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# The Dynamic Localization and Novel Functions of the Ipl1/Aurora Protein Kinase

**THESE**

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par

**Stéphanie Buvelot**

De Nyon (Vaud)

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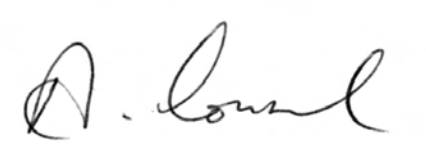

Prof. Andreas Conzelmann

Le Doyen

Prof. Dionys Baeriswyl

**DECLARATION FOR THE FACULTY**

Stéphanie Buvelot

12, ch. Veyrassat

1180 Rolle

Rolle, le 28 novembre

Aux personnes concernées,

**Sujet: Thèse présentée à l'Université de Fribourg, Suisse, pour l'obtention du grade de *Doctor rerum naturalium***

Mesdames, Messieurs,

Par la présente, je certifie que j'ai rédigé ma thèse intitulée "The Dynamic Localization and Novel Functions of the Ipl1/Aurora Protein Kinase" moi-même et sur la base d'un travail personnel sans aide illicite.

Stéphanie Buvelot

À Sue, sans qui rien n'aurait été possible.

À maman, sans qui je n'aurais pas eu la force de finir.

À Chris, sans qui rien de tout cela n'aurait eu de sens.

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## CHAPTER 1. ABSTRACT

Proper chromosome segregation is a crucial event for accurate cell division. Defects in chromosome segregation lead to genetic instability and aneuploidy and are correlated with cancer. The laboratory studies chromosome segregation in the budding yeast *Saccharomyces cerevisiae*. Since this process is highly conserved among eukaryotes, studies in yeast will provide fundamental understanding of this mechanism. Ipl1p is the budding yeast member of the highly conserved family of Ipl1/Aurora protein kinases, which play a role in chromosome segregation, the spindle checkpoint and cytokinesis. In addition, a number of studies have demonstrated that the human Aurora protein kinases are oncogenes. Defects in the regulation of the Ipl1/Aurora kinases leads to aneuploidy resulting in genomic instability. It is therefore important to better understand the regulation and functions of this protein kinase in order to elucidate details about the mechanisms that lead to genomic instability.

In an attempt to learn more about Ipl1p regulation and functions, I decided to examine Ipl1p localization carefully. I found that Ipl1p localizes to kinetochores from G1 to metaphase. Ipl1p then leaves the kinetochores and transfers onto the whole spindle. It is probably transported away from the kinetochores on microtubules. Ipl1p then accumulates at the spindle midzone where it regulates spindle disassembly. I propose that Ipl1p is a general regulator of microtubules plus ends.

Finally, in a different study, I found that mutations in Ipl1p and the spindle checkpoint allow sister chromatid separation and spindle elongation in the absence of the APC (anaphase promoting complex) function. Data presented here suggest that the spindle checkpoint may directly inhibit the Esp1p protease.

## 1.2. RÉSUMÉ

La ségrégation exacte des chromosomes est un événement indispensable pour une division cellulaire fidèle. Des anormalités dans la ségrégation des chromosomes résultent en une instabilité génomique et une aneuploïdie et sont corrélées avec le cancer. Le laboratoire étudie la ségrégation des chromosomes chez la levure de bière *Saccharomyces cerevisiae*. Étant donné que ce processus est hautement conservé parmi les eucaryotes, des études chez la levure vont fournir une compréhension fondamentale de ce mécanisme. Ipl1p est, chez la levure, le membre de la famille hautement conservée de protéine kinase : Ipl1/Aurora qui joue un rôle dans la ségrégation des chromosomes, le point de contrôle du fuseau mitotique et la cytocinèse. De plus, plusieurs études ont démontré que les homologues humains de la protéine kinase Aurora sont des oncogènes. Des anormalités dans la régulation de la protéine kinase Ipl1/Aurora mènent à l'aneuploïdie qui résultant en une instabilité génomique. Il est, par conséquent, important de mieux comprendre la régulation et les rôles de cette protéine kinase dans le but d'élucider des détails à propos des mécanismes qui mènent à l'instabilité génomique.

Afin d'en apprendre plus sur la régulation et sur la fonction d'Ipl1p, j'ai décidé d'examiner soigneusement la localisation d'Ipl1p. J'ai trouvé qu'Ipl1p se trouve aux kinétochores de G1 à la métaphase. Ensuite, la protéine quitte les kinétochores et se déplace sur le fuseau mitotique. Elle est probablement transportée des kinétochores au fuseau sur les microtubules. Ipl1p s'accumule au milieu du fuseau mitotique où la protéine régule la dépolymérisation du fuseau mitotique. Je propose qu'Ipl1p est un régulateur général des extrémités positives des microtubules.

Finalement, dans une autre étude, j'ai trouvé que des mutations dans Ipl1p et le point de contrôle du fuseau mitotique permettent la séparation des chromatides et

l'élongation du fuseau mitotique en l'absence de la fonction du complexe promouvant l'anaphase. Les données présentées ici suggèrent que le point de contrôle du fuseau mitotique peut directement inhiber la protéase Esp1p.

# CHAPTER 2. GENERAL INTRODUCTION

## 2.1. The cell division cycle

A human organism consists of about $10^{14}$ cells which all originate from a single fertilized egg. In order to grow and maintain the differentiated status of adult tissues, more than $10^{15}$ cell divisions occur during the life of a human being. Thus, the survival of an organism depends on the accuracy of each cell division. During every cell division, the cell must duplicate its content and then divide in two. The cell division cycle is called the cell cycle. It has been divided into four phases: G1, S, G2 and M (see Figure 2.1). During phase S (S = synthesis), the genome is duplicated. This is tightly regulated to make sure that each chromosome gives rise to the perfect copy of itself. The cell can then proceed into mitosis also called M-phase (M) during

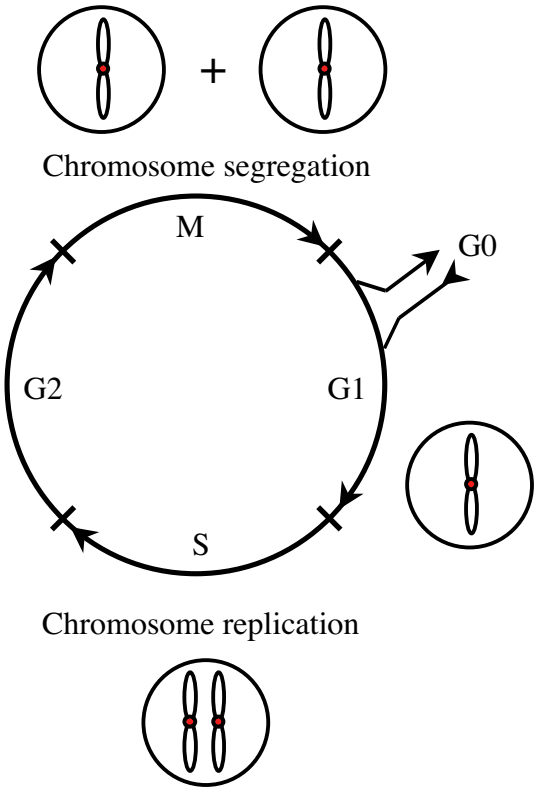


**Figure 2.1.** The cell cycle from the chromosome point of view. Centromeres of chromosomes are shown in red. See text for details.

which chromosomes are segregated equally into daughter cells (see below). G1 (G = gap) is the interval between the completion of mitosis and the beginning of S-phase and G2 is the interval between S-phase and the beginning of mitosis. These two phases provide time for the cell to grow and to prepare for the next phase. During G1 the cell monitors its environment and its size to decide whether it is going to enter the cell cycle or enter a specialized resting stage (G0), which is the final differentiated state of most cells in an adult organism.

**2.2. The yeast cell cycle**

Budding yeast divides by forming a bud that will grow to almost the size of the mother cell before it gets cleaved off at cytokinesis. The size of the bud provides an indication of the cell cycle stage. Figure 2.2. represents a cartoon of the budding yeast cell cycle. Budding yeast undergo a closed mitosis, which means that they do not break down their nuclear envelope during mitosis. The SPBs (spindle pole bodies, represented by a yellow dot) are the microtubule organizing centers in budding yeast. They are the organelles that nucleate microtubules and are embedded in the nuclear envelope having faces in both the nucleus and the cytoplasm. Recent studies have shown that the kinetochores (represented by a red dot in Figure 2.2) from the 16 chromosomes cluster together close to the SPBs during most of the cell cycle (Goshima and Yanagida, 2000; He et al., 2000). In G1 phase of the cell cycle when cells are unbudded, there is only one SPB and next to it one cluster of kinetochores (see Figure 2.2). In S-phase, the cell has a small bud. The duplicated SPBs separate to form the mitotic spindle (for review, see Winey and O'Toole, 2001). In prometaphase (medium size bud), the kinetochores are under such tension that they undergo precocious separation, while the sister arms remain held together (Biggins and

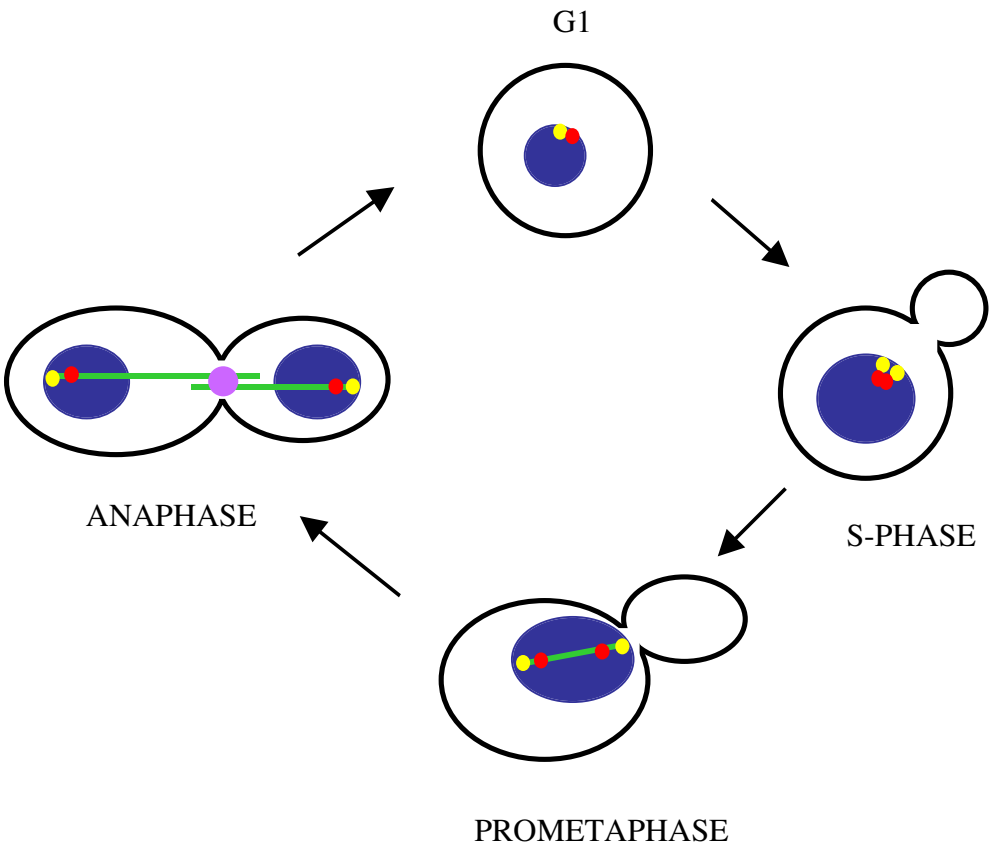


**Figure 2.2.** Cartoon of the yeast cell cycle. DNA is shown in blue (●), SPBs are represented by a yellow dot (○), kinetochores by a red dot (●), the midzone by a purple dot (●) and the spindle by a green line (╲).

Murray, 2001; Goshima and Yanagida, 2000; He et al., 2000; He et al., 2001; Pearson et al., 2001; Tanaka et al., 2000). At this point, co-localization of a kinetochore protein with a SPBs marker makes it possible to distinguish kinetochores from SPBs. In anaphase, the cell has a large bud. The SPBs have segregated away from each other and DNA segregation and spindle elongation have occurred. Again, the kinetochores are held in close proximity to the SPBs. The midzone (shown in purple) is the middle of the nucleus where the plus ends of non-kinetochore microtubules overlap (Winey et al., 1995). The midzone is thought to play a critical role in anaphase B by providing a structure that stabilizes the microtubules at the central region (Maddox et al., 2000; Pellman et al., 1995; Winey et al., 1995).

## 2.3. Chromosome segregation

Faithful chromosome segregation is a crucial event for maintenance of genetic information. Chromosome segregation defects can lead to the genetic instability that is associated with birth defects and tumors and is thought to be a major factor in the evolution of cancer (Lengauer et al., 1997; Lengauer et al., 1998). During S-phase, the chromosomes replicate and linkage is established between the two sister chromatids. The mitotic spindle then forms and microtubules attach to the chromosomes via kinetochores, multiprotein complexes assembled onto centromeric DNA (see below). Once the sister kinetochores attach to microtubules from opposite poles, the linkage between the sister chromatids is dissolved and the chromosomes segregate at anaphase, giving rise to two cells with the same genetic information. For the metaphase to anaphase transition to occur, accurate chromosome segregation depends on three parameters: the proper regulation of microtubule dynamics, functional kinetochores and the correct establishment and destruction of the linkage between sister chromatids. Defects in any of these steps can lead to genomic instability.

## 2.4. Sister chromatid separation

Several proteins involved in sister chromatid linkage have been identified (for review see Nasmyth, 2002). A conserved complex, the cohesins, is required to establish and maintain the link between sisters. In budding yeast, the Mcd1/Scc1 protein is part of this complex (Guacci et al., 1997; Michaelis et al., 1997). Other members of this complex are Smc1p, Smc3p and Scc3p (Losada et al., 1998; Michaelis et al., 1997). Although the behavior of the cohesin complex varies between organisms, the key events seem to be conserved. The regulation of sister chromatid

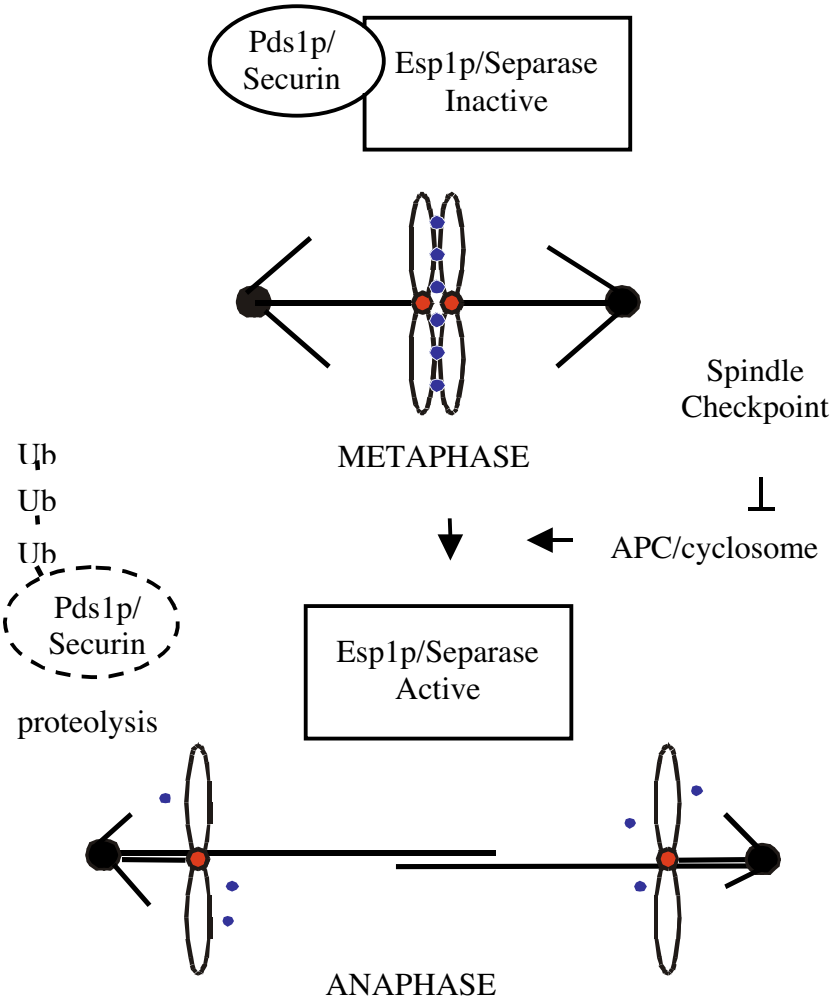


**Figure 2.3.** During metaphase, sister chromatids are held together by cohesin (blue dots) and the Esp1p/separase protease is inactive because the inhibitor Pds1p/securin is bound. Anaphase is initiated when the APC is activated, leading to the ubiquitination and subsequent degradation of securin. The activated separase cleaves cohesin, allowing the spindle to pull sister chromatids to opposite poles at anaphase.

separation in budding yeast will be explained here (see Figure 2.3), and differences with higher eukaryotes will be discussed in chapter 2.7. Sister chromatid separation is initiated by the proteolytic cleavage of the Scc1p protein by the Esp1p/separase protease (Uhlmann et al., 1999; Uhlmann et al., 2000). The Esp1p/separase is normally in a complex with the Pds1p/securin and is kept inactive (Uhlmann et al., 1999). The APC (anaphase promoting complex; see below), is a multiprotein ubiquitin ligase (for reviews, see Peters, 2002; Zachariae and Nasmyth, 1999) that catalyses ubiquitin-mediated proteolysis. It is responsible for degradation of the

Pds1p/securin at the metaphase-to-anaphase transition, leading to Esp1p activation (Ciosk et al., 1998; Cohen-Fix et al., 1996; Funabiki et al., 1996b; Stratmann and Lehner, 1996; Yamamoto et al., 1996). Chromosome segregation is monitored by the spindle checkpoint, a surveillance mechanism that prevents cells from separating their sister chromatids until chromosome alignment is complete (for review, see Millband et al., 2002). The checkpoint acts by inhibiting the APC (Fang et al., 1998a; Hwang et al., 1998; Li et al., 1997).

### 2.5. Regulation of the APC

Sister chromatid separation is closely regulated and most of the key players in this process are themselves regulated by phosphorylation.

In mitotic cells, two forms of the APC exist, $APC^{CDC20}$ and $APC^{CDH1}$, which differ in their association with the WD40-containing proteins, (Cdc20p and Cdh1p (or Hct1p) respectively), time of activation and substrate specificity (Fang et al., 1998b; Kotani et al., 1999; Kramer et al., 1998; Lim et al., 1998; Schwab et al., 1997; Visintin et al., 1997; Zachariae et al., 1998a). Cdc20p and Cdh1p bind to the APC and have been shown to determine its substrate specificity (Burton and Solomon, 2001; Hilioti et al., 2001). $APC^{CDC20}$ is active in early mitosis, where it is responsible for the degradation of Pds1p/securin. The $APC^{CDC20}$ is regulated by phosphorylation (for review see Morgan, 1999). The cyclin-dependent kinase Cdc28p, Cdk1 in other organisms, is a key regulator of cell cycle progression in budding yeast (for review see Mendenhall and Hodge, 1998). It is responsible for the phosphorylation of APC components leading to activation of the $APC^{CDC20}$ (Rudner and Murray, 2000). On the other hand, $APC^{CDH1}$ is activated in late mitosis and is required for the mitotic exit, where it promotes degradation of several proteins including the mitotic cyclins and

the microtubule associated protein Ase1 (see below) (Juang et al., 1997; Kramer et al., 1998; Murray, 1995; Schwab et al., 1997; Zachariae et al., 1998a).

### 2.6. Regulation of Pds1p, Esp1p and Scc1p

Because activation of Esp1p has to be regulated in a strict temporal order to prevent missegregation, various modes of regulation exist. Key to its regulation is Pds1p, which plays a dual role in regulating Esp1p activity. In addition, to inhibiting Esp1p, it is also required to promote its efficient nuclear localization and activation (Hornig et al., 2002; Jensen et al., 2001; Kumada et al., 1998). Pds1p is phosphorylated by the cyclin-dependent kinase Cdc28p. The phosphorylation of Pds1p by Cdc28p is important for efficient binding to Esp1p and for promoting the nuclear localization of Esp1p (Agarwal and Cohen-Fix, 2002). However, the Pds1p/Esp1p complex still forms with a low affinity in the absence of Cdc28p phosphorylation, suggesting that other factors contribute to the Pds1p/Esp1p interaction (Agarwal and Cohen-Fix, 2002). In addition to its function in an unperturbed cell cycle, Pds1p is phosphorylated in the presence of DNA damage via the DNA damage checkpoint pathway in a Mec1-and Chk1- dependent manner (Cohen-Fix and Koshalnd, 1997; Sanchez et al., 1999).

There is also a securin-independent mechanism that regulates the cleavage of the cohesin Scc1p by separase. Scc1p is phosphorylated by the Polo-like kinase Cdc5p shortly before its cleavage. This phosphorylation is required for efficient cleavage of Scc1p by Esp1p/separase (Alexandru et al., 2001). Therefore, even in the absence of securin, the cleavage of Scc1p is tightly regulated.

Finally, separase has been shown to be regulated by a dual mechanism in *Xenopus* egg extracts: binding of securin and phosphorylation. The cyclin dependent

kinase CDK1 phosphorylates separase, leading to its inactivation (Stemmann et al., 2001). In this organism, separase activation at the metaphase-anaphase transition requires the removal of both securin and an inhibitory phosphate group. However, the phosphorylation sites are not conserved in budding yeast Esp1p/separase (Stemmann et al., 2001). In addition, no evidence for Cdk-dependent inhibition of separase exists in yeast as sister separation can proceed in the presence of high kinase activity (Surana et al., 1993). However, it is still possible that Esp1p is regulated by phosphorylation due to another kinase. Lack of S-phase cyclins *CLB5* and *CLB6* bypasses the requirement of securin degradation for anaphase but not of cohesin cleavage (Meyn and Holloway, 2000).

### 2.7. Sister chromatid separation in higher eukaryotes

Since human chromosomes are much bigger compared to yeast, it is not surprising that the regulation of sister chromatid separation is more complex. In higher eukaryotes, the dissociation of cohesin at mitosis is a two step process that is regulated by two different pathways (Waizenegger et al., 2000). The bulk of cohesin is removed in prophase and prometaphase. This step is regulated by phosphorylation of cohesin subunits by the polo-like kinase (Sumara et al., 2002). Cohesin is removed without being cleaved. A small subpopulation of cohesin remains on chromosome until the onset of anaphase, preferentially at centromeres (Hauf et al., 2001; Losada et al., 2000; Waizenegger et al., 2000). It is removed from chromosomes by SCC1 cleavage, which is mediated by separase similarly to sister chromatid separation in budding yeast (Hauf et al., 2001; Waizenegger et al., 2000). Vertebrate cells contain two cohesin complexes, each containing the SMC1, SMC3 and SCC1 subunits and

either one of the Scc3 orthologs, SA1 or SA2 (Losada et al., 2000; Sumara et al., 2000). The two complexes might therefore be differently regulated.

