to undergo synchronous meiosis, subjected to four different treatments, and fixed at the indicated times for fluorescence microscopy as in B. Addition of PP1 inhibited the Ipl1 kinase activity, whereas addition of \( \beta \)-estradiol induced the production of Ndt80 (Hui Jin). Arrows indicate the time of addition.
5.4 SPBs in $P_{CLB2}IPL1$ cells contain Meiotic Specific Plaque Component

Figure 4. SPBs in $P_{CLB2}IPL1$ cells contain Meiotic Specific Plaque Component

(A) Fluorescence live-cell microscopy showing SPB segregation in wild-type (WT, HY2669) and $P_{CLB2}IPL1$ (HY2670) cells during yeast meiosis. SPBs are marked by Mpc54-GFP and Tub4-mApple. Projected images from eight z-stacks with 1-μm optical sectioning are shown. Exposure time for each optical section was 60 ms for the wild type and 100 ms for the mutant. Time lapse was 4 min for the wild type and 10 min for the mutant. Note that Mpc54-GFP signal intensity increased over time and became strongest on MI SPBs. (C) Percentage of cells showing Tub4-mApple foci that were positive with Mpc54-GFP 10 h after induction of meiosis. At least 100 cells were counted for each strain.
5.5 SPBs in $P_{CLB2}\text{IPL1}$ Cells are Functional and Form Multi-Polar Spindles during Meiosis II

A
WT

B
$P_{CLB2}\text{IPL1}$

C

D

E

F

26
Figure 5. SPBs in $P_{CLB2}IPL1$ Cells are Functional and Form Multi-Polar Spindles during Meiosis II

(A–B) Live-cell fluorescence microscopy showing SPB and microtubule spindle dynamics in WT (HY1737) and $ipl1$ mutant (HY1738) during yeast meiosis. SPBs are marked by Spc42-GFP, microtubules by Tub1-mApple. Projected images from seven z-stacks with 1-µm optical sectioning are shown. Exposure time for each optical section was 60 ms. Time zero was defined as the point of SPB separation in MI. Time in minutes is shown below each frame. Time-lapse was 2 min for the wild-type cell and 3 min for the $P_{CLB2}IPL1$ cell. Note that sister SPBs form a doublet configuration before MI separation in $P_{CLB2}IPL1$ cells. Red, Spc42; green, Tub1. Bar, 2 µm. (C) Spindle length as determined by pole-to-pole distance from WT and $P_{CLB2}IPL1$ cells as shown in A and B. Distance in three dimensions was measured. MI spindle, purple; MII spindle, other colors. (D) The morphology of separated SPBs during yeast meiosis. Cells were induced to undergo synchronous meiosis, subjected to high-pressure freezing and freeze substitution, serially sectioned, and visualized under EM. (D) Wild type (HY1423C). Three representative sections are shown (D1–D3). Two SPBs were found in this cell. Note that a bipolar spindle connects the two SPBs found in sections D1 and D3 (Thomas Giddings, CU). (E) $P_{CLB2}-IPL1$ (HY1423). Three representative sections are shown (E1–E3). Three SPBs were found in this mutant cell. Note that microtubules emanating from the SPB in section E3 are branched. The layered SPB structure appears normal in the mutant.
5.6 Promotion of SPB Separation by Cdc5 during Yeast Meiosis

A

WT

Cdc5-GFP
Tub2-GFP
Merge

B

$P_{CLB2-IPL1}$

Cdc5-GFP
Tub2-GFP
Merge

C

$WT$ $P_{CLB2-IPL1}$

Relative Cdc5-GFP intensity

D

$WT$ $P_{CLB2-IPL1}$

Cdc5-GFP
Tub2

$WT$ $P_{CUP1-CDC5}$

Cdc5-GFP
Tub2

E

$WT$ $P_{CLB2-CDC5}$ $P_{CUP1-CDC5}$ $P_{CLB2-IPL1}$

Percentage of cells

Hours after induction of meiosis
Figure 6. Promotion of SPB Separation by Cdc5 during Yeast Meiosis

(A–B) Live-cell fluorescence microscopy showing Cdc5 localization in WT (HY1993) and ipl1 mutant (HY2037) during meiosis. Cdc5 was tagged with GFP. SPB was marked by Tub4-mApple. Projected images from seven z-stacks with 1-μm optical sectioning are shown. Exposure time for each optical section was 60 ms. Time lapse was 2 min. Note that 66 min after sister-SPB separation five Tub4 foci were formed in the mutant. Arrows point toward SPB and Cdc5 foci. Red, Tub4; green, Cdc5. Bar, 2 μm. (C) Relative intensity of Cdc5-GFP in live meiotic cells. The Cdc5-GFP intensity of an area made up of 400 pixels is plotted against time. This area is centered on the SPBs. The background intensity of Cdc5 is about 110. (D) Western blot showing Cdc5-GFP production during yeast meiosis from WT (HY1993), P_{CLB2}IPL1 (HY2037), and P_{CUP1}CDC5 (HY2076) cells. The level of Tub2 serves as a loading control. To induce P_{CUP1}CDC5 expression, 60 μM (final concentration) of CuSO₄ was added to the sporulation medium after induction of meiosis. (E) The number of Tub4-GFP foci in fixed samples from WT (HY1881C), P_{CLB2}CDC5 (HY2161), P_{CLB2}CDC5 P_{CLB2}IPL1 (HY2458), and P_{CUP1}CDC5 (HY2169) cells. Yeast cells were induced to undergo synchronous meiosis and fixed at the indicated times. Tub4-GFP foci were scored by fluorescence microscopy. Averages from two independent experiments are shown.
REFERENCES


BIOGRAPHICAL SKETCH

Katelan Shirk is a former graduate student at The Florida State University from the Cellular and Molecular Department. She also holds a B.S. degree in Biological Science from The Florida State University. Katelan is from a military family and has lived in Korea, St. Louis, Missouri, and most recently Omaha, Nebraska. She enjoys to paint, sketch, and build furniture. Katelan’s life goals include writing a book and traveling to every continent. Katelan will continue to fulfill her love of research and will pursue higher education with attaining a PhD in molecular biology.