### 2.8. Sister chromatid independent functions of Esp1p

In addition to its function in sister chromatid separation, Esp1p/separase has been shown to play a role in mitotic exit, being part of the FEAR (Cdc Fourteen Early Anaphase Release) network. The Cdc14p phosphatase plays a pivotal role in mitotic kinase inactivation through its dual action in promoting proteolytic degradation of mitotic cyclins and accumulation of the Cdk inhibitor Sic1 (Visintin et al., 1998). During G1, S, G2 and early M phase, Cdc14p is held in the nucleolus by its inhibitor Ctf1/Net1. Then, during anaphase it is released from the nucleolus (Shou et al., 1999; Visintin et al., 1999). The MEN (mitotic exit network) is a complex signal transduction pathway that, upon activation, leads to the release of Cdc14p from the nucleolus (Jaspersen et al., 1998) (for review see Bardin and Amon, 2001). A recent study has shown that, a second pathway, the FEAR network, is responsible for Cdc14p release from the nucleolus during early anaphase, and that the MEN maintains it in the released state during late anaphase (Jensen et al., 2002; Stegmeier et al., 2002). The FEAR network is composed of Esp1p/separase, the polo-like kinase Cdc5p, Spo12p and the kinetochore protein, Slk19p (Stegmeier et al., 2002). Slk19p is cleaved by separase in anaphase, even though the function of this cleavage is not known (Sullivan et al., 2001).

A third function for the separase Esp1p has been proposed in regulating anaphase spindle dynamics even though the exact nature of this function is controversial. In anaphase, Esp1p localizes to the spindle and spindle midzone, which is the middle of the mitotic spindle where non-kinetochore microtubules overlap in

both budding and fission yeast (Ciosk et al., 1998; Funabiki et al., 1996a; Jensen et al., 2001; Kumada et al., 1998). Esp1p has been shown to play a role in stabilizing the mitotic spindle during anaphase (Sullivan et al., 2001; Uhlmann et al., 2000). In addition, the structure of the spindle midzone is altered in the absence of Esp1p (Sullivan et al., 2001). However, it is not clear whether this is a direct function of Esp1p or whether this is a secondary consequence of another defect. Jensen et al. found that Esp1p plays a role in spindle elongation and proposed that problems in spindle elongation in *esp1* mutants lead to defective anaphase spindle (Jensen et al., 2001). In contrast, Severin et al. found no function in spindle elongation for Esp1p and therefore propose that Esp1's role is to stabilize anaphase spindles (Severin et al., 2001b). The difference between those two studies will be discussed in chapter 5.

Although not all Esp1p functions are clearly understood. Esp1p plays many key roles during mitosis of the cell cycle.

### 2.9. The spindle checkpoint

As mentioned above, the spindle checkpoint is a surveillance mechanism that arrests cells in metaphase when all the chromosomes are not correctly attached to opposite poles. The conserved components of the checkpoint were originally identified in budding yeast and include the Mad proteins (Mad1-3p), Bub1p and Bub3p and the Mps1 protein kinase (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). A second checkpoint, containing the Bub2 protein, monitors the correct delivery of DNA into the daughter cell (Alexandru et al., 1999; Bardin et al., 2000; Fesquet et al., 1999; Li, 1999).

The spindle checkpoint proteins are kinetochore components or are recruited to kinetochores during a checkpoint arrest, suggesting that the checkpoint signal is

generated at the kinetochore (Bernard et al., 1998; Chen et al., 1996; Taylor and McKeon, 1997). The spindle checkpoint monitors the interaction between kinetochores and microtubules. It has been shown that microtubule attachment to kinetochores (Rieder et al., 1995; Waters et al., 1998) as well as tension generated at the kinetochore by microtubule forces (Li and Nicklas, 1995; Severin et al., 2001b; Shonn et al., 2000; Stern and Murray, 2001) are sensed by the spindle checkpoint. Recently, the Ipl1/Aurora protein kinase has been shown to play a role in the spindle checkpoint specifically when kinetochores are not under tension (Biggins and Murray, 2001). However, many details about the spindle checkpoint pathway still need to be elucidated to understand how the checkpoint regulates chromosome segregation.

### 2.10. Microtubules

The spindle is composed of microtubules, polymers of α- and β- tubulin heterodimers that self-assemble into tubulin heterodimers (Nogales et al., 1999). Tubulin dimers are arranged longitudinally to form protofilaments, 12-15 of which are joined through lateral association to form a 25 nm tube. Microtubules are dynamic polymers that grow and shrink by addition or loss of tubulin dimers from the ends of the microtubules (for review see Desai and Mitchison, 1997). They undergo dynamic instability, a behavior where both polymerizing and depolymerizing microtubules exist in the same population and interconvert (Mitchison and Kirschner, 1984). The dynamics of microtubule polymerization increase in mitosis (Belmont et al., 1990). Dynamic instability comes from the fact that β-tubulin has GTPase activity. When the dimers are incorporated into a microtubule, the GTPase activity is stimulated (Stewart et al., 1990), leading to a conformational change from straight (GTP-tubulin) to

curved (GDP-tubulin) (Hyman et al., 1995). Microtubules in the growth state maintain a cap of stabilizing GTP-tubulins (Drechsel and Kirschner, 1994). When the cap is lost, GDP-tubulins are exposed at the microtubule plus end, leading to a curved conformation of the microtubules, which promotes rapid shrinkage (Caplow et al., 1994; Mandelkow et al., 1991) (for review see Desai and Mitchison, 1997).

### 2.11. The spindle from metaphase to anaphase

The spindle contains two types of microtubules: those that bind to kinetochores (kinetochore microtubules) and those that interdigitate with each other (non-kinetochore microtubules). The budding yeast metaphase mitotic spindle consists of 32 kinetochores microtubules (16 from each SPBs) and 8 overlapping non-kinetochore microtubules (4 from each SPBs) and is about 1.5 to 2 μm long (for review see Desai and Mitchison, 1997) (Winey et al., 1995). The minus ends of the microtubules are bound to the SPB and the plus ends are distal to the SPB. During metaphase, the nucleus migrates to the bud neck (nuclear migration) and the spindle aligns parallel to the mother-bud axis (spindle orientation) (Cottingham and Hoyt, 1997; Shaw et al., 1997) (for review see Segal and Bloom, 2001). Anaphase then occurs and is composed of two phases: anaphase A, where the sister chromatids move toward the SPBs, and anaphase B, where the SPBs move apart from each other. Anaphase B is biphasic: it has a first rapid phase of elongation and a second slow one (Straight et al., 1997; Yeh et al., 1995). When the spindle has reached its maximal length of approximately 10 μm, the spindle disassembles starting at the midzone (Maddox et al., 2000). In budding yeast, microtubules disassembly occurs only at the plus end of the microtubule (Maddox et al., 2000).

Proper regulation of microtubule dynamics is critical for a number of cellular events. The kinetochores bind to the plus end of microtubules, and must therefore maintain attachment to a microtubule end that is alternately growing and shrinking. During anaphase, chromosome movement to the poles is largely dependent on the ability of kinetochores to follow the shrinking plus end of microtubules (Rieder and Alexander, 1990; Skibbens et al., 1993). The interpolar microtubules are carefully controlled to promote spindle elongation during anaphase and then switch to a state of depolymerization at the plus ends to disassemble the spindle (Maddox et al., 2000; Straight et al., 1998). The regulation of cytoplasmic astral microtubules is required for spindle positioning and nuclear fusion (Cottingham and Hoyt, 1997; Maddox et al., 1999; Shaw et al., 1997).

### 2.12. Motor proteins

Regulation of microtubule polymerization during the cell cycle is regulated by a balance between microtubule-destabilizing and –stabilizing factors. The destabilizing factors include KIN I, a family of motor proteins, and the stabilizing factors include a large group of microtubule-associated proteins (MAPs).

Motor proteins use the energy derived from ATP hydrolysis to generate work. Among these are molecular motors belonging to the kinesin and the dynein superfamilies. The common feature of kinesins is a conserved catalytic core, called the motor domain, which contains microtubule-binding sites and ATP-binding sites (for review see Vale and Fletterick, 1997). Three subfamilies exist based on the position of the kinesin-related motor domain within the protein. The KIN N subfamily has the motor domain at the N-terminus of the protein and moves predominately towards microtubule plus ends. The KIN C subfamily has the motor domain at the C-

terminus of the protein and generally move towards the minus end. Finally, the KIN I subfamily, which contains an internal motor domain, have a microtubule destabilizing activity but do not actually have motor activity (Desai et al., 1999). Dyneins are structurally unrelated to kinesins but they also use the hydrolysis of ATP to glide along microtubules. They move only in a minus-end-directed manner (Wang et al., 1995).

### 2.13. Motor proteins in *Saccharomyces cerevisiae*

The budding yeast genome encodes six kinesin-related proteins and a single dynein (Dyn1p). Five of the six kinesin-related proteins have been implicated in mitotic spindle function: Cin8p, Kip1p, Kip2p, Kip3p and Kar3p. All single deletions are viable, due to extensive overlap in function between these motors. It is therefore not easy to attribute distinct functions to each motor. Cin8p and Kip1p are members of the BimC subclass and are required for formation and maintenance of the bipolar spindle (Hoyt and Geiser, 1996; Kashina et al., 1997). They are thought to be plus end directed motors based on homology with the BimC family even though no direct evidence for this directionality exists. In addition, they make a major contribution to anaphase B spindle elongation (Saunders et al., 1995; Straight et al., 1998). Cin8p localizes to kinetochores throughout the cell cycle and is found at the midzone in late anaphase (He et al., 2001; Hoyt et al., 1992). Kip2p plays a role in cytoplasmic microtubule stabilization (Huyett et al., 1998). The directionality of this motor has not been studied yet. Kar3p antagonizes the pole-separating activity of the BimC motors during spindle assembly and elongation (Saunders et al., 1997; Saunders and Hoyt, 1992). It is also thought to participate in the positioning of the spindle (Cottingham and Hoyt, 1997; DeZwaan et al., 1997). Kar3p has been shown to be a minus end

directed motor, consistent with its motor domain being at the C-terminus of the protein (Endow et al., 1994). Finally, Dyn1p, the only dynein, is believed to act on cytoplasmic microtubules and to participate in nuclear migration (Eshel et al., 1993; Li et al., 1993; Yeh et al., 1995). In addition, Dyn1p also plays a role in spindle assembly and elongation together with Cin8p and Kip1p (Saunders et al., 1995; Yeh et al., 1995).

Kip3p is a member of the KINI family (Severin et al., 2001a). This family of motors has been shown to have microtubule destabilizing activity in several organisms (Desai et al., 1999; Garcia et al., 2002; Kline-Smith and Walczak, 2002; Moores et al., 2002; Walczak et al., 1996). In budding yeast, Kip3p is required for spindle disassembly: Straight et al. showed that *kip3Δ* mutant cells have a spindle breakdown delay (Straight et al., 1998), consistent with Kip3p localization to the midzone in anaphase (DeZwaan et al., 1997). In addition, Kip3p also regulates cytoplasmic microtubules and plays a role in the migration of the nucleus to the bud neck and the proper alignment of the mitotic spindle before anaphase (Cottingham and Hoyt, 1997; DeZwaan et al., 1997).

### 2.14. Non-motor microtubule associated proteins in *Saccharomyces cerevisiae*

In budding yeast, several non-motor microtubule associated proteins exist, however for the purpose of this work, two are relevant: Stu2p and Ase1p. Stu2p is the budding yeast member of the protein family that includes *Schizosaccharomyces pombe* Dis1 (Nabeshima et al., 1998), human TOGp (Charrasse et al., 1998), *Caenorhabditis elegans* ZYG-9 (Matthews et al., 1998) and *Xenopus* XMAP215 (Tournebize et al., 2000). Members of this protein family have been shown to promote microtubule growth in other organisms (Charrasse et al., 1998; Cullen et al.,

1999; Matthews et al., 1998; Tournebize et al., 2000). In *Xenopus* and budding yeast, this family has been shown to oppose the action of the KIN1 subfamily of kinesins (Severin et al., 2001a; Tournebize et al., 2000). Stu2p is an essential microtubule protein in budding yeast (Wang and Huffaker, 1997). It localizes to kinetochores, cytoplasmic microtubules and the spindle midzone in anaphase (He et al., 2001; Kosco et al., 2001; Wang and Huffaker, 1997). It promotes microtubule dynamics (Kosco et al., 2001) and plays a role in spindle orientation, metaphase chromosome alignment and spindle elongation (Kosco et al., 2001; Severin et al., 2001a).

Ase1p is a non-essential microtubule-associated protein (Juang et al., 1997). It contains the common features of MAPs from other organisms even though it does not have homology to known MAPs (Pellman et al., 1995). It localizes to the midzone in anaphase (Pellman et al., 1995). The protein is degraded by APC-mediated proteolysis and a non-degradable version of the protein delays spindle disassembly (Juang et al., 1997). In addition, it cooperates with Bik1p, the budding yeast homolog of the human MAP protein CLIP-170 to promote spindle elongation (Pellman et al., 1995; Pierre et al., 1992).

### 2.15. Kinetochores

The kinetochore is a multiprotein complex that assembles on the centromeric DNA (for review Cheeseman et al., 2002b). The budding yeast *Saccharomyces cerevisiae* centromere consists of approximately 125 basepairs of DNA that can be divided into three major regions (Clarke and Carbon, 1980): CDEI, CDEII, and CDEIII. More than 40 proteins are known kinetochore proteins so the following list is not exhaustive. *CEN* DNA is thought to be wrapped around a specialized nucleosome containing Cse4p, a histone H3 variant (Meluh et al., 1998). CDEIII, which is

essential for kinetochore function, binds a complex called CBF3 (for review see Pidoux and Allshire, 2000). The CBF3 complex is composed of four proteins including Ndc10p (Lechner and Carbon, 1991). Additional complexes localizing to the kinetochore are: the four subunit Ndc80 complex (Janke et al., 2001; Wigge and Kilmartin, 2001) and the twelve subunit Cft19 complex (Cheeseman et al., 2002a; Ghosh et al., 2001; Goshima and Yanagida, 2000; Measday et al., 2002; Ortiz et al., 1999). The DASH complex is a nine subunit complex that contains the Dam1 protein (Cheeseman et al., 2002a; Janke et al., 2002; Li et al., 2002). This complex is thought to be more distal from the DNA and play a role in microtubule attachment, being necessary to establish and maintain bi-orientation (Janke et al., 2002; Jones et al., 2001; Li et al., 2002). In addition, components of the DASH complex localize along the length of the mitotic spindle where they play a role in spindle integrity (Cheeseman et al., 2001; Hofmann et al., 1998; Jones et al., 1999). The Slk19 protein localizes to kinetochores. It also localizes at the spindle midzone in anaphase and plays a role as a spindle microtubule-stabilizing protein both at the kinetochores and at the midzone (Zeng et al., 1999). Finally, the Ipl1/Aurora protein kinase localizes to kinetochores (see below).

### 2.16. The Aurora protein kinase family

The yeast Ipl1 and the *Drosophila* Aurora proteins are the founding members of a conserved serine/threonine protein kinase family (Ipl1/Aurora) that are key regulators of chromosome segregation and cytokinesis (Chan and Botstein, 1993; Glover et al., 1995) (for reviews see Shannon and Salmon, 2002; Stern, 2002). In mammals, the Aurora family is further subdivided into 3 families: Aurora A, B and C (for review, see Nigg, 2001). Aurora A is required to maintain the separation of

centrosomes and to make a bipolar spindle; it localizes at the centrosome (Glover et al., 1995). Aurora C is mainly expressed in the testis and has not been well characterized yet (Kimura et al., 1999). Aurora B is the most extensively studied. It exhibits a "chromosomal passenger" localization pattern, where it localizes to the chromosomes and kinetochores, transfers to the spindle, and eventually accumulates at the spindle midzone and midbody (Bischoff et al., 1998; Murata-Hori et al., 2002; Petersen et al., 2001; Schumacher et al., 1998; Terada et al., 1998).

Aurora A and B in humans have been shown to be oncogenes and to be amplified in many colorectal and breast cancer cell lines (Bischoff et al., 1998; Zhou et al., 1998). In addition, expression of an activated Aurora B kinase can transform Rat1 fibroblasts and NIH3T3 cells *in vitro* as well as cause tumors in nude mice (Bischoff et al., 1998). These data suggest that defects in the regulation of the Ipl1/Aurora kinases can lead to genomic instability.

### 2.17. Ipl1/Aurora binding partners

Aurora B has been shown to interact with the INCENP protein (inner centromere protein) and with the Survivin/Bir1 protein in several organisms, including budding yeast (Adams et al., 2000; Bolton et al., 2002; Cheeseman et al., 2002a; Kaitna et al., 2000; Kim et al., 1999; Leverson et al., 2002; Morishita et al., 2001; Rajagopalan and Balasubramanian, 2002; Speliotes et al., 2000). Defects in INCENP localization, which was the first chromosomal passenger proteins described (Cooke et al., 1987), disrupt Aurora B localization, suggesting that at least one function of the interaction may be to localize Aurora B to mitotic structures (Adams et al., 2000). In addition, in budding yeast the INCENP homolog, called Sli15p, plays a role in stimulating Ipl1p's kinase activity (Kang et al., 2001; Kim et al., 1999).

Survivin/Bir1 is a conserved protein of unknown function that contains baculoviral inhibitor-of-apoptosis repeats (BIR) (Ambrosini et al., 1997). The BIR motifs were first identified in proteins acting in the apoptotic pathway of cell death (Deveraux and Reed, 1999). Recent studies have shown that some of the BIR-containing proteins are also important for other fundamental cellular processes. Survivin/bir1 are also chromosome passenger proteins and they also play a role in localizing Ipl1/Aurora and stimulating its kinase activity (Bolton et al., 2002; Morishita et al., 2001; Rajagopalan and Balasubramanian, 2002; Skoufias et al., 2000; Speliotes et al., 2000; Uren et al., 2000; Yoon and Carbon, 1999). The budding yeast member of this family is called Bir1p (Yoon and Carbon, 1999). Aurora, INCENP and Survivin work together as a complex to accomplish their essential functions in chromosome segregation and cytokinesis (Adams et al., 2000; Bolton et al., 2002; Kaitna et al., 2000; Kim et al., 1999; Leverson et al., 2002; Morishita et al., 2001; Rajagopalan and Balasubramanian, 2002; Speliotes et al., 2000).

### 2.18. Ipl1p In *Saccharomyces cerevisiae*

In budding yeast, there is a single Aurora protein kinase, Ipl1p (Chan and Botstein, 1993; Francisco et al., 1994). Ipl1p, which stands for increase in ploidy, has been isolated in screens for chromosome segregation defects (Biggins et al., 2001; Chan and Botstein, 1993). The protein contains a highly conserved C-terminal catalytic domain and a divergent N-terminal domain. All the members of the Ipl1/Aurora family have a potential destruction box at the C-terminus of the protein, which is known to direct APC-dependent degradation (Giet and Prigent, 1999; Glotzer et al., 1991). In addition, they all contain a conserved phosphorylation motif RRXT in the activation loop that confers positive regulation in a number of kinases

(Boulton et al., 1990; Giet and Prigent, 1999; Hanks and Quinn, 1991). However, it is unclear whether those two domains play any function in the regulation of Ipl1p.

In budding yeast, as in higher eukaryotes, Ipl1p is required for chromosome segregation. In *ipl1* mutant cells, sister chromatids are pulled to the same spindle pole instead of segregating to opposite poles even though sister chromatid separation occurs normally (Biggins et al., 1999; Kim et al., 1999) (Figure 2.4). Wild type and

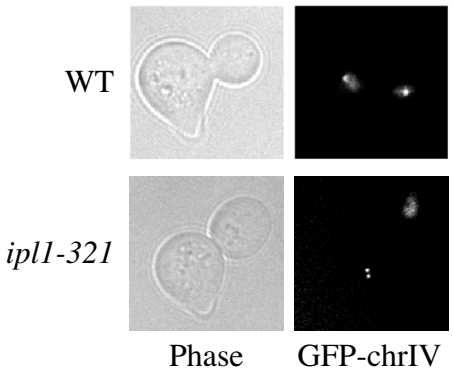


**Figure 2.4.** *ipl1-321* mutant cells missegregate their chromosome. Wild type and *ipl1-321* cells were shifted to the restrictive temperature (37ºC) for 3 h. and harvested for microscopy. Sister chromatids were visualized by GFP-lacI binding to lactose operators integrated 12 kb from the centromere of chromosome IV. In wild type cells, sister chromatids always segregate to opposite poles (top panel) while in *ipl1-321* cells sister chromatids travel to a single pole in the majority of cells (bottom panels).

temperature sensitive *ipl1* mutant cells expressing GFP (green fluorescent protein)-tagged chromosome IV were shifted to the non-permissive temperature for 3 hours. After fixation, the cells were analyzed by microscopy. Whereas, in wild type anaphase cells, sister chromatids segregated to opposite poles, in *ipl1* mutant cells, the sister chromatids travel to the same pole, even though they have separated. Experiments *in vitro* and *in vivo* suggested that the segregation defect might be due to an inability of an *ipl1* mutant to release mono-oriented kinetochore-microtubules attachment in order to make the correct bi-oriented attachments (Biggins et al., 1999; Tanaka et al., 2002). In addition to chromosome segregation, Ipl1p is also required for the spindle checkpoint when kinetochores are not under tension (Biggins and Murray, 2001). Therefore, despite a major chromosome segregation defect, *ipl1* mutant cells do not activate the checkpoint. Figure 2.5 represents a model for Ipl1p function in chromosome segregation. When kinetochores attach to microtubules from the same

pole, mono-oriented attachments are generated. Although kinetochore attachment is fulfilled, no tension is generated at the kinetochore and the spindle checkpoint is activated. Ipl1p releases the incorrect microtubule-kinetochore attachment, allowing the cell to make a bi-oriented attachment. Accordingly, as in higher eukaryotes, Ipl1p has been shown to localize to kinetochores and to the spindle (Biggins and Murray, 2001; Biggins et al., 1999; He et al., 2001; Kang et al., 2001; Tanaka et al., 2002).

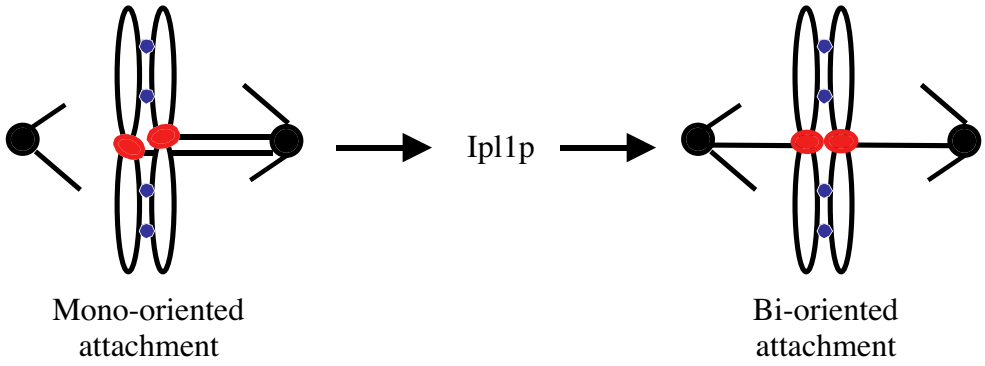


**Figure 2.5.** Model for Ipl1p's functions. Kinetochores are represented by red dots and cohesins by blue dots. When kinetochores attach to microtubules from the same poles (mono-oriented attachment, shown on the left) a tension defect is sensed. Ipl1p then releases kinetochore-microtubule attachment allowing the cell to generate bi-oriented attachment.

Finally, it has been suggested that Ipl1p is opposed by the protein phosphatase I activity of Glc7p in budding yeast (Francisco and Chan, 1994; Francisco et al., 1994). Not surprisingly, protein phosphatase I is required for proper chromosome segregation (Hisamoto et al., 1994; Sassoon et al., 1999).

### 2.19. The Ipl1/Aurora substrates

A number of Ipl1/Aurora substrates have been identified in various organisms, however the relevance of most of them is undetermined. A putative Ipl1p consensus site has recently been determined ({RK}x{TS}{ILV}) (Cheeseman et al., 2002a). However, since this is such a recent discovery, most of Ipl1p potential substrates have

not yet been confirmed by mutation of the phosphorylation site. In *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* histone H3 is phosphorylated by Ipl1p or its respective homologs (Giet and Glover, 2001; Hsu et al., 2000). However, the function of this phosphorylation is unclear, as unphosphorylatable form of H3 does not have a detectable phenotype. In fission yeast, Bir1, the Survivin homolog, has been shown to be an *in vitro* substrate for the Ipl1/Aurora kinase (Leverson et al., 2002). In budding yeast, Ndc10p, a kinetochore protein, has been reported to be a substrate for Ipl1p *in vitro* (Biggins et al., 1999). In human cells, CENP-A, the histone H3- like homolog, has been shown to be a substrate of the Aurora B kinase. This phosphorylation seems to be implicated in cytokinesis, as non-phosphorylable CENP-A have defects in cytokinesis (Zeitlin et al., 2001). Finally, a recent study in budding yeast identified six different kinetochore proteins as Ipl1p targets: Dam1p, Spc34p, Ask1p, Ndc80p, Sli15p, the INCENP homolog and Ipl1p itself (Cheeseman et al., 2002a; Li et al., 2002) (Kang et al., 2001). Dam1p, Spc34p and Ask1p are all members of the DASH complex (see above) (Janke et al., 2002; Li et al., 2002). Phosphorylation of this complex and especially of Dam1p by Ipl1p plays an essential role in the transition from mono-oriented to bi-oriented attachment (Cheeseman et al., 2002a). Taken together, as the list of Ipl1p potential substrates is growing, future experiments will have to address which of the targets are essential for the different functions of Ipl1p.

### 2.20. Aim of my thesis

As described above, vertebrate homologues of the Ipl1/Aurora protein kinase show a distinct subcellular localization during the cell cycle. In an attempt to learn more about the regulation of this protein kinase and to study potentially new

functions, I focused on the regulation of Ipl1p localization throughout the cell cycle. This is described here in addition to a new function for Ipl1p that came from the localization studies. The last chapter investigates a new function of the spindle checkpoint in regulating sister chromatid separation and spindle elongation in the absence of APC function.

# CHAPTER 3. REGULATION OF Ipl1p LOCALIZATION

## 3.1 SUMMARY

Ipl1p localizes to kinetochores in metaphase but is absent from the kinetochores in anaphase and instead localizes to the spindle. I decided to study Ipl1p localization throughout the cell cycle in more detail. I found that Ipl1p localizes to kinetochores from G1 to metaphase, it then transfers to the spindle and accumulates at the midzone and travels back to the poles. Its localization is therefore similar to chromosomal passenger proteins and therefore Ipl1p seems more closely related to the Aurora B family members. Ipl1p anaphase localization is going to be discussed in more details in chapter 4. In this chapter I am going to investigate the requirements for Ipl1p leaving the kinetochores at metaphase. Ipl1p plays an essential role in setting up bi-orientation. It has been proposed that Ipl1p acts by releasing mono-oriented attachments in order to generate bi-orientation, the state when sister chromatid are attached to microtubules from opposite poles. The protein therefore has to be tightly regulated in order to stop its action once bi-orientation is established. It is possible that part of the regulation is obtained by regulating its localization, as it is so dynamic. I therefore reasoned that elucidating the regulation of its relocalization from kinetochores to the spindle might be important for understanding this protein. I found that relocalization is independent of tension establishment, cohesin unloading, APC function and checkpoint inactivation. From the experiments presented here, I propose that Ipl1p is probably transported away from the kinetochores; however, the precise mechanism is still unclear (see discussion). In addition, complex formation with Bir1p is necessary for optimal transport.

## 3.2. RESULTS

### 3.2.1. Ipl1 is a chromosomal passenger protein

The Ipl1 protein kinase is required for chromosome segregation and the spindle checkpoint when kinetochores are not under tension (Biggins and Murray, 2001; Biggins et al., 1999). To learn more about Ipl1p functions, I examined the localization of endogenous Ipl1p fused to GFP at its C-terminus in living cells, using bud size as a marker for cell cycle stage (Figure 3.2.1.A). In unbudded G1 cells, Ipl1-GFP localizes as a dot. In some G1 cells, Ipl1p also localizes as a small line (data not shown). In small budded S-phase cells, Ipl1p localizes as a distinct dot that separates into two discrete dots in medium budded metaphase cells and corresponds to precociously separated kinetochores (Biggins and Murray, 2001; Goshima and Yanagida, 2000; He et al., 2000; He et al., 2001; Pearson et al., 2001; Tanaka et al., 2000). In large budded cells, Ipl1-GFP exhibits dynamic localization patterns: in some cells, Ipl1-GFP is on the whole spindle but absent from the kinetochores that are clustered at the poles as previously reported (Tanaka et al., 2002). I also found two sites of Ipl1p localization that had not been seen before: Ipl1-GFP is found at the spindle midzone, the region of the spindle where interpolar microtubules interdigitate, as well as in small tufts that vary in shape near the spindle poles at telophase. This likely represents Ipl1p bound to the remnants of depolymerized microtubules (Winey et al., 1995).

Since the Ipl1-GFP fusion created a temperature sensitive protein, I also localized endogenous Ipl1p using anti-Ipl1p antibodies (see Figure 4.2.2 for antibody specificity) by performing immunofluorescence on chromosome spreads, the detergent-insoluble residue of yeast spheroplasts (Loidl et al., 1998) (Figure 3.2.1.B). Ipl1p co-localizes with the epitope tagged kinetochore protein Cse4-myc12 in G1 and

S phase (data not shown) and in metaphase when Cse4p-myc12 separates into two discrete dots (Figure 3.2.1.B). In anaphase, Ipl1p exhibited localization patterns similar to Ipl1-GFP and co-localized with tubulin to the spindle (Figure 3.2.1.B) and spindle midzone (data not shown). Therefore, GFP-tagged Ipl1p shows an identical localization pattern as the endogenous protein and is similar to Aurora B and other "chromosomal passenger" proteins: it localizes to kinetochores from G1 to metaphase and then to the spindle and the spindle midzone but is absent from the kinetochores. Ipl1p localization at the midzone is discussed in more detail in Chapter 4. In the current chapter, I am going to investigate the requirement(s) for Ipl1p leaving the kinetochores. As it seems that Ipl1p leaving the kinetochores happens between metaphase and anaphase, several events could be responsible for its delocalization: the establishment of tension (3.2.2.), the unloading of the cohesin complex (3.2.3.), the inactivation of the checkpoint (3.2.4.) etc… In addition, it is possible that the protein is being transported away from the kinetochores by an active mechanism or that it is being degraded at the kinetochores and that another pool is loaded onto the spindle. I set out to test these possibilities.

### 3.2.2. Ipl1p localizes to kinetochores under tension

The laboratory previously found that Ipl1p localizes to kinetochores that are not under tension and that it is required for the spindle checkpoint at this time (Biggins and Murray, 2001). Once tension is established, Ipl1p needs to be inactivated to allow cell cycle progression. Although it was reported that Ipl1p no longer co-localizes with the kinetochore protein Ndc10p at metaphase (Tanaka et al., 2002), I always found Ipl1p localized in discrete dots. I therefore repeated the Ipl1-YFP and Ndc10-CFP co-localization experiment published by Tanaka et al. In

asynchronously growing cells, Ipl1-YFP and Ndc10-CFP always co-localized as characteristic kinetochore dots in S-phase and metaphase cells (Figure 3.2.2.A). In addition, they also co-localized as a line that looks like a short spindle in some cells (Figure 3.2.2.A, early anaphase). Since the pole-to-pole distance is longer than a metaphase spindle, it likely represents cells that are initiating anaphase. However, as previously reported (Tanaka et al., 2002), in late anaphase Ipl1-YFP and Ndc10-CFP show a different localization pattern. Ipl1-YFP localizes onto the whole spindle in a punctate manner. Ndc10-CFP localizes to the whole spindle as well, but it also localizes with the kinetochores that are clustered at the poles, as seen by strong staining at the poles. In the merged image, it is clear that both proteins co-localize to the spindle but that only Ndc10p is found at the kinetochores near the poles. This confirms that in late anaphase Ipl1p has left the kinetochores. It is to note that sometimes a partial co-staining between Ipl1-YFP and Ndc10-CFP can be observed near one of the two poles. This could represent Ipl1p having incompletely left the kinetochores in one of the two clusters or co-localization between Ipl1p and Ndc10p on the spindle very close to the kinetochores. I do favor the later hypothesis as in most cases co-staining of the two proteins near one pole is not observed. Co-localization studies between Ipl1p a kinetochore protein that does not localize to the spindle such as Cse4p will be necessary to elucidate this point.

I also analyzed Ipl1p localization in a population of metaphase-arrested cells by depleting the Cdc20 protein that activates the APC and found that Ipl1-YFP and Ndc10-CFP always co-localize (Figure 3.2.2.B). Although the majority of cells show two distinct dots that represent the kinetochores, approximately one-third of the cells exhibit co-localization in a line similar to certain cells in the asynchronous population (data not shown). This may mean that Ipl1p can transfer to the spindle in this arrest,

or that these cells do not have clustered kinetochores. Ipl1p also discretely localizes to kinetochores when cells are arrested in metaphase by overexpression of the Mps1 protein kinase that causes cells to constitutively activate the spindle checkpoint (Hardwick et al., 1996) (data not shown). Therefore, although Ipl1p leaves kinetochores after metaphase, it is still bound to kinetochores that are under tension, indicating that the establishment of tension is not responsible for Ipl1p relocalization. I use relocalization as a general term and it does not imply any mechanism.

### 3.2.3. Ipl1p localization is not dependent on cohesion

Since Ipl1p left kinetochores after the establishment of tension, I tested whether the unloading of the cohesin complex was required for Ipl1p leaving the kinetochores. In *Schizosaccharomyces pombe*, the cohesin complex is required for Ark1 (the Ipl1p homolog in this organism) localization to kinetochores (Morishita et al., 2001). I therefore analyzed Ipl1p localization in cells in the absence of cohesion. To do this, I used a strain that expressed one of the proteins of the cohesin complex: Scc1p under the control of a conditional promoter, the galactose inducible promoter: *pGAL*. *pGAL-SCC1* cells expressing Ipl1-GFP, grown in galactose were arrested in G1 using α-factor for 2.5 hours. The cells were then shifted to glucose for 1 hour to repress the expression of Scc1p. They were then released into the cell cycle in glucose to repress Scc1p. Time points were taken and cells were analyzed by microscopy. Cell cycle position was determined by bud size. Figure 3.2.3. shows that Ipl1-GFP is able to load onto kinetochores in the absence Scc1p (S-phase and metaphase pictures). The kinetochores cluster less well under these conditions and therefore the signal is more diffuse than normally seen (see Figure 3.2.3). In addition, chromosome segregation is abnormal so the intensity of the two clusters is not always equal (data

not shown) (Guacci et al., 1997; Michaelis et al., 1997). I analyzed cell viability during the experiment and found that the cells were dying, indicating that Scc1p is truly depleted. Under these conditions, Ipl1-GFP is still able to transfer onto the spindle at anaphase. No pole signal is observed, demonstrating that Ipl1p has left the kinetochore at that point. Finally, Ipl1-GFP is found near the poles in telophase. I also analyzed Ipl1p localization in an *scc1* mutant (*mcd1-1*) background by chromosome spreads using anti-Ipl1p antibodies (Guacci et al., 1997). Under those conditions, I also found normal Ipl1p loading onto the kinetochores (data not shown). However, the relocalization to the spindle was impossible to assess due to the fragility of the spindle in this background. The spindle is intrinsically fragile and breaks down soon after elongating (Severin et al., 2001b). In addition, the chromosome spread technique does not always fully preserve the spindle. Therefore, I could not detect intact spindles in the spreads that would allow us to determine whether Ipl1p had transferred onto the spindle. In conclusion, Ipl1p is able to load onto the kinetochores in the absence of the cohesin complex. In addition, the relocalization from kinetochores to the spindle occurs normally in the absence of cohesion, as judged by the *pGAL-SCC1* experiment, indicating that Ipl1p relocalization from kinetochores does not require the cohesin complex. The apparent difference between budding and fission yeast is discussed below.

### 3.2.4. Checkpoint inactivation does not regulate Ipl1p relocalization to the spindle

Because tension establishment and cohesin unloading did not regulate Ipl1p relocalization to the spindle, I next tested whether the inactivation of the spindle checkpoint was required for Ipl1p relocalization. I therefore analyzed the effect of

checkpoint inactivation on Ipl1p localization in a condition where Ipl1p would normally stay on the kinetochores. Nocodazole is a microtubule depolymerizing drug. In the presence of nocodazole, the spindle is depolymerized leading to the activation of the spindle checkpoint, and therefore a metaphase arrest. It was previously published that Ipl1p localizes to the kinetochores under these conditions (Biggins and Murray, 2001). Wild type and *mad1Δ* mutant cells expressing Cse4-myc to mark the kinetochores were arrested in G1 using α-factor and then released into the cell cycle in the presence of nocodazole. After 2.5 hours, the cells were fixed and processed for chromosome spreads (see Figure 3.2.4.). In parallel, cells were analyzed by microscopy to ensure that wild type cells were arrested as large budded cells whereas a majority of *mad1Δ* mutant cells had rebudded, indicating that the cells are checkpoint deficient. As I harvested the cells 2.5 hours after G1 arrest and as I observed a large amount of rebudded the cells, the *mad1Δ* mutant cells where no longer in metaphase. The cells were at a cell cycle stage that was after metaphase and so, at least in a portion of cells, Ipl1p should no longer localize to kinetochores. The chromosome spreads were stained with DAPI to recognize DNA (far right), with anti-Ipl1p antibodies to recognize Ipl1p (shown in green) and with anti-myc to recognize Cse4-myc (shown in red). The merge (shown in yellow, far right) shows that Ipl1p localizes to kinetochores in wild type cells in the presence of nocodazole as previously reported. In addition, Ipl1p also localizes to the kinetochores in *mad1Δ* mutant cells. Therefore, the checkpoint is not required for Ipl1p localization to kinetochores. In addition, the presence of microtubules seems to be important for Ip1lp leaving the kinetochores as Ipl1p is unable to leave the kinetochores in a *mad1Δ* mutant in the absence of microtubules even though other cell cycle events occur normally.

### 3.2.5. Ipl1p does not leave the kinetochores in a strain with a mutant Bir1p.

In budding yeast, Ipl1p forms a complex at the kinetochores with the INCENP homolog, Sli15p and the Survivin homolog, Bir1p (Cheeseman et al., 2002a; Kim et al., 1999). I decided to analyze whether complex formation was required for Ipl1p localization to the kinetochores and/or transfer onto the spindle. Only the effect of a mutation in Bir1p was studied. *BIR1* is an essential gene. I tried to delete the gene by disrupting the N-terminus of the protein. However, cells containing the *bir1ΔN-terminus* mutation do not show a major growth defect suggesting that deleting the N-terminus of the protein does not inactivate the whole protein and that I created a mutant allele of Bir1p. Alternatively it is possible that the strain contains a suppressor. Even though the exact reason for survival of this strain is unclear, I studied the localization of Ipl1p in this background. Cells with a mutant version of Bir1p (*bir1ΔN-terminus*) expressing Ipl1-GFP were analyzed by microscopy in an asynchronously growing population. Ipl1p localizes normally to kinetochores in G1, S-phase and metaphase (Figure 3.2.5. and data not shown). In anaphase, Ipl1p localizes to the spindle as seen in a wild type background. However, in all anaphase cells observed, there is strong staining at the poles of the spindle most likely corresponding to kinetochores. Therefore, even though Ipl1p localizes to the spindle it has not left the kinetochores. In telophase cells, Ipl1p still localizes to kinetochores as well as the depolymerized spindle. It is noteworthy that chromatin immunoprecipitation studies will be required to confirm that the strong pole staining observed in the *bir1* mutant cell corresponds to kinetochores and not SPBs. However, with the current knowledge it most likely corresponds to kinetochores. In conclusion, in a background mutant for Ipl1p's partner Bir1p, Ipl1p does not leave the kinetochore properly but is still able to localize to the spindle.

### 3.2.6. Ipl1p's kinase activity is not required for its localization.

I next wanted to investigate whether Ipl1p's kinase activity was required for its localization. To do this, I used an allele of Ipl1p (*ipl1-as5*) where the ATP binding site of the protein kinase of interest is mutated (a gift from the Shokat laboratory). *ipl1-as5* is mutated at the methionine at position 18, which is changed to a glycine. The Shokat and Morgan labs have shown that these mutants are selectively sensitive to inhibitors that inactivate the mutant kinase but do not affect the wild type kinase activity (Bishop et al., 2000; Shah et al., 1997). We have determined that the *ipl1-as5* mutant is sensitive to the kinase inhibitors 1-naphthyl-PP1 (1-NA, Figure 3.2.7) and 1-naphthylmethyl-PP1 (1-NM) (data not shown). S. Tatsutani has also shown that the kinase activity is abolished in the presence of the inhibitor 1-NA (S. Tatsutani personal communication).

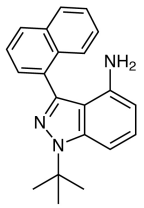


**Figure 3.2.7.** Structure of the inhibitor that was used to inhibit the *ipl1-as5* allele.

I therefore tagged this mutant with GFP to determine whether Ipl1p's kinase activity was required for its localization. *ipl1-as5-GFP* mutant cells were arrested in G1 using α-factor and then released into the cell cycle in the presence of inhibitor (1-NA) to inactivate Ipl1-as5p. In a parallel experiment, S. Biggins has shown that the concentration of inhibitor used resulted in cell death. Time points were taken and the cells were analyzed by microscopy. Bud size was used to determine the cell cycle stage. Ipl1-as5p was able to load onto kinetochores (data not shown), implying that the kinase activity of Ipl1p was not necessary for its loading onto kinetochores. In

metaphase, Ipl1-as5 localized as either one dot (see Figure 3.2.6.A top), two dots of unequal intensity (Figure 3.2.6.A bottom) or more rarely as two dots of equal intensity as normally seen (data not shown). This is due to the fact that Ipl1p is required for setting up bi-orientation. In the absence of Ipl1p activity, most kinetochore attachments are mono-oriented and therefore unable to show precocious separation. This shows that Ipl1p-as5p has been inactivated in this experiment. In anaphase, Ipl1-as5p localizes to the whole spindle but is absent from the kinetochores as seen for Ipl1p, indicating that Ipl1p is able to relocalize to the spindle independently of its kinase activity. In telophase, Ipl1p localizes to the poles. Therefore, Ipl1p's kinase activity is not required for Ipl1p loading onto the kinetochore or for its relocalization from the kinetochores to the spindle.

Ipl1p has a characteristic N-terminal domain and a catalytic C-terminal kinase domain. I was interested in determining what region(s) of the protein mediates its different localization patterns. To do this, I first split the protein into two regions: the N-terminus and the catalytic domain and tested which region could localize. Wild type cells (Figure 3.2.6.B, top row), cells containing an extra copy of full-length Ipl1-myc under the control of the galactose promoter (*pGAL-myc12-IPL1,* second row), cells containing an extra copy of the N-terminus of Ipl1p under the control of the galactose promoter (*pGAL-myc12-IPL1-N-terminus*, third row) and cells containing an extra copy of the catalytic domain of Ipl1p under the control of the galactose promoter (*pGAL-myc12-IPL1-catalytic domain,* fourth row) were processed for immunofluorescence after one hour of induction. They were stained with anti-Tub1p to recognize the spindle (far left), with anti-myc to recognize Ipl1-myc (shown in red) and with DAPI to recognize DNA (shown in blue). The merge between Ipl1-myc and the DNA is shown in purple on the far right. The staining is specific to Ipl1-myc, as

an untagged strain does not show any signal (top row). When overexpressed, Ipl1p localizes to the entire nucleus. Because the protein is overexpressed to high levels, it is not possible to see distinct kinetochore localization. Full-length Ipl1p also localizes to the spindle (second row). The N-terminus localizes similarly to full-length (third row) and is found in the entire nucleus. In addition, the N-terminus localizes to the spindle. In contrast, the catalytic domain is not able to localize properly (fourth row), and is found all over the cell. In conclusion, the N-terminus of the protein contains the information for localization. However, due to the strong overexpression, kinetochore localization cannot be determined with this assay.

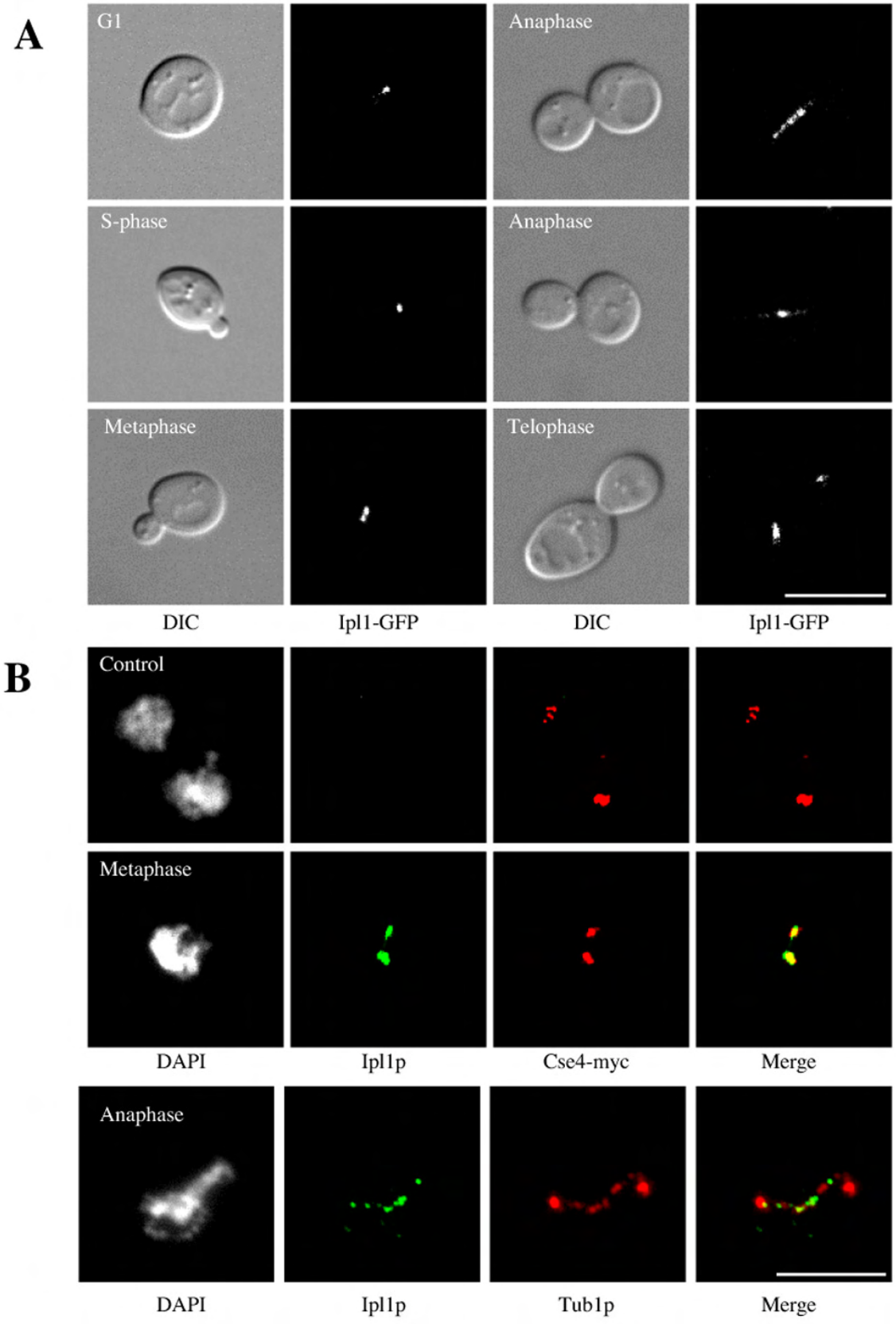
A
G1
Anaphase
S-phase
Anaphase
Metaphase
Telophase
DIC
Ipl1-GFP
DIC
Ipl1-GFP
B
Control
Metaphase
DAPI
Ipl1p
Cse4-myc
Merge
Anaphase
DAPI
Ipl1p
Tub1p
Merge

**Figure 3.2.1.** Ipl1p is a chromosomal passenger protein. (A) Live microscopy was performed on cells containing Ipl1-GFP (SBY556). DIC pictures are shown to the left of each corresponding fluorescence picture. In G1, S-phase and metaphase cells, Ipl1p localizes in a discrete dot corresponding to kinetochores. In anaphase and telophase cells, Ipl1p localizes along the whole spindle (top right), at the spindle midzone (middle right) or near the spindle poles (bottom right). (B) Chromosome spreads (SBY617) were stained with DAPI to recognize DNA (left panels), with or without (control) anti-Ipl1p antibodies, and with anti-myc antibodies to recognize the kinetochore protein Cse4-myc12. In the bottom row, spreads were stained with anti-Tub1p antibodies to recognize the spindle. The merged images (right panels) show that endogenous Ipl1p exhibits the same localization pattern as Ipl1-GFP and localizes to the kinetochores in metaphase and to the spindle and spindle midzone during anaphase. Bar: 10 μm.

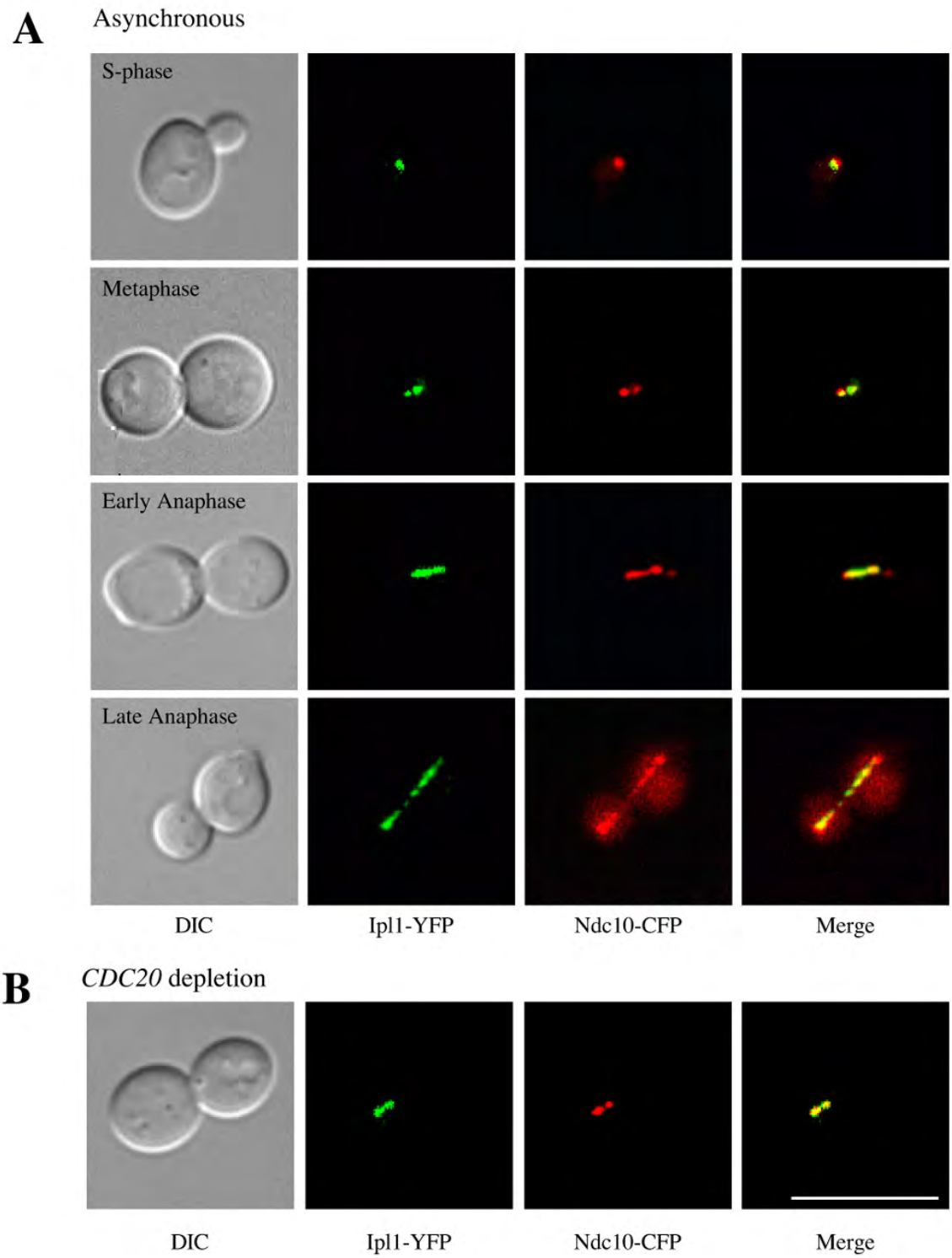
A
Asynchronous
S-phase
Metaphase
Early Anaphase
Late Anaphase
DIC
Ipl1-YFP
Ndc10-CFP
Merge
B
CDC20 depletion
DIC
Ipl1-YFP
Ndc10-CFP
Merge

**Figure 3.2.2.** Ipl1p localizes to kinetochores until after metaphase. (A) Microscopy was performed on cells containing Ipl1-YFP (shown in green) and Ndc10-CFP (shown in red) (SBY1246). DIC pictures are shown on the far left. The merged image (yellow, far right) shows that Ipl1p and Ndc10p co-localize in S-phase (first row), in metaphase cells where kinetochores are precociously separated (second row) and in early anaphase cells (third row). However, in late anaphase (fourth row), Ipl1-YFP does not localize to kinetochores. (B) *pGAL-CDC20* cells containing Ipl1-YFP and Ndc10-CFP (SBY1246) were arrested in metaphase by shifting the cells to glucose media. The merged microscopy images show that Ipl1p and Ndc10p also co-localize in metaphase-arrested cells. Bar: 10 μm.

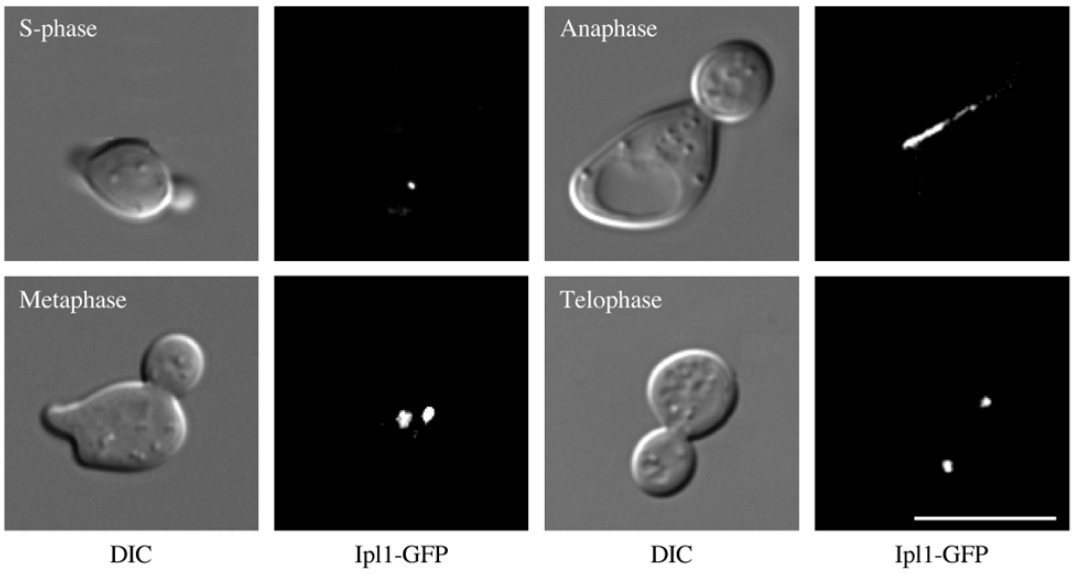


**Figure 3.2.3.** Ipl1p localization is normal in *pGAL-SCC1* cells. *pGAL-SCC1* cells expressing Ipl1-GFP (SBY1475) were grown in galactose, arrested in G1 using α-factor for 2.5 h, were shifted to glucose for 1 h. The cells were then released into the cell cycle in glucose. Time points were taken and cells were analyzed by microscopy. Cell cycle position was determined by bud size. Ipl1p is able to load onto kinetochores without Scc1p (S-phase, metaphase) and to transfer onto the spindle normally (Anaphase). Bar: 10 μm.

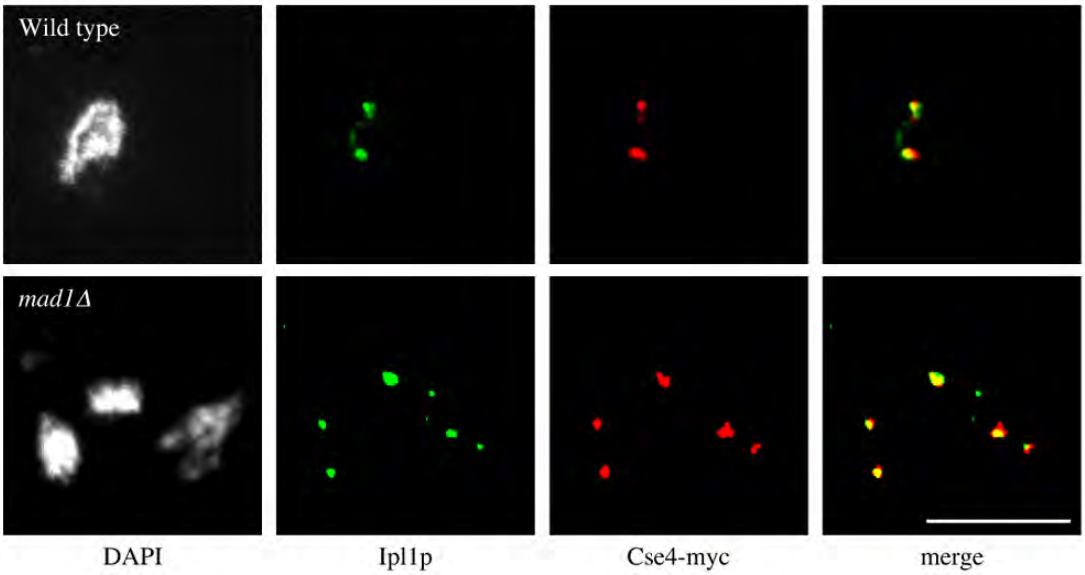


**Figure 3.2.4.** Checkpoint inactivation does not regulate Ipl1p relocalization to the spindle. Wild type (SBY1323, top row) and *mad1Δ* mutant (SBY1326, bottom row) cells expressing Cse4-myc were arrested in G1 using α-factor and then were released into the cell cycle in the presence of nocodazole. After 2.5 hours, the cells were fixed and processed for chromosome spreads. The chromosome spreads were stained with DAPI to recognize DNA (far left), with anti-Ipl1p to recognize Ipl1p (shown in green) and with anti-myc to recognize Cse4-myc (shown in red). The merge (shown in yellow, far right) shows that Ipl1p localizes to kinetochores in the presence of nocodazole even when the checkpoint is inactive. Bar: 10 μm.

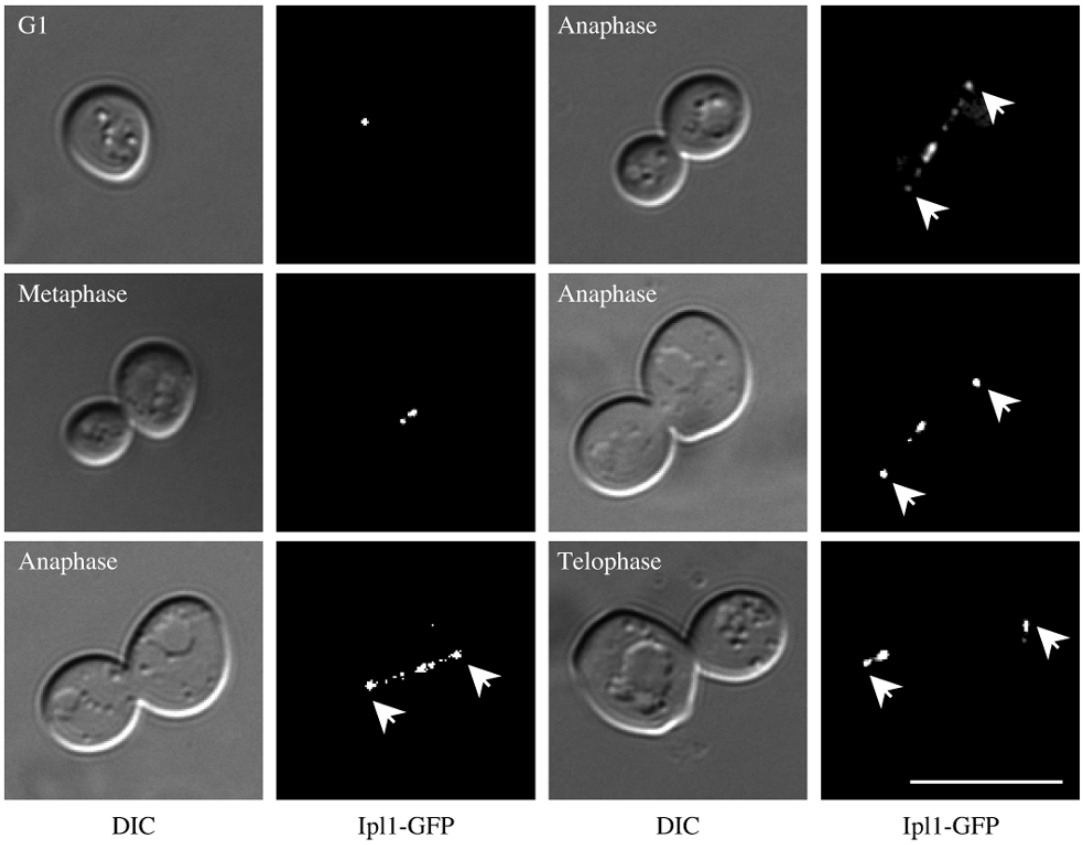


**Figure 3.2.5.** Ipl1p does not leave the kinetochores in a Bir1p mutant strain. Cells with a mutant version of Bir1p (*bir1ΔN-terminus,* SBY896) expressing Ipl1-GFP were analyzed by microscopy. Ipl1p localizes normally to kinetochores in G1 and in metaphase. In anaphase, Ipl1p transfers onto the spindle but it does not leave the kinetochore properly. In telophase cells, Ipl1p is still localized to kinetochores as well as the depolymerized spindle. Arrowheads point kinetochore localization in anaphase and telophase cells. Bar: 10 μm.

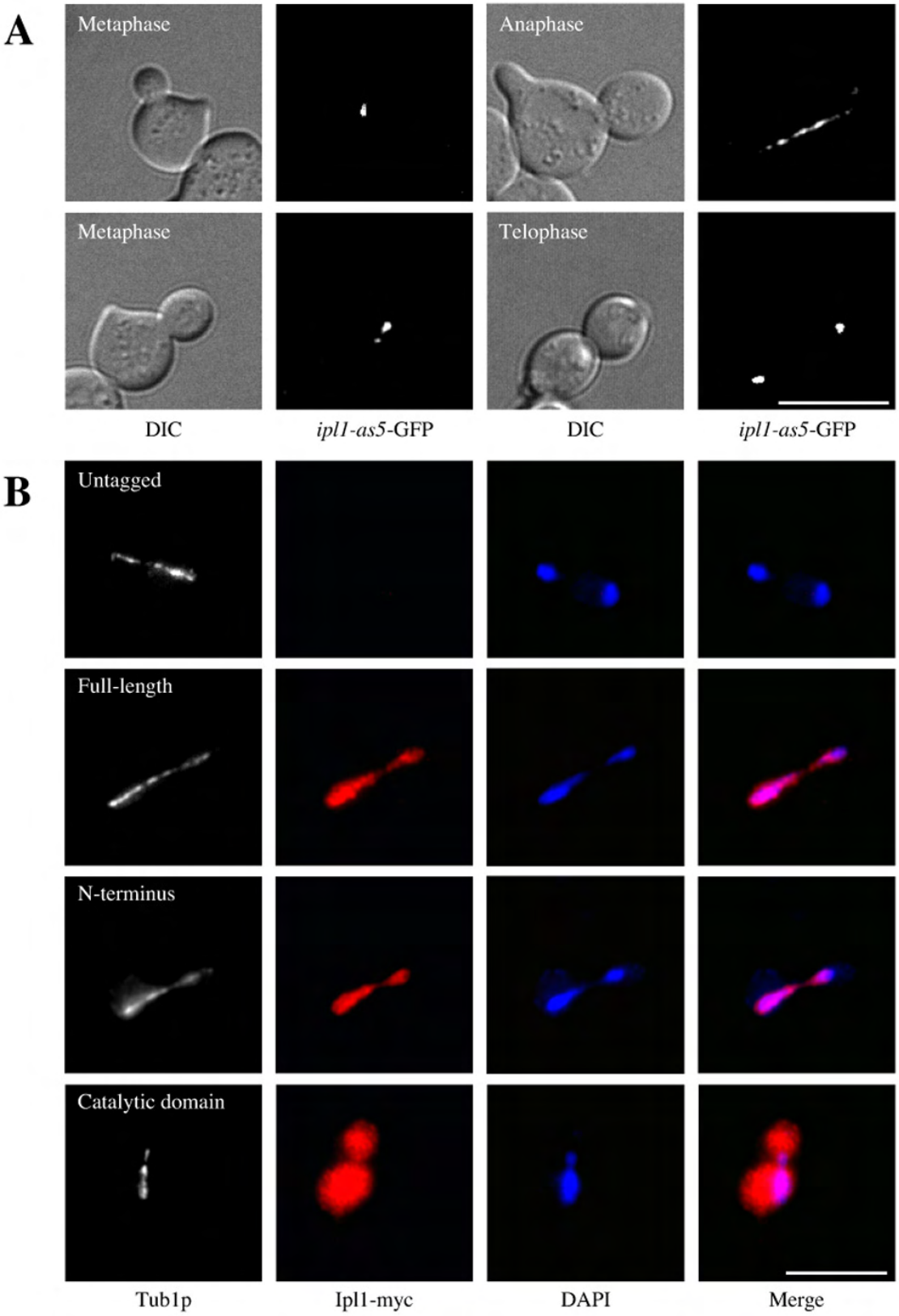
A
Metaphase
Anaphase
Metaphase
Telophase
DIC
ipl1-as5-GFP
DIC
ipl1-as5-GFP
B
Untagged
Full-length
N-terminus
Catalytic domain
Tub1p
Ipl1-myc
DAPI
Merge

**Figure 3.2.6.** Ipl1p's kinase activity is not required for its localization. (A) *ipl1-as5-GFP* mutant cells (SBY1356) were arrested in G1 using α-factor and then released in the cell cycle in the presence of 50 μM inhibitor (1-NA). Time point were taken and the cells were analyzed by microscopy. *ipl1-as5* is able to load onto kinetochores and to transfer onto the spindle without kinase activity. (B) Wild type cells (SBY3, top row), cells containing an extra copy of Ipl1-myc full length under the control of the galctose promoter (*pGAL-myc12-IPL1,* SBY736, second row), cells containing an extra copy of the N-terminus of Ipl1p under the control of the galactose promoter (*pGAL-myc12-IPL1-N-terminus*, SBY735, third row) and cells containing an extra copy of the catalytic domain of Ipl1p under the control of the galactose promoter (*pGAL-myc12-IPL1-catalytic domain,* SBY676, fourth row) grown in raffinose, were induced with 4% galactose for 1 hour. After fixation, the cells were processed for indirect immunofluorescence. They were stained with anti-Tub1p to recognize the spindle (far left), with anti-myc to recognize Ipl1-myc (shown in red) and with DAPI to recognize DNA (shown in blue). The merge is shown in purple on the far right. The staining is specific to Ipl1-myc as an untagged strain does not show any signal (top row). When overexpressed, full-length Ipl1p localizes to the whole nucleus and co-localize with the spindle (second row). The N-terminus alone localizes similarly to full-length (third row), whereas the catalytic domain does not localize properly (fourth row). Bar: 10 μm.

## 3.3. DISCUSSION

### 3.3.1. Ipl1p leaves the kinetochores after tension is established

I show that Ipl1p exhibits a localization pattern similar to "chromosomal passenger proteins". During prometaphase, Ipl1p monitors kinetochore tension and promotes microtubule release of mono-oriented kinetochore attachments, thus ensuring that sister kinetochores establish bi-orientation prior to chromosome segregation (Biggins and Murray, 2001; Tanaka et al., 2002). It had been proposed that Ipl1p delocalized from kinetochores at metaphase, providing an attractive mechanism for inactivating the kinase when tension and bi-orientation are established (Tanaka et al., 2002). However, I found that both Ipl1-GFP and endogenous Ipl1p localize to kinetochores in metaphase, when tension is established, both in an asynchronous population and in a population of cells arrested in metaphase. My data agrees with the observation that a mammalian Aurora B-GFP fusion protein left kinetochores 0.5 min after the initiation of anaphase, well after tension was established (Murata-Hori et al., 2002). K. Tanaka and T. U. Tanaka recently found that Ipl1p is on metaphase kinetochores by chromatin immunoprecipitation, thus reconciling our data (personal communication). In addition, I found that tension is not necessary for Ipl1p relocalization since Ipl1p leaves the kinetochore in the absence of tension: in cells lacking cohesins. In conclusion, the establishment of tension is neither sufficient nor necessary for Ipl1p to leave kinetochore. However, it is possible that the establishment of tension regulates Ipl1p by other mechanism such as regulating its kinase activity for example.

### 3.3.2. The cohesin complex is not required for Ipl1p localization

I found that Ipl1p does not require the presence of cohesin to localize to kinetochores from G1 to metaphase. This is not surprising since Ipl1p activates the spindle checkpoint in cells lacking cohesion (Biggins and Murray, 2001). So it would be expected that the active protein would be properly localized. However, this is in contrast to *Schizosaccharomyces pombe* where the Ipl1p homolog (Ark1) does not localize onto kinetochores in the absence of cohesins (Morishita et al., 2001). The reason for this difference is not clear. It is possible that the bigger centromeres of fission yeast that make several microtubule attachments, have a more complex 3-D structure. More proteins may therefore be involved in setting up this structure, including cohesins. In contrast, the point centromeres of budding yeast that attach to only one microtubule may have a less complex 3-D structure such that cohesins are not involved.

In addition, Ipl1p localization throughout the cell cycle is normal in the absence of cohesin: in the *pGAL-SCC1* experiment Ipl1p leaves the kinetochores after metaphase and relocalizes to the spindle. This suggests that the cohesin complex is not required for Ipl1p relocalization. This idea is strengthened by the fact that Ipl1p relocalization does not occur in the *mad* mutant cells in nocodazole even the cohesin complex has been correctly loaded and unloaded in this case. Taken together this suggests that Ipl1p relocalization is independent of cohesins. This is in contradiction with conclusions of a paper from Vagnarelli et al (Vagnarelli and Earnshaw, 2001) where they say that cohesin unloading is necessary for INCENP and Aurora B relocalization. However, the authors only found a correlation between the degree of sister separation and the delocalization of INCENP or Aurora B. By no mean does this show a direct link between cohesin unloading and INCENP relocalization. It is

however possible that cohesin regulates the INCENP/Aurora complex differently in different organisms.

### 3.3.3. The spindle checkpoint does not regulate Ipl1p relocalization

Two experiments suggest that the spindle checkpoint does not affect the localization of Ip1lp as well. First, as mentioned above, the spindle checkpoint is activated in *pGAL-SCC1* cells. However, no significant delay in Ipl1p relocalization was observed, suggesting that the checkpoint does not affect it. It must be noted however that only live microscopy could assess whether Ipl1p relocalization occurs with wild type kinetics in these cells. From my experiment I can say that the relocalization of Ipl1p occurs while the checkpoint is active. Second, I found that Ipl1p stays on kinetochores in a *mad* mutant in nocodazole where the spindle checkpoint is inactive. As no relocalization was observed in the absence of the checkpoint, this would suggest that checkpoint probably does not regulate Ipl1p relocalization.

In addition, in *mad* mutant cells in the presence of nocodazole, cell cycle progression occurs normally even though chromosome segregation cannot happen. Cell cycle events such as APC activation, Pds1p destruction, cohesin loading and unloading, Clb2p destruction etc. occur normally in those cells (Li and Murray, 1991). As Ipl1p does not relocalize, this suggests that these events are not required for Ipl1p to leave the kinetochores. However I cannot exclude that they are necessary in combination with other factors.

### 3.3.4. Ipl1p is probably transported away from kinetochores

One important conclusion that comes from the *mad* mutant experiment is that microtubules play an essential role in Ipl1p relocalization, as it does not occur in their absence. My favorite model based on these results is that Ipl1p is transported away from the kinetochores using the microtubules. Indeed, it is not likely that the pool of Ipl1p at the kinetochores is being destroyed and that another pool localizes onto the spindle because you would expect the signal to disappear in *mad* mutants in nocodazole. However, I cannot formally exclude this possibility, as it is conceivable that the potential destruction machinery for Ipl1p needs the presence of microtubules in order to get to Ipl1p at the kinetochores. I favor the transport model, where Ipl1p is transported away from the kinetochores after metaphase, as it is the simplest explanation of those results. This correlates well with a recent report from cultured cells showing that centromeric Aurora B was transported to the midzone (Murata-Hori and Wang, 2002).

How is Ipl1p transported away from kinetochores? It is worth noting that Ipl1p can directly bind microtubules *in vitro* (Kang et al., 2001). However no non-motor domain proteins have been shown to move along microtubules on their own. In addition, Ipl1p's kinase activity is not required for its relocalization since *ipl1* mutants are able to leave the kinetochore. It is therefore unlikely that Ipl1p moves along microtubules on its own, suggesting active transport away from the kinetochore by a motor protein. In *Drosophila melanogaster*, Rough deal, a protein of the metaphase checkpoint, was found to be transported away from kinetochore by the motor protein dynein (Wojcik et al., 2001). No homolog of Rough deal exists in budding yeast. In cultured cells the Mad2 and BubR1 checkpoint proteins have been shown to be transported away from the kinetochores by the dynein/dynactin complex (Howell et

al., 2001). In budding yeast no evidence exist that Dyn1p, the only dynein, acts in the nucleus. However, it is possible that the nuclear localization of Dyn1p has been missed in previous studies. Several motor proteins have been localized to the kinetochores including Cin8p (Hoyt et al., 1992). However, Cin8p is thought to be a plus end directed motor and a motor transporting Ipl1p away from the kinetochores would need to be minus end directed. Kar3p is the only motor in budding yeast that has been shown to be minus end directed and will therefore be a likely candidate for transporting Ipl1p away from the kinetochores (Endow et al., 1994).

Alternatively, it is possible that other proteins are responsible for Ipl1p relocalization. Here I found that complex formation with Bir1p plays an important role in Ipl1p relocalization, as Ipl1p does not leave kinetochores normally in a *bir1* mutant. However, as the exact nature of this mutation is unknown, I cannot determine whether complex formation is essential. It is noteworthy that quantification would be necessary to determine whether Ipl1p relocalization from the kinetochore is impaired or totally abolished in the *bir1* mutant strain, *id est* whether a portion of Ipl1p is relocalized or not. If no Ipl1p relocalization of Ipl1p occurs in the *bir1* mutant background that would suggest that relocalization is not an essential function as the cells are viable. Further characterization of the mutant strain will be required to determine the function of the relocalization and the role played by complex formation in Ipl1p relocalization. The two models are not necessarily mutually exclusive, as complex formation might be needed in order for the whole complex to be transported away from the kinetochores by a motor protein.

## 3.4. FUTURE PLANS

### 3.4.1. Does Ipl1p directly relocalize from kinetochores to the spindle?

The data presented here suggested that Ipl1p is transported away from the kinetochores by an active mechanism. However, it is not clear whether the same pool of protein localizes at the kinetochores and at the midzone. One way to address that will be to localize Ipl1p-RFP (red fluorescent protein) during one cell cycle. RFP has very slow folding properties (Baird et al., 2000), slower than a yeast cell cycle. So, newly synthesized RFP does not fold and fluoresce in the first cell cycle (Pereira et al., 2001). Cells expressing Ipl1-RFP will be arrested in G0 by letting the cells spend 4 days in stationary phase. The stationary cells will then be diluted into fresh medium and followed by time-lapse microscopy. The cells will show kinetochore localization of Ipl1-RFP. If the Ipl1-RFP signal is lost at anaphase, I will conclude that a new pool of Ipl1p localizes to the spindle. However, if the spindle is marked with Ipl1-RFP signal in anaphase, no strong conclusion can be made. Indeed, it is possible that the Ipl1p already present on the kinetochores is transported to the spindle. However, it is equally possible that Ip1l protein from the previous cell cycle is still present in the cytoplasm and localize to the spindle in anaphase.

As a complementary approach, FRAP (florescence redistribution after photobleaching (Maddox et al., 2000)) will be used. Ipl1-GFP at the kinetochores will be bleached right before anaphase initiation and the recovery of the signal will be followed. If no recovery is observed, this would show that the same protein that localized to the kinetochore transfers onto the spindle. One problem with this approach is that it is not known how dynamic Ipl1p is at the kinetochore in metaphase. If it is very dynamic, it will be hard to bleach the kinetochore signal right prior anaphase initiation to test the transport hypothesis. In a recent study on Aurora

B in cultured cells the authors used FRAP to demonstrate that Aurora B is very dynamic at the centromeres in metaphase and that it then transfers along midzone microtubules (Murata-Hori and Wang, 2002), suggesting that this will be a viable approach.

Another interesting experiment would be to see whether Ipl1p can localize to the spindle without prior localization to the kinetochores. To abolish kinetochore function I will use an *ndc10* mutant strain. Ndc10p is a core component of the kinetochore and in its absence no kinetochore protein tested is able to localize to kinetochores, including Ipl1p (Goh and Kilmartin, 1993; He et al., 2001). *ndc10* mutant cells will be arrested in G1 using α-factor at the permissive temperature and then released into the cell cycle at the restrictive temperature thus inactivating kinetochore function. Ipl1p will then be localized by chromosome spreads to determine whether it is found on the spindle or not without prior kinetochore localization. If Ipl1p is able to localize to the spindle this would argue against a direct transport from the kinetochore to the spindle. One caveat is that as Ndc10p shows a localization pattern very similar to Ipl1p and is a potential substrate for Ip1lp. Thus, it might be implicated in regulating its localization. Therefore, if Ip1lp is not found on the spindle, it would not necessary imply that it needs to localize to the kinetochore prior to localizing to the spindle or possibly that Ndc10p plays a direct role in relocalizing Ipl1p from the kinetochore to the spindle.

### 3.4.2. Are motor proteins responsible for Ipl1p transport?

If I can formally show that Ipl1p is transported away from the kinetochores, I will attempt to determine the mechanism used. Some obvious candidates for transporting Ipl1p away from kinetochores are motor proteins. As discussed in the

introduction, six motor proteins in budding yeast play a role in mitosis: Cin8p, Kip1p, Kip2p, Kip3p, Dyn1p and Kar3p. Kip2p and Dyn1p are thought to be cytoplasmic proteins and are therefore not likely candidates. However, in the literature several precedents exist for dynein transporting checkpoint proteins away from the kinetochores making of Dyn1p a good candidate (Howell et al., 2001; Wojcik et al., 2001). Cin8p and Kip1p are thought to be plus end directed motors and would therefore not likely contribute to transport of Ipl1p away from the kinetochores, which is a minus end directed movement. However, the evidence for the plus directionality of these motors this is based on structure homology and so their directionality will still have to be tested. Kip3p is thought to have microtubule depolymerizing activity and no motor activity pre se (Severin et al., 2001a). Kar3p is the only motor to have been shown to be minus end directed and will therefore be tested first (Endow et al., 1994). I will therefore localize Ipl1-GFP in the absence of the different motor proteins by live microscopy to determine whether they affect Ipl1p relocalization from kinetochore to the spindle. I will test all the different motors, as no formal prove exist that they are not implicated in Ipl1p relocalization. Although *cin8Δ* and *kar3Δ* mutants activate the spindle checkpoint, the arrest is eventually bypassed and it is possible to analyze anaphase movement in those strains (Straight et al., 1998) (P. Maddox personal communication). These studies will give interesting information even if no motors affect Ipl1p relocalization. Indeed, Ipl1p localization to the spindle is punctate and, as shown in chapter 4, shows a dynamic pattern on the spindle, (see chapter 4.4.). If no single deletion of a motor protein shows any defect I will test double deletions.

### 3.4.3. Role of Ipl1p complex formation in its relocalization

I show here that formation of a complex between Ipl1p and Bir1p is important for Ipl1p relocalization. However the nature of the *BIR1* allele used here is unclear. I will therefore repeat the experiment using a strain conditionally expressing Bir1p. Such cells expressing Ipl1-GFP will be arrested in G1 using α-factor, depleted of Bir1p and then released into the cell cycle. The localization of Ipl1-GFP will be monitored by microscopy. This will determine whether Bir1p is necessary for Ipl1p relocalization from the kinetochore to the spindle. In addition, it will be interesting to determine whether the other member of the complex, Sli15p, plays a role in Ip1lp relocalization. Similar experiments will be conducted using a strain that conditionally expresses Sli15p.

### 3.4.4. Dissection of Ipl1p localization domains

I found here that the localization information was contained in the N-terminus of the protein. This is somewhat surprising because this is the most divergent part of the protein but Ipl1p's homologues in other organisms show a similar localization pattern. However, it is possible that a small domain or a phosphorylation site that might be well conserved is required for correct localization. One caveat of the strategy used is that because the protein was overexpressed to very high levels it was not possible to assess kinetochore localization. In addition, the endogenous full-length protein being present, it is possible that the N-terminus localizes normally due to dimerization. I will therefore repeat the experiment using the endogenous promoter of Ipl1p. Cells will express the endogenous Ipl1p in addition to each different domain of Ipl1p tagged to GFP under the control of the endogenous promoter. This new assay will allow us to test both kinetochore and spindle localization. I will further truncate

the domains to determine which domain(s) is responsible for the kinetochore and/or spindle localization. If such a domain is found, I will delete it in Ip1lp and determine the phenotype associated with its mislocalization. It should be noted that I might not find the exact domain as small domains on their own might not fold properly and therefore not localize properly. One caveat that remains is the fact that the endogenous protein always being present, the domain might localize properly through dimerization. We have not been able to find any condition allowing to eliminate the endogenous protein: with all the conditional promoters tested, Ipl1 protein is still present. I will therefore perform co-immunoprecipitation experiments to determine whether the potential localization domain is able to dimerize with the endogenous protein. The potential localization domain will be tagged with the myc epitope and immunoprecipitated. I will then determine whether the endogenous protein was co-immunoprecipitated by immunoblotting with anti-Ipl1p antibodies. If the endogenous protein does not co-immunoprecipitate that would suggest that a localization domain has been found. If it does, more work will need to be done to determine whether the domain found is implicated in localization or not.

# CHAPTER 4. Ipl1p REGULATES SPINDLE DISASSEMBLY

## 4.1. SUMMARY

In the preceding chapter I found that Ipl1p displays several localization patterns in anaphase, being found on the spindle and at the spindle midzone. In this chapter I am going to discuss a new function for Ipl1p in anaphase. Using time-lapse microscopy, I found that Ipl1p has a specific function in mitotic spindle disassembly that is separable from its previously identified roles in chromosome segregation and the spindle checkpoint. Ipl1-GFP transfers from kinetochores to the spindle after metaphase and accumulates at the spindle midzone late in anaphase. The Ipl1 protein levels and kinase activity are high in anaphase, and two independent tests determined that *ipl1* mutants have hyperstable microtubules. As the spindle disassembles, Ipl1p follows the plus ends of the depolymerizing spindle microtubules. Many Ipl1p substrates co-localize with Ipl1p to the spindle midzone, identifying additional proteins that may regulate spindle disassembly. I propose that Ipl1p regulates both the kinetochore and interpolar microtubule plus ends to regulate its various mitotic functions.

## 4.2. RESULTS

### 4.2.1. Ipl1p accumulates at the spindle midzone and then follows the plus ends of the depolymerizing spindle microtubules

As discussed in chapter 3, I found that Ipl1p can be found both on the whole spindle and accumulated at the midzone in anaphase. Therefore, I was interested in determining the precise order of these localization patterns during anaphase. In order to do this, I performed live microscopy on cells expressing Ipl1-GFP. Asynchronously grown cells were observed by time-lapse microscopy and pictures

were taken every 15 seconds (Figure 4.2.1.A). I found that, at anaphase, Ipl1-GFP is distributed along the entire spindle length in a punctate pattern (0'). Ipl1p then accumulates at the spindle midzone (8.5'), splits into two distinct dots (11.75') and then travels back to the poles (13.25').

Since Ipl1p localizes to the spindle midzone very late in anaphase and then travels back to the poles, I considered the possibility that it might be following the plus ends of the depolymerizing spindle microtubules. To test this idea, I performed live microscopy on cells co-expressing Tub1-CFP (tubulin) and Ipl1-GFP. Although CFP and GFP have overlapping spectrums, the Ipl1-GFP and Tub1-CFP signals were easily discernable (see Figure 4.2.1.B). It was not possible to use the non-overlapping spectrum of YFP because the Ipl1-YFP signal was not strong enough to perform time-lapse imaging of cells. I started imaging a cell when Ip1lp (shown in green) localized to the midzone of a long spindle (shown in red) (0'). After 1 minute, the Ipl1p signal splits and there was no longer any tubulin signal in the center of the spindle implying that the spindle is starting to dissemble. As the spindle depolymerized toward the poles, the Ipl1p signal always localized near the plus end of the spindle microtubules (2'). At the end of spindle breakdown, the remaining tubulin at the pole co-localized with Ipl1p (3.5'). Therefore, Ipl1p accumulates at the spindle midzone in late anaphase and then follows the plus ends of the depolymerizing microtubules back to the poles.

### 4.2.2. Ipl1p protein levels and kinase activity are high at anaphase

To determine how the Ipl1 protein levels correlated with the dynamic localization patterns I observed, I generated antibodies against a recombinant glutathione-S-transferase-Ipl1 fusion protein. The affinity-purified antibodies

recognized a single major band in yeast lysates that migrates just above the 45 kD marker that increases in intensity when Ipl1p is overexpressed from the inducible galactose promoter (Figure 4.2.2.A). The antibodies also recognize additional bands that correspond to epitope-tagged Ipl1-myc12 (Figure 4.2.2.A, lane 3), indicating the antibodies specifically recognize Ipl1p. Depending on the gel conditions, there is also a minor upper band that represents a cross-reacting band.

We analyzed Ipl1 protein levels at anaphase. To do this, cells arrested in metaphase by Cdc20p depletion were released into the cell cycle and time points were taken every 10 min. An immunoblot was performed using anti-Ipl1p antibodies as well as anti-Tub1p antibodies to assess equal loading. We found that Ipl1p levels are low in the metaphase arrest and start peaking 10 minutes after the release corresponding to anaphase (Figure 4.2.2.B).

Sean Tatsutani developed an Ipl1p kinase assay to analyze Ipl1p's kinase activity during the cell cycle. Wild type Ip1lp and the Ipl1-321 protein that has reduced kinase activity at high temperatures (Biggins et al., 1999) were immunoprecipitated with anti-Ipl1p antibodies and then incubated with the histone-fold domain of the kinetochore protein Cse4 in a kinase reaction *in vitro*. The majority of Ipl1p present in the yeast lysates (Figure 4.2.2.C, pre) was depleted by the antibody (Figure 4.2.2.C, post). When the immunoprecipitations (Figure 4.2.2.C, IP) were used for kinase assays, Cse4p was radiolabeled in the presence of wild type Ipl1p, but not the kinase inactive Ipl1-321 protein (Figure 4.2.2.C), showing that the assay specifically reflects an Ipl1p-associated kinase activity.

We analyzed Ipl1p kinase activity as cells exited mitosis by releasing cells arrested in metaphase by Cdc20p depletion and taking time points every 10 min. Ipl1p was immunoprecipitated and the kinase activity was monitored against the substrate

Cse4p. The amount of Ipl1 protein present in the kinase assays was determined by quantitative immunoblotting. To calculate the Ipl1p specific activity, the relative amount of radioactive phosphate incorporated into Cse4p was normalized to the relative amount of Ipl1 protein immunoprecipitated at each time point (Figure 4.2.2.D). To determine the corresponding cell cycle position, we monitored the budding index and spindle disassembly (Figure 4.2.2.D). We found that the Ipl1p kinase activity increases as cells leave metaphase, peaking just prior to spindle disassembly.

### 4.2.3. *ipl1-321* mutant cells are defective in spindle disassembly

The dynamic localization of Ipl1p on spindles, tracking back to the plus ends, and the peak in Ip1l protein levels and kinase activity at anaphase suggested that Ipl1p might regulate spindle function. To test this, I performed live cell imaging of wild type and *ipl1-321* mutants containing Tub1-GFP. Cells were synchronized in G1 with α-factor and then released to 35°C to inactivate Ipl1-321p and time-lapse images were captured every minute. The start of spindle elongation was used as a reference for anaphase initiation and examples of the time-lapse data for a wild type and an *ipl1-321* mutant cell are shown in Figure 4.2.3.A. Spindle elongation was quantified by measuring the length of the spindle every minute after the initiation of anaphase B (Figure 4.2.3.B). In wild type cells, I observed biphasic spindle elongation (Straight et al., 1997; Yeh et al., 1995). Wild type spindles reach about 8.4 μm in length approximately 14 minutes after the initiation of anaphase B and then disassemble. In *ipl1-321* mutant cells, spindle elongation occurs with kinetics similar to wild type cells. However, the spindle continues to grow to a length of approximately 10.4 μm, delaying spindle disassembly for about 6 minutes, a 42% increase in the duration of

anaphase B. In two of the 10 cells analyzed, this results in a spindle that is forced to bend when reaching the cell membrane (see Figure 4.2.3.A, panel A*).

The structure of the spindle did not appear grossly altered. If the intensity of the spindle staining along its length is analyzed in a wild type cell, an increase in staining is observed at the central spindle due to overlapping microtubules (Severin et al., 2001b). It had been reported that mutants that cause spindle fragility showed a decrease in the central staining (Severin et al., 2001b). Therefore, I reasoned that an *ipl1-321* mutant cell that showed hyperelongated spindle might have an increase in central staining. However, this was not observed (data not shown). It is however possible that a more careful analysis of the spindle structure, such as electron-microscopy, will reveal a defect in spindle structure that is not detectable by fluorescence microscopy.

### 4.2.4. *ipl1-321* mutant cells are defective in spindle orientation

The *ipl1-321* mutant cells also exhibit a spindle orientation defect that I quantified by measuring the angle between the spindle axis and the mother-bud axis every minute starting at metaphase. In wild type cells, it takes less than 6 min for the spindle to orient itself on the mother-bud axis, whereas it takes more than 11 min in *ipl1-321* mutant cells (data not shown). In addition, the spindle moves in a single direction towards the mother-bud axis in 80% of wild type cells. In contrast, 80% of the spindles in *ipl1-321* mutant cells "flipped" at least once (Figure 4.2.3.C). The spindle started orienting towards the mother-bud axis but continued past it instead of stopping and then moved in the other direction to finally orient along the mother-bud axis. The angles by which the spindle axis deviated from the mother-bud axis were

also much greater in the *ipl1-321* mutants than in wild type cells. For example, one spindle turned 180° before orienting correctly in one of the *ipl1-321* mutant cells.

Spindle positioning is regulated by cytoplasmic microtubules (for review see Segal and Bloom, 2001). I therefore analyzed cytoplasmic microtubule length and number in the *ipl1-321* mutant cells in the movies described above. The number of cytoplasmic microtubules was not altered in *ipl1-321* mutant cells in anaphase. One microtubule per SPB was observed in both wild type and in *ipl1-321* mutant cells as reported (Gupta et al., 2002). The number of microtubules per SPB was not assessed at other phases of the cell cycle. The cytoplasmic microtubules are slightly longer in *ipl1-321* mutant cells as compared to wild type cells (data not shown). However, only a few cytoplasmic microtubules were measured and a more extensive analysis will be needed to determine whether this difference is significant or not.

### 4.2.5. The role of Ipl1p in spindle disassembly is an independent function

Since Ipl1p is required for chromosome segregation and the spindle checkpoint, I tested whether the defect in spindle disassembly was a consequence of defects in these functions. We previously showed that if bipolar spindle assembly occurs prior to Ipl1p inactivation, chromosome segregation is normal (Biggins and Murray, 2001). I therefore arrested cells containing Tub1-GFP in metaphase by depleting the Cdc20 protein, shifted the cells to the restrictive temperature to inactivate Ip1lp, and then released them into the cell cycle. Aliquots were taken every 5 min and cells with a spindle pole-to-pole distance corresponding to a late anaphase cell (equal or greater than 9 μm) were analyzed for the presence or absence of a spindle. Since tubulin is always at the SPB, the pole-to-pole distance can be measured regardless of whether a spindle is present. Spindle disassembly occurred in 68% of

Cdc20 depleted cells, while only 36% of the Cdc20 depleted *ipl1-321* double mutant cells underwent spindle breakdown (Figure 4.2.4.A). In addition, I never saw any defect in spindle elongation in either strain. This result suggests that the spindle breakdown defect in *ipl1-321* mutants is independent from Ipl1p's role in chromosome segregation. I also analyzed DNA segregation to ensure chromosome segregation occurred normally in both strains (data not shown). In addition, I found that the *ipl1-321* mutant cells remained viable when released from metaphase (data not shown), indicating that the spindle disassembly defect does not uncover an essential function.

The difference in spindle disassembly between wild type and *ipl1-321* mutant cell populations released from a metaphase arrest is similar to that observed when cells are released from G1 and chromosome segregation is defective. I analyzed this by synchronizing wild type and *ipl1-321* mutant cells containing Tub1-GFP in α-factor, releasing them to 37°C and analyzing spindle disassembly as described above. While 78% of the wild type cells underwent spindle breakdown, only 40% of *ipl1-321* mutant cells had disassembled their spindles at this time (Figure 4.2.4.B). Therefore, the spindle disassembly defect occurs in *ipl1-321* mutants when chromosome segregation is normal or defective, indicating that the role for Ipl1p in spindle disassembly is independent from its function in chromosome segregation.

Since mutants defective in the mitotic exit network exhibit a spindle breakdown delay (Stegmeier et al., 2002), I tested whether *ipl1-321* mutant cells delayed mitotic exit by monitoring the destruction of Clb2p, the major mitotic B-type cyclin. Cells from the experiment described in Figure 4.2.4.A. were collected every five min and then immunoblotted with anti-Clb2p antibodies (Figure 4.2.4.C). Clb2p degradation occurred with similar kinetics in wild type and *ipl1-321* mutant cells,

indicating Ipl1p is not required for mitotic exit.

Ipl1p also has a function in the spindle checkpoint when kinetochore tension is not generated (Biggins and Murray, 2001), so I tested whether other spindle checkpoint genes are also required for spindle disassembly. I analyzed the dynamics of spindle elongation and breakdown in *mad1*Δ and *mad2*Δ strains containing Tub1-GFP by live microscopy. The average spindle length at each minute after anaphase B initiation in 10 cells for each strain is shown in the graph in Figure 4.2.4.D. I found that the dynamics of spindle elongation and breakdown in wild type, *mad1*Δ and *mad2*Δ strains were similar (Figure 4.2.4.D). In addition, I did not detect the presence of hyperelongated spindles or spindle positioning defects in *mad1*Δ or *mad2*Δ mutant cells (data not shown). Therefore, a function in spindle disassembly is not a general property of all checkpoint proteins, although it is possible that other spindle checkpoint proteins I did not examine have a role in spindle disassembly. Taken together, these data suggest that Ipl1p's role in spindle disassembly is independent from its roles in chromosome segregation and the spindle checkpoint and identifies a previously unknown function for this protein kinase.

### 4.2.6. *ipl1-321* mutant cells have hyperstable microtubules

Since *ipl1-321* mutants are defective in spindle breakdown, I tested whether their microtubules are hyperstable by analyzing growth on the microtubule-depolymerizing drug benomyl. 5-fold serial dilutions of wild type and *ipl1-321* mutant cells were spotted on media with or without benomyl at the permissive temperature (Figure 4.2.5.A). I was not able to assay the defect at higher temperatures, where the *ipl1-321* allele might be more defective, due to growth defects. Whereas both strains grow equally well on rich media, *ipl1-321* cells grow

better than wild type cells on media containing benomyl, indicating they have hyperstable microtubules.

I confirmed that *ipl1-321* mutants affect microtubule stability by a second test. Mutants defective in APC function arrest in metaphase with short spindles and sister chromatids held together (Zachariae et al., 1996). When sister chromatid cohesion is released in the absence of APC function by a mutation in the Mcd1/Scc1 cohesion protein, spindle elongation occurs in the presence of high levels of the Pds1 and Clb2 proteins (Michaelis et al., 1997). This leads to fragile spindles where the spindle elongates but breaks down abnormally fast, creating an "anaphase-like prometaphase" (Severin et al., 2001b). I therefore tested whether the addition of an *ipl1-321* mutation could stabilize the fragile spindles. I used a *cdc26Δ* strain, which leads to temperature sensitive inactivation of the APC, in combination with the cohesin mutation *mcd1-1* to create fragile spindles. *cdc26*Δ *mcd1-1* and *cdc26*Δ *mcd1-1 ipl1-321* mutant cells containing Tub1-GFP were shifted to the restrictive temperature for 4 hours to arrest the cells in anaphase-like prometaphase and then analyzed for the presence or absence of a spindle. As previously reported, I found that nearly all *cdc26*Δ *mcd1-1* mutants (99%) underwent spindle breakdown (Figure 4.2.5.B) (Severin et al., 2001b). However, only 35% of the *cdc26*Δ *mcd1-1 ipl1-321* mutant cells underwent spindle breakdown, showing that the addition of an *ipl1-321* mutation stabilizes the spindle.

### 4.2.7. Ipl1p and Kip3p act in the same pathway

To determine how Ipl1p promotes spindle disassembly, I tested whether it acted in the same pathway as a known regulator of spindle breakdown, the motor protein Kip3 (Straight et al., 1998). Spindle elongation and breakdown kinetics were analyzed in *kip3Δ* and *kip3Δ ipl1-321* mutants by time-lapse microscopy as described

in Figure 4.2.3.A. I measured spindles at each time point and found that *ipl1-321*, *kip3Δ* and *kip3Δ ipl1-321* mutant cells exhibit similar delays in spindle breakdown (Figure 4.2.6.A). This suggests that Ipl1p and Kip3p act in the same pathway to promote spindle disassembly at the end of anaphase. Since Kip3p localizes to the spindle midzone, I tested whether Ipl1p kinase activity was required for Kip3p localization (DeZwaan et al., 1997). Kip3p was C-terminally tagged with GFP and analyzed by microscopy in wild type and *ipl1-321* mutant cells (Figure 4.2.6.B). The cells were shifted to the restrictive temperature (37°C) for one hour to inactivate Ipl1-321p. I found that Kip3p localization to the spindle midzone is not altered in *ipl1-321* mutant cells at the non-permissive temperature. I next tested whether Kip3p was required for Ipl1p localization to the midzone by analyzing Ipl1-GFP localization in a *kip3Δ* background and found that Ipl1p localization is not altered by the absence of Kip3p. Therefore, Ipl1p localize to the spindle midzone independently of Kip3p and Kip3p localization to the midzone does not require Ipl1p's kinase activity. In addition, I have not found any evidence that Kip3p is phosphorylated by Ipl1p (data not shown). So even though the two proteins act in the same pathway, Ipl1p does not appear to directly act on Kip3p.

### 4.2.8. The midzone is not grossly altered in an *ipl1-321* mutant cell

The fact that Kip3p localized normally to the midzone in an *ipl1-321* mutant cell suggested that the midzone structure was still partially functional in an *ipl1-321* mutant cell. I therefore decided to extend the analysis to other midzone proteins to determine whether the structure was truly unaffected. I tested two other midzone proteins for their presence at the midzone in an *ipl1-321* mutant cell: the microtubule associated proteins Ase1 and Stu2. Both proteins have been implicated in anaphase

spindle stability and they are therefore potential candidates for Ipl1p's substrate for spindle disassembly. Wild type and *ipl1-321* mutant cells containing Ase1 or Stu2 fused with GFP at the C-terminus were analyzed by microscopy. The cells were shifted to the restrictive temperature for 1 hour to inactivate Ipl1-321p. In anaphase, Ase1p localizes at the midzone, in a broader region than Ipl1p, as previously reported (see Figure 4.2.7.A). The localization of Ase1p was not affected in an *ipl1-321* mutant cell. In both backgrounds, Ase1-GFP was absent in G1 cells. Stu2p localizes to the midzone in late anaphase cells, as previously described (Kosco et al., 2001). This localization pattern was unaltered in an *ipl1-321* mutant cell. In conclusion, both proteins localize to the midzone in *ipl1-321* mutant cells, suggesting that the structure of the midzone is intact in these cells. In addition, if Ipl1p regulates one of these proteins, it does not act on their localization.

I then wanted to determine whether Ipl1p itself was able to localize to the midzone when mutated. To test that, I localized *ipl1-as5-GFP* in anaphase as described in Figure 3.2.6.A. Cells expressing *ipl1-as5* tagged at the C-terminus with GFP were arrested in G1 using α-factor. The cells were released into the cell cycle in the presence of inhibitor to inactivate Ipl1-as5p. Time points were taken and *ipl1-as5-GFP* localization was determined. In late anaphase cells, Ipl1-as5p localized to the midzone (Figure 4.2.7.B), indicating that the kinase activity is not required for Ipl1p localization to the midzone. In addition, when the same cell was observed after spindle depolymerization had started, Ipl1-as5-GFP localized to the end of the depolymerizing spindle. DNA segregation was analyzed to ensure that Ipl1-as5p had been inactivated. DNA segregation was unequal in *ipl1-as5-GFP* mutant cells. The unequal segregation was similar to unequal DNA segregation observed in an *ipl1-321* mutant cells, indicating that the Ipl1-as5p had been inactivated in this experiment.

Localization of Ipl1-321p by chromosome spreads using anti-Ipl1p antibodies confirmed that an inactive version of Ipl1p is able to localize at the midzone (data not shown).

### 4.2.9. A number of Ipl1p potential substrates are at the midzone

Because I identified a novel localization pattern for Ipl1p to the midzone, I tested whether proteins that Ipl1p regulates also localize to the spindle midzone. First, I tested Cse4p which we have shown here is an Ipl1p substrate *in vitro*. Localization of a Cse4-GFP fusion by live microscopy showed that the protein localizes to the kinetochores at all cell cycle stages and does not transfer to the spindle (data not shown). I next analyzed the localization of C-terminal GFP fusions to the Ndc10, Sli15 and Dam1 proteins (Figure 4.2.7.C). Ndc10-GFP localized to the midzone in late anaphase cells in addition to the previously reported spindle and kinetochore localization (Goh and Kilmartin, 1993; Lechner and Carbon, 1991). I found that Sli15-GFP also accumulates at the spindle midzone and exhibits the same localization pattern as Ipl1p throughout the entire cell cycle (Figure 4.2.7.C and data not shown). Various laboratories have reported that Dam1p localizes to kinetochores throughout the cell cycle and to the mitotic spindle (He et al., 2001; Hofmann et al., 1998; Jones et al., 2001). Here I show that Dam1-GFP also localizes to the spindle midzone in anaphase cells. Therefore, the majority of known Ipl1p substrates localize to the spindle midzone, providing a number of potential candidates for Ipl1p regulation of spindle disassembly at the end of anaphase.

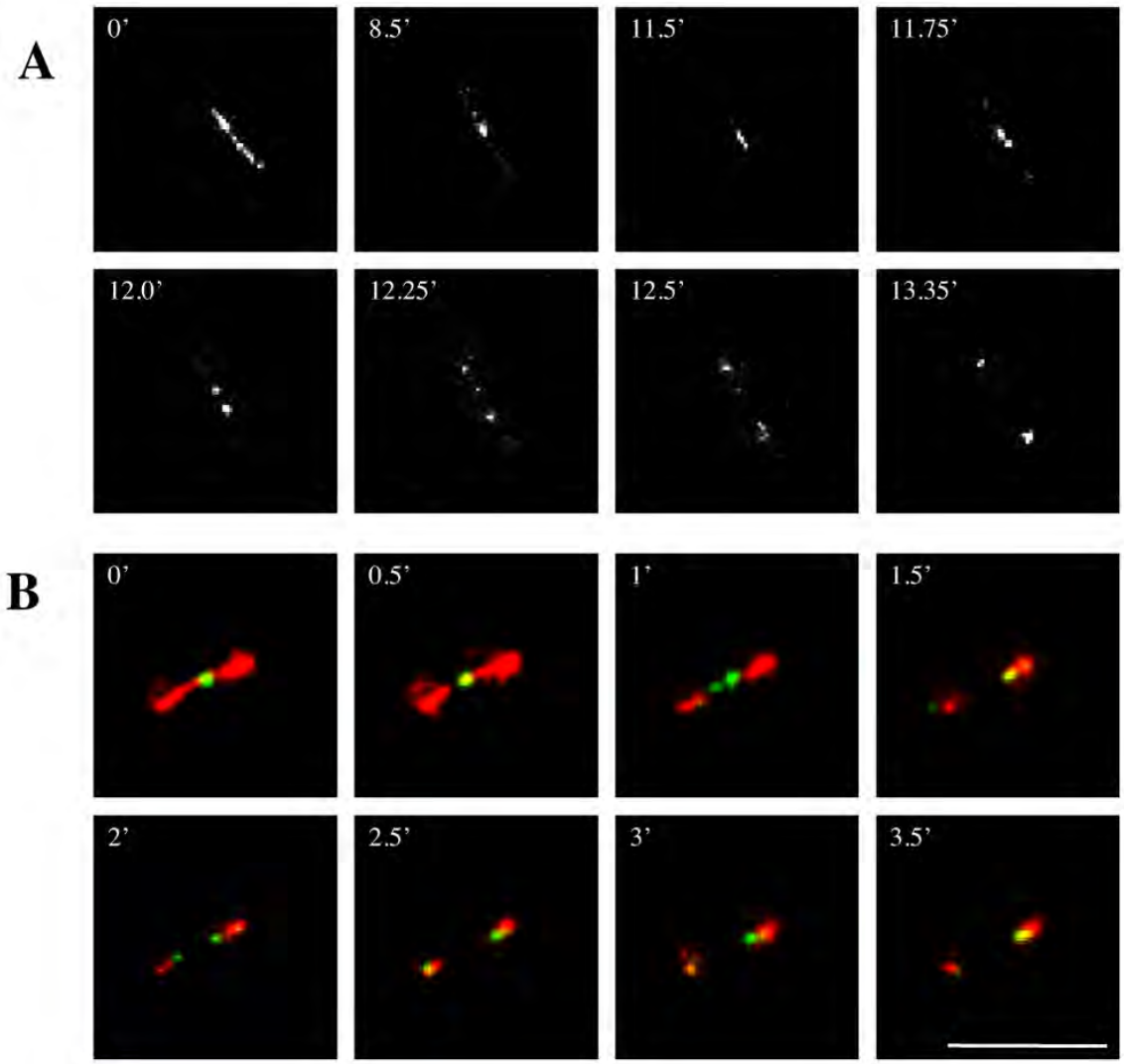


**Figure 4.2.1.** Ipl1p follows the plus ends of the depolymerizing spindle. (A) Time-lapse live microscopy was performed on anaphase cells expressing Ipl1-GFP (SBY556). 5 Z sections at 0.5 μm intervals were acquired every 15 s. Selected frames show that Ipl1p first localizes to the whole spindle (0'), accumulates at the spindle midzone (8.5'), splits (11.75') and then tracks back to the spindle poles (13.25'). The same pattern was observed in 10 independent cells. (B) Live image analysis was performed on cells expressing Ipl1-GFP and Tub1-CFP (SBY1036). Every 30 s, 5 Z sections at 0.5 μm intervals were acquired while alternating between the two channels (FITC and CFP). The deconvolved movie shows Tubulin in red, Ipl1p in green, and the overlapping signal in yellow. Before spindle disassembly, Ipl1p localizes to the spindle midzone (0'). When the spindle starts breaking down (1'), the Ipl1p signal splits and Ipl1p follows the plus ends of the depolymerizing spindle (2') until it reaches the spindle poles (3.5'). The same pattern was observed in 10 independent cells. Bar: 10 μm.

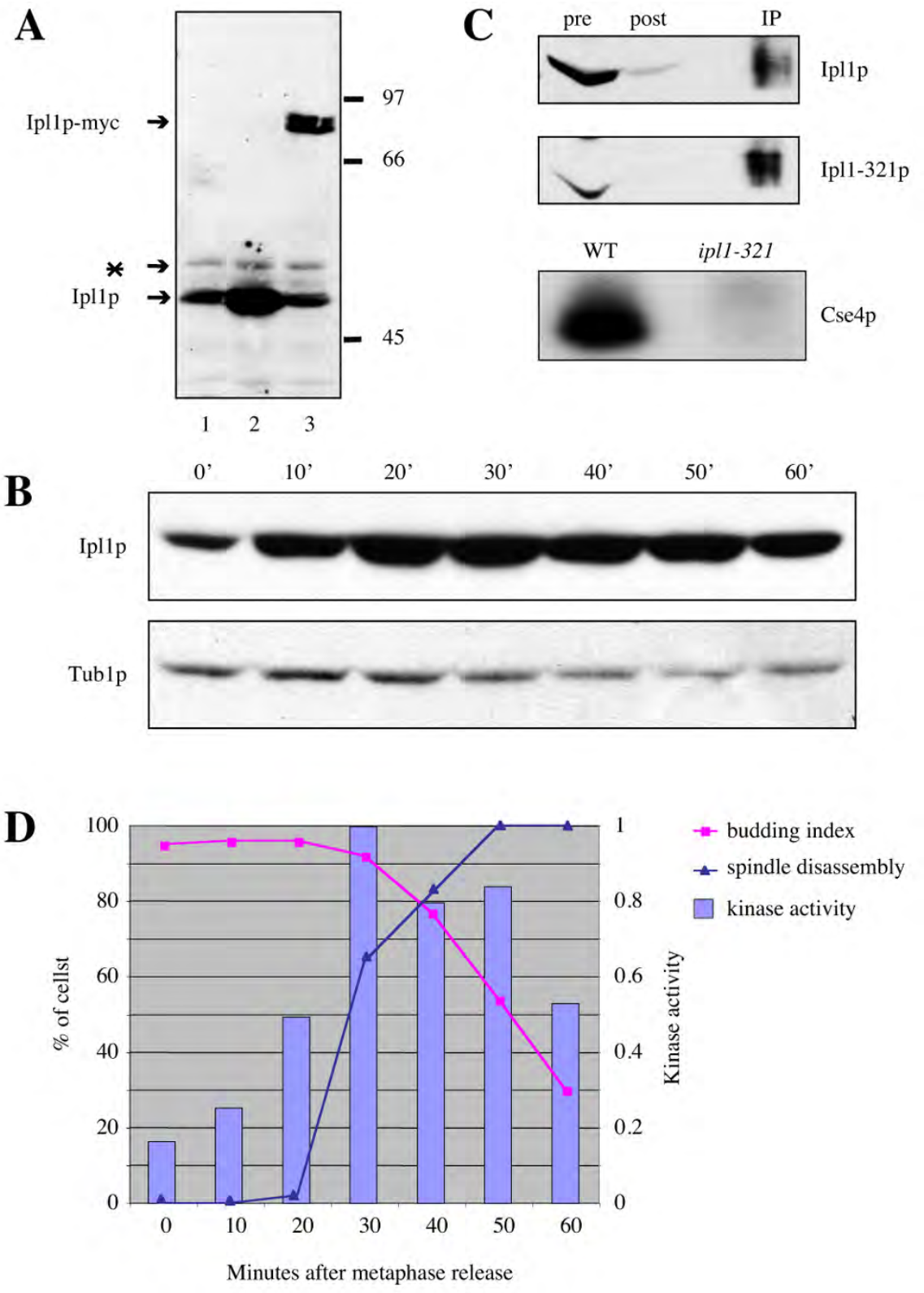
A
Ipl1p-myc
*
Ipl1p
97
66
45
1 2 3
C
pre post IP
Ipl1p
Ipl1-321p
WT ipl1-321
Cse4p
B
0' 10' 20' 30' 40' 50' 60'
Ipl1p
Tub1p
D
% of cellst
Kinase activity
budding index
spindle disassembly
kinase activity
Minutes after metaphase release

**Figure 4.2.2.** Ipl1p protein levels and kinase activity are high when spindles disassemble. (A) Antibody specificity. Anti-Ipl1p antibodies were used for immunoblotting on lysates containing endogenous Ipl1p (lane 1, SBY3), overexpressed Ipl1p (lane 2, SBY554) and endogenous Ipl1p plus an Ipl1-myc12 fusion protein (lane 3, SBY736). * indicates a background band. (B) Ipl1p protein levels increase at anaphase. *pGAL-CDC20* cells expressing Tub1-GFP (SBY952) were synchronized in metaphase by growth in glucose for 3 h. They were then released into galactose media and aliquots were taken every 10 min and immunoblots were performed using anti-Ipl1p antibodies. Tub1p antibodies were used to assess equal loading. (C) Ipl1p kinase assay. Ip1lp was immunoprecipitated from a wild type (SBY3) and *ipl1-321* mutant strain (SBY322) and then incubated with the histone-fold domain of the Cse4 kinetochore protein in a kinase reaction *in vitro*. The majority of Ipl1p present in the lysates before the immunoprecipitation (pre), was removed (post) and similar amounts of protein were used in the kinase assay (IP). The autoradiogram (lower panel) shows that Cse4p is radiolabeled in the presence of wild type Ipl1p but not Ipl1-321 mutant protein. (D) Ipl1p kinase activity peaks prior to spindle disassembly. *pGAL-CDC20* cells expressing Tub1-GFP (SBY952) were synchronized in metaphase by growth in glucose for 3 h. They were then released into galactose media and aliquots were taken every 10 min and kinase assays were performed with the substrate Cse4p *in vitro*. The specific activity was calculated by normalizing the relative amount of radioactive phosphate incorporated into Cse4p to the relative amount of Ipl1p immunoprecipitated at each time point. The amount of Ipl1p immunoprecipitated in each kinase reaction was determined by quantitative immunoblotting using the LI-COR Biosciences Odyssesy infrared imaging system. Microscopy was performed to determine the percent budding (✿) and the percent spindle disassembly (✪) and the specific kinase activity (blue bars, arbitrary units). Ipl1p kinase activity increases prior to spindle breakdown.

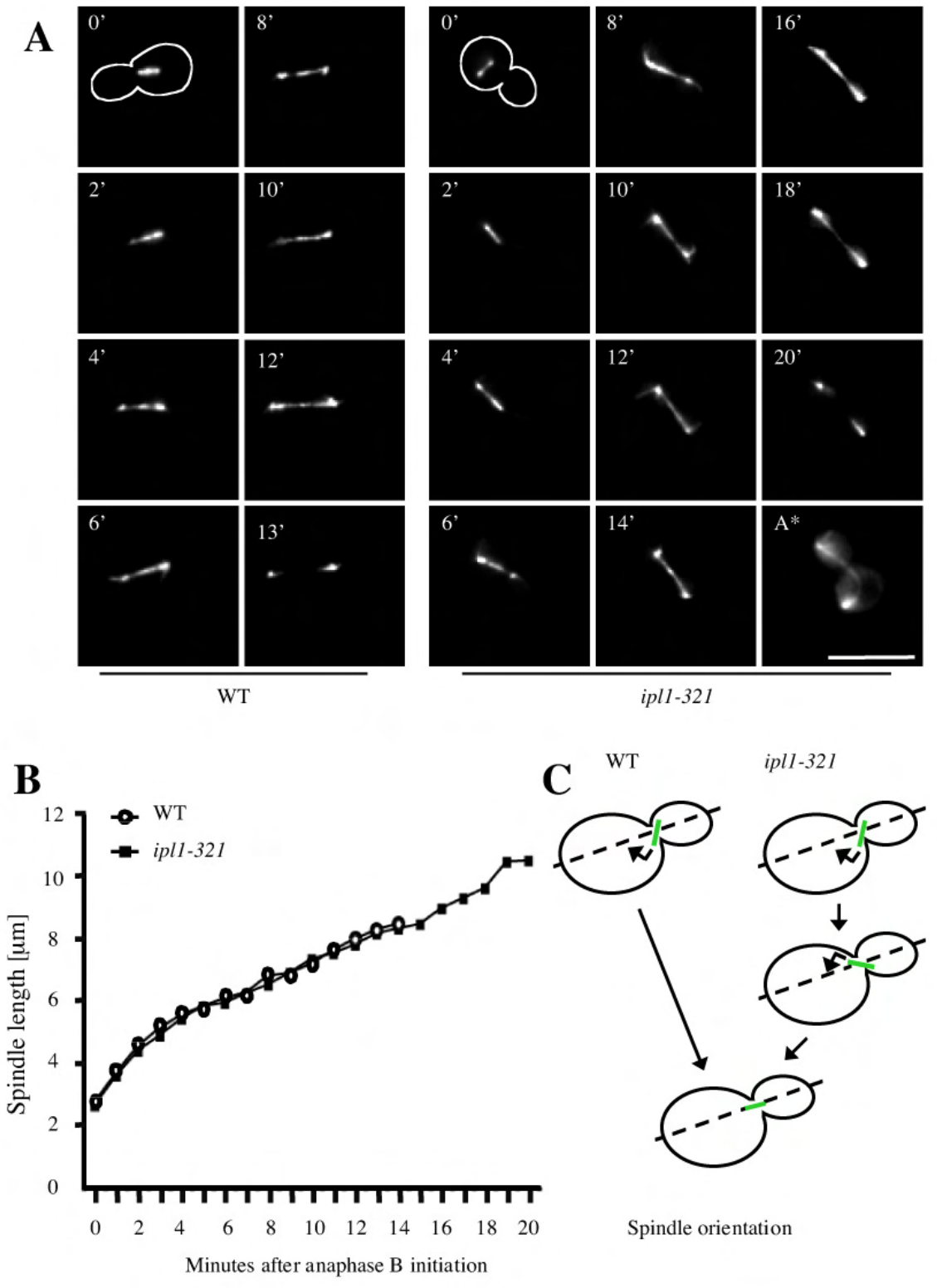
A
0'
8'
2'
10'
4'
12'
6'
13'
0'
8'
16'
2'
10'
18'
4'
12'
20'
6'
14'
A*
WT
ipl1-321
B
WT
ipl1-321
Spindle length [µm]
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
18
20
Minutes after anaphase B initiation
C
WT
ipl1-321
Spindle orientation

**Figure 4.2.3.** *ipl1-321* mutants are defective in spindle disassembly. (A) Live microscopy was performed on wild type (SBY130, left) and *ipl1-321* mutant cells (SBY97, right) containing Tub1-GFP that were released from α–factor at 35°C. 8 Z sections at 0.5 μm intervals were acquired every minute. Images of the spindle in a single cell are shown every 2 min after the initiation of anaphase (time 0'). An outline of the cell is shown at time 0'. Spindle disassembly is delayed in *ipl1-321* cells and the spindle orientation changes during the initial phases of anaphase. A hyperelongated spindle in an *ipl1-321* mutant cell is shown in A*. (B) The spindle length at each time point was measured and the averages of 10 cells for each strain are graphed. Spindles disassemble in wild type cells (●) 14 min after anaphase B initiation while *ipl1-321* mutant cells (✿) take 20 min. (C) The angle between the spindle axis and the mother-bud axis was measured on the cells in (B). 80% of wild type spindles move in a single direction while 80% of *ipl1-321* mutant cells flip past the mother-bud axis at least once. Bar: 10 μm.

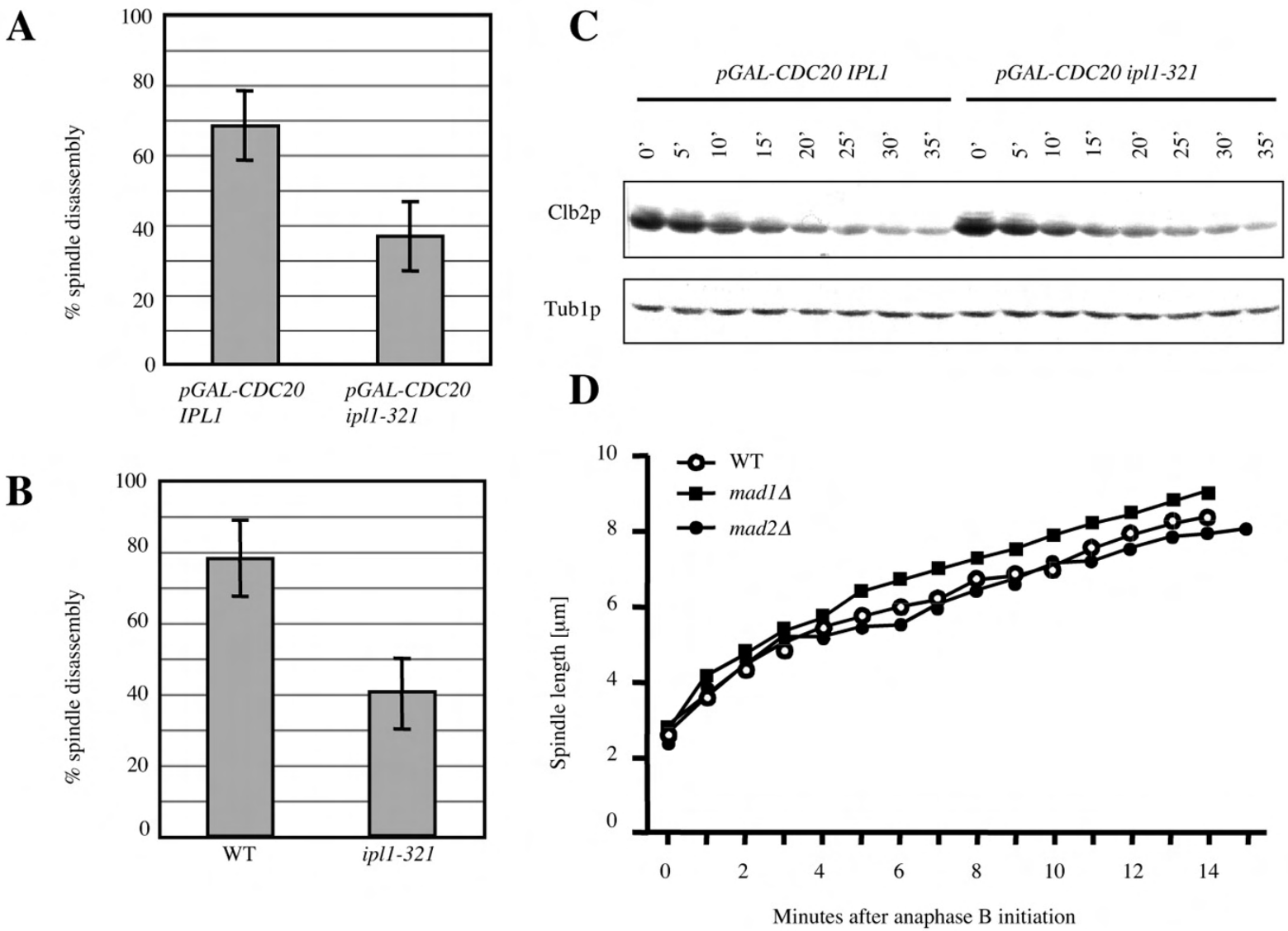
A
100
80
60
40
20
0
% spindle disassembly
pGAL-CDC20 IPL1
pGAL-CDC20 ipl1-321
B
100
80
60
40
20
0
% spindle disassembly
WT
ipl1-321
C
pGAL-CDC20 IPL1
pGAL-CDC20 ipl1-321
0’ 5’ 10’ 15’ 20’ 25’ 30’ 35’ 0’ 5’ 10’ 15’ 20’ 25’ 30’ 35’
Clb2p
Tub1p
D
WT
mad1Δ
mad2Δ
Spindle length [µm]
10
8
6
4
2
0
0 2 4 6 8 10 12 14
Minutes after anaphase B initiation

**Figure 4.2.4.** Ipl1p's role in spindle disassembly is independent from its roles in sister chromatid segregation and the spindle checkpoint. (A) *pGAL-CDC20* (SBY952) and *pGAL-CDC20 ipl1-321* (SBY943) cells containing Tub1-GFP were shifted to glucose to arrest cells in metaphase and then shifted to 37°C inactivate Ipl1-321p. Cells were then released into galactose media at 37°C to restore Cdc20 protein synthesis in the presence of α-factor to arrest cells in the following G1. The percentage of cells with a pole-to-pole distance greater than 9 μm were monitored for the presence or absence of a spindle. Spindle disassembly occurred in 68% of *pGAL-CDC20* cells compared to 36% of *pGAL-CDC20 ipl1-321* mutant cells released from metaphase. (B) Wild type (SBY130) and *ipl1-321* (SBY97) cells containing Tub1-GFP were released from α factor (T=0) into the restrictive temperature (37°C). Time points were taken at 60, 70, and 80 minutes after release and monitored for the presence or absence of a spindle as in (A). In wild type cells, 78% of the spindles have depolymerized compared to only 40% of *ip1l-321* mutant spindles. The bars represent the 95% confidence interval. (C) Cells from (A) were taken every 5 min and Clb2p and Tub1p (loading control) protein levels were monitored by immunoblotting. Clb2p levels decline with similar kinetics in both strains indicating that *ipl1-321* mutant cells exit mitosis normally. (D) Wild type (⭘, SBY130), *mad1Δ* (✿, SBY1423) and *mad2Δ* (●, SBY1422) strains containing Tub1-GFP were analyzed for spindle elongation and disassembly as described in Figure 4.2.3.B. *mad1Δ* and *mad2Δ* mutants disassemble spindles with the same kinetics as wild type cells, indicating that a spindle disassembly defect is not a general property of all checkpoint mutants.

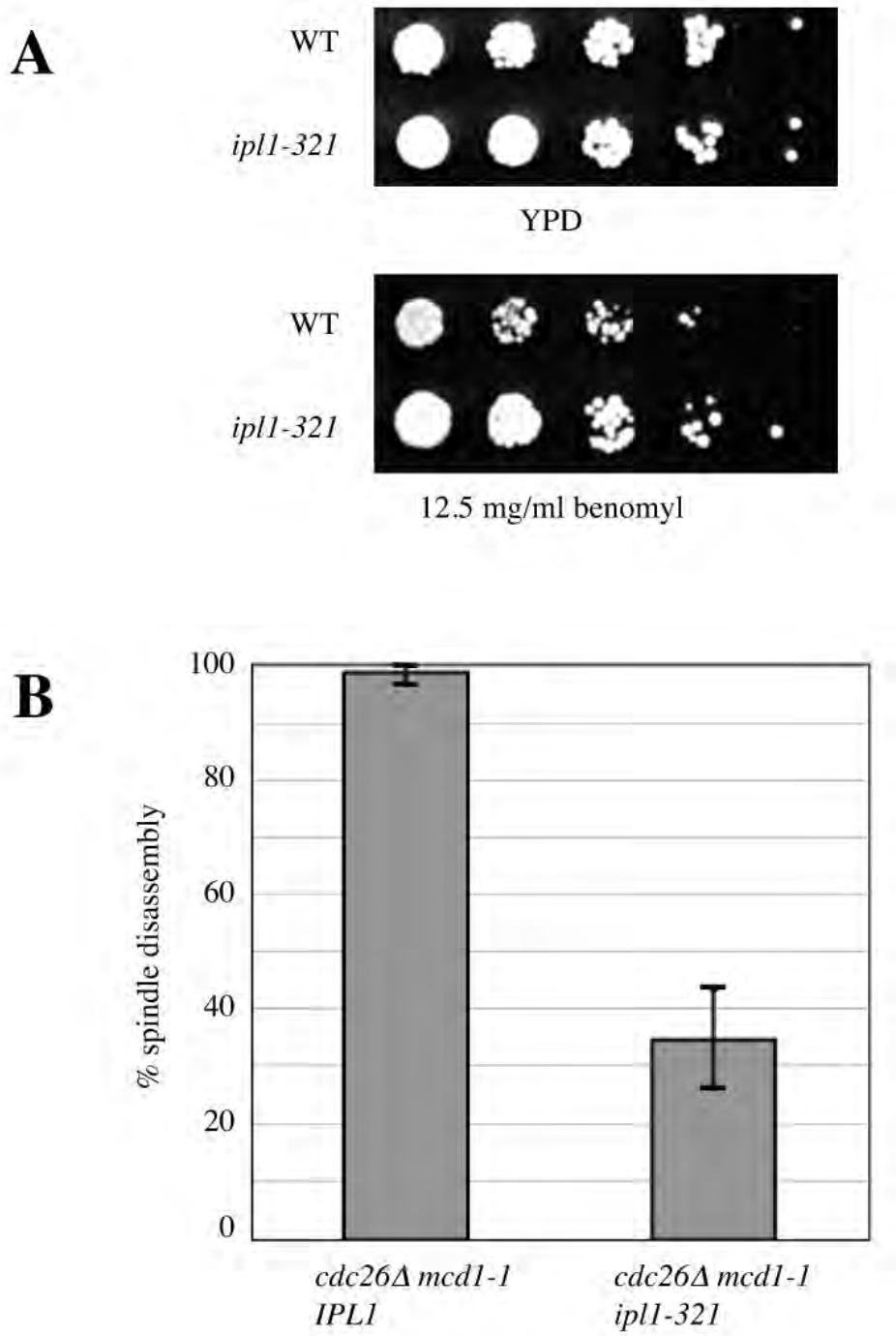


**Figure 4.2.5.** *ipl1-321* mutants have hyperstable microtubules. (A) 5 fold serial dilutions of wild type (SBY214) and *ipl1-321* (SBY322) cells were spotted on rich media (YPD, top panel) or YPD containing 12.5 mg/ml of benomyl (bottom panel) and grown at 23°C. *ipl1-321* cells grow better than wild type cells on media containing the microtubule-depolymerizing drug benomyl, indicating that they have hyperstable microtubules. (B) *cdc26Δ mcd1-1* (SBY965) and *cdc26Δ mcd1-1 ipl1-321* (SBY964) cells were shifted to 37°C to arrest cells in "anaphase-like prometaphase" and the percentage of spindle disassembly was quantified. The majority of spindles in the *cdc26Δ mcd1-1* strain (99%) have disassembled compared to the *cdc26Δ mcd1-1 ipl1-321* cells (35%). The bars represent the 95% confidence interval.

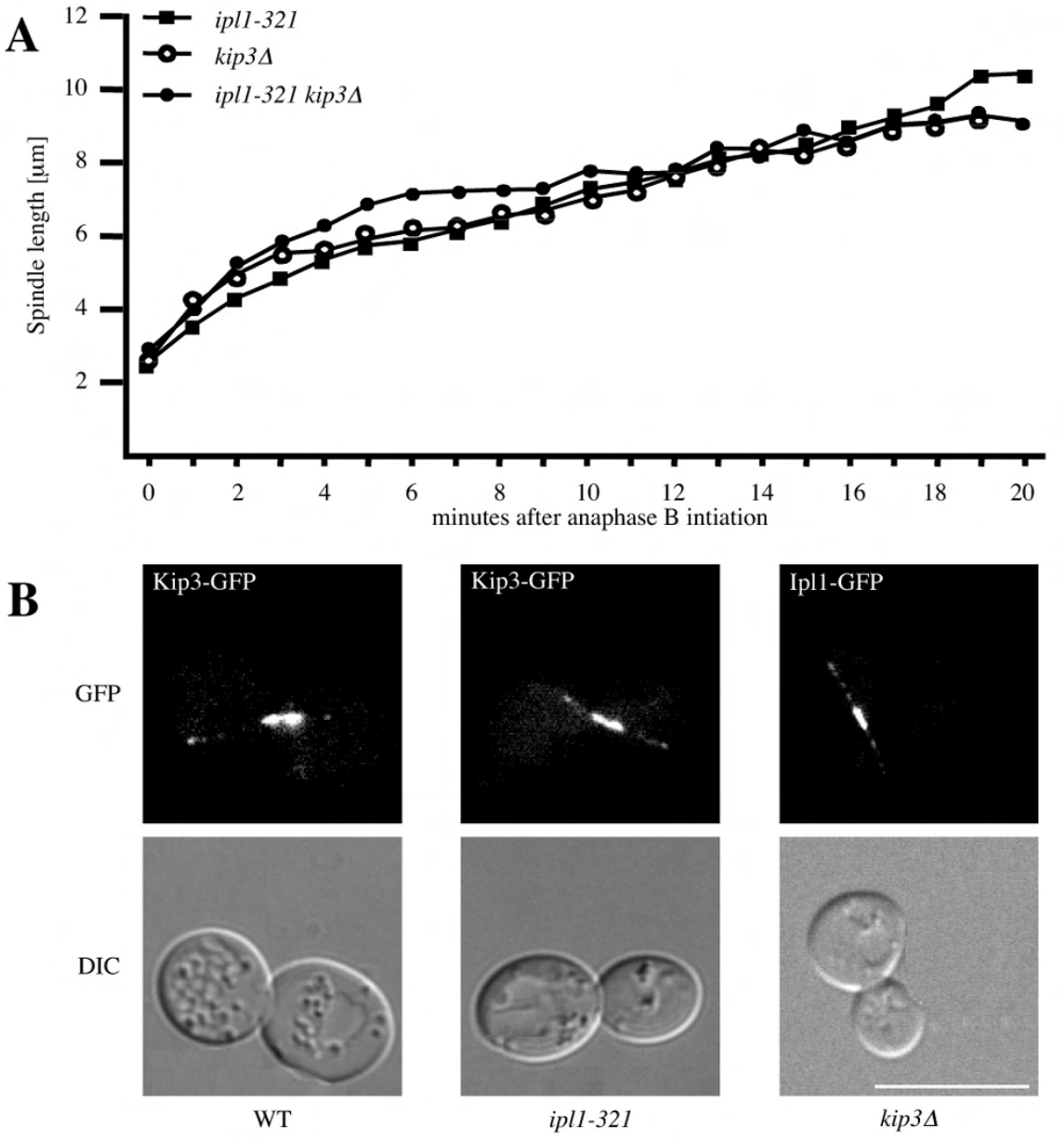


**Figure 4.2.6**. Ipl1p and Kip3 act in the same pathway. (A) *ipl1-321* (✿, SBY97), *kip3Δ* (✪, SBY1538) and *kip3Δ ipl1-321* (●, SBY1539) strains containing Tub1-GFP were analyzed for spindle elongation and breakdown as described in Figure 4.2.3. The average spindle length for 10 cells (6 for *kip3Δ ipl1-321*) of each strain was measured and graphed. All 3 mutants have a similar delay in spindle disassembly suggesting that they act in the same pathway. (B) Ipl1p and Kip3p do not require each other to localize to the spindle midzone. Live microscopy was performed on cells containing endogenous Kip3p fused to GFP at the C-terminus in a wild type (SBY1355) or *ipl1-321* strain (SBY1276) that had been shifted to 37°C for 30 min to inactivate Ipl1p-321. Live microscopy was also performed on Ipl1-GFP in a *kip3Δ* background (SBY1543). DIC pictures are shown to the top of each corresponding fluorescence picture. Both proteins are able to localize to the spindle midzone in the absence of the other protein. Bar: 10 µm.

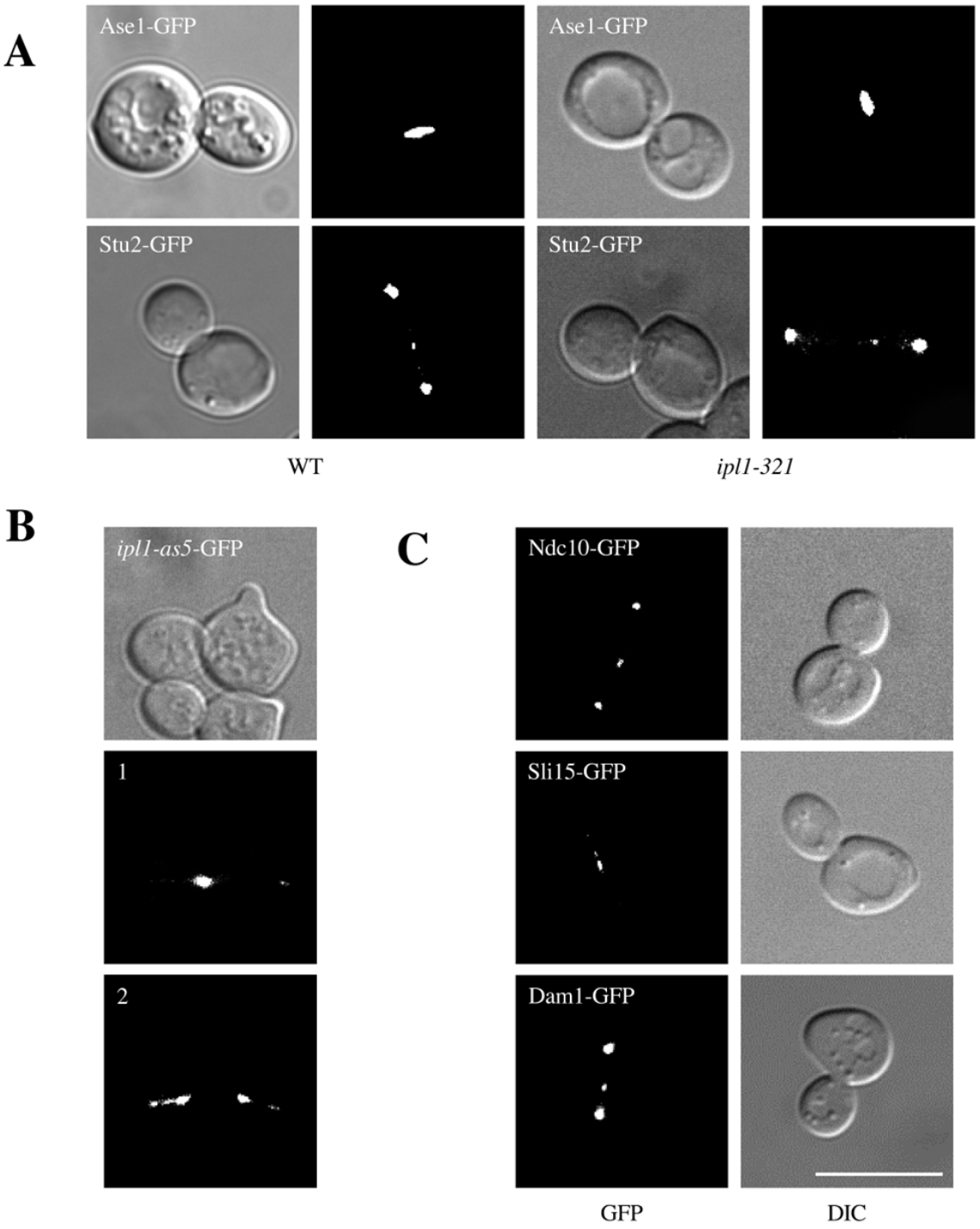
A
Ase1-GFP
Ase1-GFP
Stu2-GFP
Stu2-GFP
WT
ipl1-321
B
ipl1-as5-GFP
1
2
C
Ndc10-GFP
Sli15-GFP
Dam1-GFP
GFP
DIC

**Figure 4.2.7.** The midzone is not grossly altered in an *ipl1-321* mutant and a number of Ipl1p potential substrates are at the midzone. (A) The midzone is not grossly altered in an *ipl1-321* mutant. Live microscopy was performed on cells containing endogenous Ase1p (top row) or Stu2p (bottom row) fused to GFP at the C-terminus in a wild type background (SBY1162: Ase1-GFP, SBY1447: Stu2-GFP, left) and in an *ipl1-321* background (SBY1802: Ase1-GFP, SBY1448: Stu2-GFP, right). The DIC image is shown on the left of each corresponding fluorescent image. Cells were shifted to 37°C for 1 hour to inactivate Ipl1-321p. Both proteins localize at the midzone in the absence of Ipl1p. (B). *ipl1* mutant localize at the midzone in anaphase. *ipl1-as5-GFP* mutant cells (SBY1356), arrested in G1 using α-factor, were released in the cell cycle in the presence of 50 μM inhibitor (NA-1), as in Figure 3.2.6.A. Time point were taken and the cells were analyzed by microscopy. The DIC picture (top row) shows that the cell is in late anaphase. At this time, *ipl1-as5-GFP* localizes at the midzone (florescent picture 1). After a little while, *ipl1-as5-GFP* was seen on the depolymerzing spindle. An *ipl1* mutant protein is therefore able to localize at the midzone normally. (C) Some Ipl1p substrates localize to the spindle midzone. Live microscopy was performed on strains containing endogenous Ndc10p (SBY539), Sli15p (SBY875) or Dam1p (SBY1115) fused to GFP at the C-terminus. The fluorescence images (left panels) show that all three Ipl1p substrates localize to the spindle midzone. The corresponding DIC pictures are shown on the right. Bar: 10 μm.

## 4.3. DISCUSSION

I have shown that the Ipl1/Aurora protein kinase plays a role in spindle microtubule disassembly and spindle orientation. Ipl1p's role in spindle disassembly is not a consequence of a delay in mitotic exit or a prior defect in chromosome segregation. In addition, it is unlikely due to a defect in the spindle checkpoint since the *mad1* and *mad2* checkpoint mutants exhibited normal spindle disassembly. This is the first study examining the effects of spindle checkpoint mutants on anaphase spindle dynamics and it suggests that the spindle checkpoint does not directly regulate microtubules in metaphase and anaphase. Ipl1p localizes to the spindle midzone in anaphase and tracks the plus ends of the depolymerizing spindle microtubules, suggesting it may directly regulate microtubule plus ends. I found that a number of kinetochore proteins localize to the midzone, suggesting the plus ends of the interpolar microtubules may be regulated in a manner similar to kinetochores. I propose that Ipl1p is a general microtubule plus end regulator and that its function in spindle disassembly in anaphase is similar to its function in promoting bi-orientation in prometaphase.

### 4.3.1 Ipl1p's role in spindle orientation

In addition to a role in spindle breakdown, live microscopy also revealed that *ipl1* mutants misorient their spindles in metaphase. The angles by which the spindle deviates from the mother-bud axis are greater in *ipl1* mutant cells and their spindles take longer to orient than in wild type cells. A β-tubulin mutant that has hyperstable microtubules has orientation defects similar to *ipl1* mutants (Gupta et al., 2002). So it is possible that Ipl1p acts on cytoplasmic microtubules in a similar manner to nuclear microtubules to promote spindle orientation. However, the defects observed in the β-

tubulin mutants and in *ipl1* mutants are not exactly the same. β-tubulin mutants show a decrease in microtubules dynamics, indicated by a decrease in recovery after photobleaching (FRAP) (Gupta et al., 2002). *ipl1* mutation does not affect the total turnover of microtubules as recovery after photobleaching is normal in an *ipl1* mutant cell (data not shown). It is possible that Ipl1p's role it to promote microtubule depolymerization and not to regulate the turnover of tubulin dimers. Alternatively it is possible that only a fraction of microtubules are affected in an *ipl1* mutant cell, only the kinetochore microtubules for example, and that does not constitute a defect that can be seen by FRAP. In addition, I never observed endogenous Ipl1p in the cytoplasm, although I cannot exclude that there may be a small pool of cytoplasmic staining that I could not detect. When overexpressed, Ipl1-GFP is found in the cytoplasm (Kim et al., 1999). It is therefore possible that the orientation problems I observed may be indirect. For instance, the hyperstable nuclear microtubules may lead to changes in the cytoplasmic microtubule dynamics that result in orientation defects. It is also possible that Ipl1p regulates a protein in the nucleus affecting its function in the nucleus as well as in the cytoplasm. It will take further work to understand whether the spindle orientation problem is a direct function for the Ipl1p kinase.

*kip3Δ* mutants have also been reported to have a spindle positioning defect. The defects in both *kip3Δ* and *ipl1* mutants were similar in that the "flipping" of the spindle was also observed in *kip3Δ* mutant cells. However the defects in both mutants were not equivalent as *kip3Δ* mutant cells have defect in nuclear migration in addition to a defect in spindle orientation. The addition of the *ipl1* mutation did not increase the defect in spindle orientation of the *kip3Δ* mutant cells. Therefore, if Ipl1p plays a specific role in spindle positioning it is likely to act in the same pathway as Kip3p.

### 4.3.2. Functions of the Ipl1/Aurora protein kinase family

I show that the localization of Ipl1p to the mitotic spindle is correlated with spindle disassembly in budding yeast. Since Aurora B localizes to the spindle midzone in all organisms, this may be another conserved function of the Ipl1/Aurora protein kinase family. In other organisms, Aurora B is required for cytokinesis and this may be coupled to defects in spindle microtubule depolymerization that have not been previously noticed. In human cells, cytokinesis is regulated by the phosphorylation of CENP-A by Aurora B (Zeitlin et al., 2001). We report here that the budding yeast histone variant Cse4p is also a good substrate for Ipl1p *in vitro*. Further analysis of the effects of Cse4p phosphorylation by Ipl1p may reveal more details about spindle disassembly and/or cytokinesis in budding yeast.

I show that the dynamics of spindle elongation are not altered in *ipl1* mutant cells, unlike mutants in the Aurora-INCENP-Survivin complex in *Schizosaccharomyces pombe* (Morishita et al., 2001; Rajagopalan and Balasubramanian, 2002). This difference may be due to the number of kinetochore microtubule binding sites in each organism. In *Schizosaccharomyces pombe*, there are multiple binding sites, which results in lagging chromosomes if bi-orientation is not achieved. In budding yeast where there is a single microtubule binding site, defects in bi-orientation cannot generate lagging chromosomes. However, when a conditional dicentric chromosome is activated in budding yeast thus creating a lagging chromosome, spindle elongation is delayed (Yang et al., 1997). My study in budding yeast had the advantage that defects in bi-orientation do not interfere with spindle dynamics. To determine whether the spindle disassembly function of Ip1lp is conserved, spindle dynamics will need to be analyzed in situations where chromosome segregation is normal.

Consistent with a role in spindle disassembly, we found that Ipl1p kinase activity increases just prior to spindle breakdown. Few studies have looked at the regulation of Ipl1p homologs. In *Drosophila* and rat tissues, Aurora B protein levels and kinase activity peak during mitosis (Bischoff et al., 1998; Terada et al., 1998). However, the time points were not close enough in those studies to determine whether the peak of kinase activity corresponds to spindle breakdown. In fission yeast, Aurora B is not cell cycle regulated (Petersen et al., 2001; Leverson et al., 2002), making it unclear whether there are conserved mechanisms that regulate Ipl1/Aurora B protein levels and kinase activity. Our study also revealed that Ipl1p kinase activity is low when cells are arrested in metaphase with kinetochores under tension. This may reflect an active mechanism that regulates Ipl1p stability and/or activity once tension is established. Future work will be needed to elucidate the mechanisms that lead to changes in Ipl1p kinase activity during mitosis.

### 4.3.3. How does Ipl1p regulate spindle disassembly?

In support of a role for Ipl1p in direct regulation of microtubules, I found that *ipl1* mutants have hyperstable microtubules. The *ipl1* mutant cells are more resistant to the microtubule depolymerizing drug benomyl than wild type cells and are able to alleviate the spindle fragility of *apc mcd1* mutant cells. However, since Ipl1p is the first example of a protein that stabilizes *apc mcd1* spindles when mutated, it is not clear how it acts. Although mutations in Ipl1p affect spindle breakdown, they do not do this by grossly altering the structure of the spindle midzone since all the spindle midzone proteins I tested still localized to the midzone in an *ipl1* mutant.

My studies using live microscopy revealed a previously unidentified localization pattern for Ipl1p. At anaphase, Ipl1p is transported along the spindle to

the midzone and then tracks the plus ends of the depolymerizing spindle microtubules back to the poles. To my knowledge, the only other protein in budding yeast that exhibits this localization pattern is the Ipl1p substrate Ndc10p and suggests regulated transport of these proteins on microtubules in anaphase, possibly by motor proteins (D. Bouck and K. Bloom, personal communication). The localization to the plus ends may indicate that Ipl1p directly destabilizes microtubules like catastrophe factors, such as the KINI family of motor proteins (Desai et al., 1999). Accordingly, Ipl1p binds microtubules *in vitro* (Kang et al., 2001). However, Ipl1p does not phosphorylate microtubules or tubulin monomers in an *in vitro* kinase assay (data not shown). Ipl1p may therefore directly promote microtubule depolymerization by a phosphorylation independent mechanism, or it may instead control a microtubule-binding protein.

There are two non-essential proteins known to be involved in spindle microtubule disassembly in budding yeast: the motor protein Kip3 and the microtubule associated protein Ase1 (Juang et al., 1997; Straight et al., 1998). Ipl1p and Kip3p may act in the same spindle disassembly pathway because the double mutant exhibits the same spindle breakdown defect as each single mutant. So far I have yet to obtain evidence that Ipl1p regulates Kip3p or Ase1p (data not shown). There are other potential candidates for Ipl1p regulation that will need to be investigated, such as the midzone protein Stu2 that opposes the Kip3 protein and the Esp1p/Pds1p cell cycle regulation complex that is also found at the midzone and has a function stabilizing spindles during anaphase (Jensen et al., 2001; Severin et al., 2001a; Uhlmann et al., 2000). I also found that three known Ipl1p substrates are at the spindle midzone: Ndc10p, Sli15p and Dam1p. It is interesting to note that Dam1p was originally identified for its role in regulating spindle dynamics (Hofmann et al., 1998;

Jones et al., 1999). Therefore, a number of potential Ipl1p substrates localize to the spindle midzone and it will need to be determined whether any of these candidates also promote spindle disassembly.

### 4.3.4. The spindle midzone: a kinetochore-like structure?

Several kinetochore proteins are now known to localize to the spindle midzone in anaphase, including Stu2p, Slk19p and the motor protein Cin8 (Hoyt et al., 1992; Kosco et al., 2001; Zeng et al., 1999). Here I show four additional kinetochore proteins localizing to the midzone: the Ipl1/Aurora protein kinase, the INCENP homolog Sli15p, Dam1p and Ndc10p. Since midzone staining is difficult to detect, it may have been overlooked in a number of other localization studies and many more kinetochore proteins may be present at the midzone. Most of the spindle midzone proteins have been implicated in the regulation of spindle dynamics in anaphase by either promoting spindle elongation or spindle disassembly. An intriguing possibility is that the microtubule plus ends at the spindle midzone are regulated in anaphase similarly to the kinetochore-microtubule attachments in prometaphase. Future work will determine whether the Ipl1/Aurora protein kinase and other spindle midzone proteins are global regulators of microtubule plus ends.

## 4.4 Future Plans

### 4.4.1. How is Ipl1p accumulation to the midzone achieved?

I have shown that Ipl1p localization in anaphase is very dynamic localizing onto the entire spindle length in anaphase, accumulating at the spindle midzone in late anaphase and then tracking the plus ends of the depolymerizing spindle back to the poles (Figure 4.2.1.). Furthermore, I have also shown that Ipl1p kinase activity is not

important for this localization pattern (Figure 3.2.6. and 4.2.7.), as a kinase inactive mutant is able to accumulate at the midzone in late anaphase. It will therefore be interesting to determine the requirement for this localization. As Ipl1p is found in a punctate pattern along the spindle length, one likely possibility is that it is transported by a motor protein. To test this, I will analyze Ipl1p localization in anaphase in cells lacking a motor protein. As discussed in chapter 3.4.2, of the six motor proteins implicated in mitosis, two are thought to be plus end directed motor proteins, the direction needed to accumulate at the midzone: Cin8p and Kip1p. In addition, Cin8p does localize to the midzone in anaphase making it a likely candidate (Hoyt et al., 1992). It is interesting to note that Cin8p and Ipl1p alleles are synthetically lethal, even though the nature of this synthetic lethality is unclear (Geiser et al., 1997). I will therefore analyze the localization of Ipl1-GFP by live microscopy in anaphase in a background deleted in one of these motor proteins. If no motor is implicated in Ipl1p accumulation at the midzone, I will test the contribution of other members of the Ipl1/Sli15/Bir1 complex as in chapter 3.4.3. Ipl1-GFP localization in anaphase will be analyzed in cells depleted for one of the component of the complex.

### 4.4.2. Does Ipl1p directly regulate microtubules?

I show here that Ipl1p plays a role in spindle disassembly in late anaphase, however it is unclear whether Ipl1p acts directly on microtubules or not. Since Ipl1p is able to directly bind microtubules *in vitro* (Kang et al., 2001), I will determine whether Ipl1p is able to promote microtubule depolymerization *in vitro*. Taxol stabilized microtubules will be polymerized *in vitro* (Hyman et al., 1991). They will then be incubated with increasing amount of bacterial Ipl1p and incubated at RT. After sedimentation on a glycerol cushion, the supernatants and the pellets will be

analyzed by SDS-PAGE. Intact microtubules will appear in the pellet whereas depolymerized microtubules will appear in the supernatant (Desai et al., 1999). If Ipl1p is able to promote microtubules depolymerization, the amount of tubulin in the supernatant will increase. If not that would suggests that Ipl1p acts by regulating a minrotubule binding protein (see below).

As mentioned above, Ipl1p is able to bind microtubules *in vitro*. However it would be interesting to determine whether Ipl1p has a preference for microtubule plus ends as it is found at the plus ends of microtubules *in vivo*. To do this, I will determine whether Ipl1p binding to microtubules increases when more plus ends are present. Taxol stabilized microtubules will be polymerized *in vitro* (Hyman et al., 1991). Half of the microtubules will be mechanically sheared whereas the other half will be kept intact. Bacterial Ipl1p will then be incubated with the microtubules. After sedimentation through a glycerol cushion, I will determine whether the amount of Ip1lp precipitated is different when the microtubules are sheared or not. If more Ipl1p is precipitated when the microtubules are sheared that would suggest that Ip1lp has a preference for the ends of the microtubules. As *in vivo* Ip1lp is found at the plus ends of microtubules I would infer that Ipl1p has a preference for microtubules plus ends. If no difference is observed that would suggest that Ipl1p binds the length of the pure microtubules.

### 4.4.3. Ipl1p substrate for the spindle depolymerization function

As mentioned in the discussion, a number of potential Ipl1p substrates exist for the spindle function. This includes the motor protein Kip3, the MAPs Ase1p and Stu2p as well as Ipl1p potential substrates at the midzone Ndc10p, Sli15p and Dam1p. First I will determine whether Ipl1p phosphorylates Kip3p, Ase1p or Stu2p.

*In vivo* phosphate labeling will be performed on wild type versus *ipl1* mutant cells shifted to the restrictive temperature (37°C) for 1 hour to inactivate mutant Ipl1 protein. The phosphorylation status of the different proteins will then be analyzed by autoradiography. All three proteins contain Ipl1p potential phosphorylation sites. Kip3p has 3 potential sites, whereas both Ase1p and Stu2p have 5 potential sites. If one of the proteins is phosphorylated in an Ipl1p dependent manner, I will mutate the potential phosphorylation sites and analyze spindle dynamics in anaphase by live microscopy as done in Figure 4.2.3. to determine whether the phosphorylation by Ipl1p of this protein plays a role in spindle disassembly. In parallel, the potential phosphorylation sites of Ipl1p in Ndc10p, Sli15p or Dam1p will be mutated and spindle dynamics in anaphase will be analyzed in the resulting mutants to determine if any of these proteins plays a role in spindle disassembly. If none of the potential substrates tested play a role in spindle disassembly, more work will be needed on the regulation of spindle disassembly to determine which protein(s) Ipl1p is regulating for its spindle disassembly function.

### 4.4.4. Spindle orientation characterization

I show here that *ipl1* mutant cells have a spindle orientation defect: the spindle takes longer to align with the mother-bud axis in an *ipl1* mutant cell than wild type. I will characterize this function further to determine whether this is a new function for Ipl1p or whether this an indirect effect. I will determine whether the defect in spindle orientation can be uncoupled from Ipl1p chromosome segregation function. To do this, I will arrest cells expressing Tub1-GFP in metaphase by depleting the Cdc20 protein (*pGAL-CDC20* vs. *pGAL-CDC20 ipl1*) and then inactivate Ipl1 mutant protein by shifting to the restrictive temperature. I have shown that under those conditions,

Ipl1p function in chromosome segregation is fulfilled (Figure 4.2.5.A.). Fixed cells will then be analyzed to determine whether *ip1l* mutant cells show a defect in spindle orientation under those conditions. If this is observed, it suggests that the defect in spindle orientation is an independent function of Ipl1p. If no defect in spindle orientation can be seen, no strong conclusion can be drawn. Indeed, it could mean that Ipl1p does not play a direct role in spindle orientation or that in metaphase Ipl1p has already fulfilled its role in spindle orientation.

I will also analyze the behavior of cytoplasmic microtubules carefully over the cell cycle, as they are thought to regulated spindle orientation. I show here that the number of cytoplasmic microtubules is unaltered in anaphase. However, a recent study found that β-tubulin mutant that has orientation defects similar to *ipl1* mutants, showed an altered number of cytoplasmic microtubules in G1 (Gupta et al., 2002). I will therefore determine whether the number of cytoplasmic microtubules is altered in other phases of the cell cycle. To do this, wild type and *ipl1* mutant cells expressing Tub1-GFP will be analyzed by live microscopy throughout an entire cell cycle at the restrictive temperature. This will also allow me to determine whether the length of cytoplasmic microtubules is affected by an *ipl1* mutation. I will also determine whether the dynamics of the microtubules are affected by analyzing the rates of polymerization and depolymerization. Those studies will determine whether Ipl1p affects cytoplasmic microtubules.

I will investigate whether Ipl1p shows genetic interaction with proteins known to play a role in spindle orientation. Two pathways promoting spindle positioning have been described. One pathway containing Kar9p, Kip3p, Bim1p etc is responsible for the migration of the nucleus to the bud neck and for alignment with the mother-bud axis (Kusch et al., 2002). The second pathway containing Dyn1p, Num1p, etc is

responsible for the migration of the nucleus through the bud neck (Eshel et al., 1993; Li et al., 1993; Yeh et al., 1995)(Heil-Chapdelaine et al., 2000). The two pathways are partially redundant and spindle positioning occurs in most cases with the presence of only one pathway. In addition, other protein such as Stu2p, β-tubulin, etc have been shown to play a role in spindle positioning even though it is not clear how (Kosco et al., 2001) (Gupta et al., 2002). My preliminary experiments analyzing the double mutant *ipl1 kip3Δ* suggested that Ipl1p might act in the Kip3p pathway but further genetic evidence would be necessary to confirm this result. In addition, if Ipl1p shows specific genetic interaction with only one pathway this would strengthen the idea that this is a specific function for Ipl1p.

Taken together, these experiments should reveal how Ipl1p mechanically functions during different phases of mitosis. Results should reveal more information about this oncogene and its substrates or binding partners and should therefore help to understand the generation of genomic instability.

# CHAPTER 5. *ipl1* MUTANTS BYPASS A METAPHASE ARREST CAUSED BY INACTIVATION OF THE APC

## 5.1. SUMMARY

The spindle checkpoint is a surveillance mechanism that arrests cells in metaphase when kinetochore-microtubule attachment is impaired. It acts by inhibiting the Cdc20 specificity factor of the anaphase promoting complex. Mutations in any subunit of this complex show a metaphase arrest phenotype. The laboratory has previously published that in an *apc ipl1-321* double mutant strain the spindle elongates. I decided to analyze this phenotype further and found that sister chromatid separation occurs in an *apc ipl1-321* strain, resulting in spindle elongation. I show here that this actually represents a new function of the spindle checkpoint. The bypass of the metaphase arrest caused by a mutation in the APC is dependent on the Esp1p protease but does not depend on Pds1p/securin degradation. Even though the exact mechanism is not clear, data presented here suggest that the checkpoint acts as an inhibitor of the Esp1p protease. In this model, in an *apc* mutant, Esp1p is inhibited both by securin and by the spindle checkpoint. Inactivation of either pathway would lead to Esp1p activation resulting in sister chromatid separation and spindle elongation. I also show that even though sister chromatid separation and spindle elongation are normally coupled, they are independent processes. Spindle elongation can occur in the absence of sister chromatid separation.

## 5.2. RESULTS

### 5.2.1. The spindle elongation in an *apc ipl1* mutant cell is independent from Ipl1p's kinetochore function

The APC is a protein complex that targets mitotic inhibitors for ubiquitin dependent degradation (for reviews see Peters, 2002; Zachariae and Nasmyth, 1999). When this complex is inactivated, by a mutation in a protein of the complex (Cdc26p, Cdc20p, Cdc23p…), cells arrest in metaphase with sisters held together and short spindle (Zachariae et al., 1996). The laboratory had previously published that in *cdc23-1 ipl1-321* mutant cells, the spindle elongates (Biggins et al., 1999). This was originally thought to represent a defect in kinetochore function. I decided to analyze this phenotype more thoroughly and found that the original hypothesis was not correct as discussed below. I first repeated the experiment with another mutant of the APC complex to determine whether this was an allele specific phenomenon. Cdc26p is a heat shock protein that stabilizes the interaction of the core subunits with the rest of the particle (Zachariae et al., 1998b; Zachariae et al., 1996). It is therefore only essential at 37°C. *cdc26Δ* and *cdc26Δ ipl1-321* mutant cells expressing Tub1-GFP were grown to mid-log at the permissive temperature (23°C) and then shifted to the restrictive temperature (37°C) for 4 hours to inactivate the gene products. The cells were then fixed and analyzed by microscopy. The spindle pole-to-pole distance was measured in at least 100 cells in each background. A representative example is shown for each strain in Figure 5.2.1.A. As reported, *cdc26Δ* cells arrested as large budded cells with a short spindle (Zachariae et al., 1996). I found that 70% of *cdc26Δ ipl1-321* cells elongated their spindle (see Figure 5.2.2.), suggesting that spindle elongation is a general property of *apc ipl1-321* mutant cells.

I next wanted to determine whether the spindle elongation in an *apc ipl1-321* mutant cell really reflected Ipl1p's kinetochore function. S. Biggins had previously reported that if Ipl1p is inactivated after bipolar spindle formation chromosome segregation is normals. Therefore, Ipl1p's function at the kinetochore is completed once bipolar spindle is established (Biggins and Murray, 2001). I therefore arrested cells containing Tub1-GFP in metaphase by depleting the Cdc20 protein (*pGAL-CDC20* vs. *pGAL-CDC20 ipl1-321*) and then shifted the cells to the restrictive temperature to inactivate Ip1lp for 4 hours (Figure 5.2.1.B shows the design of the experiment). After fixation of the cells, the pole-to-pole distance was measured. A pole-to-pole distance of 1-3 μm was considered to be a short spindle, whereas a pole-to-pole distance of 4 μm and longer was considered to be a long spindle. The graph in Figure 5.2.1.C shows that, under those conditions, a majority of *pGAL-CDC20* cells (79%) arrested with a short spindle as expected for a mutation in the APC complex. In contrast, 59% of *pGAL-CDC20 ipl1-321* cells elongated their spindle. This suggests that spindle elongation in *apc ipl1-321* mutant cell is independent from Ipl1p's function in chromosome segregation. In addition, this data support the idea that spindle elongation is a general property of an *apc ipl1-321* cell as a third mutant in the APC complex (a run down in Cdc20p) shows the phenotype.

### 5.2.2. Spindle elongation in an *apc ipl1-321* mutant is a general function of the spindle checkpoint.

Because spindle elongation in an *apc ipl1-321* mutant cell did not seem to reflect Ipl1p's function in chromosome segregation, I decided to investigate whether it reflected Ipl1p's function in the spindle checkpoint. I therefore analyzed spindle elongation in an *apc* mutant combined with a spindle checkpoint mutant (*mad1Δ*). I

also analyzed two other kinetochore mutants: a mutant in the histone H3-variant Cse4p, and a mutant in a member of the CBF3 complex: Ndc10p. The two mutants differ from each other in their ability to activate the spindle checkpoint: *cse4-323* mutants are checkpoint proficient whereas *ndc10-1* mutants are checkpoint deficient (S. Biggins data not shown) (Tavormina and Burke, 1998). *cdc26Δ, cdc23-1 cse4-323, cdc26Δ ipl1-321, cdc26Δ ndc10-1* and *cdc26-100 mad1Δ* strains expressing Tub1-GFP were grown at the permissive temperature (23°C) and then shifted to the restrictive temperature (37°C) for 4 hours. After fixation of the cells, the pole-to-pole distance was measured as described above. The graph in Figure 5.2.2. shows that 78% of *cdc26Δ* cells arrest with a short spindle. *cdc26-100* and *cdc23-1* mutant cells also show a large bud arrest with a short spindle and are not significantly distinct from *cdc26Δ* (data not shown). As previously reported, 66% of *cdc23-1 cse4-323* cells are able to maintain a short spindle (Biggins et al., 2001). As mentioned above, *cdc26Δ ipl1-321* cells are unable to maintain a short spindle and 70% of the cells elongate their spindle. I found that 76% of *cdc26Δ ndc10-1* and 73% of *cdc26-100 mad1Δ* cells, like a *cdc26Δ ipl1-321* strain, are unable to maintain a short spindle in a metaphase arrest due to inactivation of the APC. In conclusion, spindle checkpoint mutants (*ipl1-321* and *mad1Δ*) and mutants unable to activate the checkpoint (*ndc10-1*) are unable to maintain a short spindle in an *apc* mutant, suggesting that spindle elongation in an *apc ipl1-321* strain might reflect Ipl1p's function in the spindle checkpoint.

### 5.2.3. The spindle elongation in an *apc ipl1-312* requires Esp1p activity

In order to further characterize spindle elongation in an *apc ipl1-321* cell, I decided to analyze DNA segregation to determine whether chromosome segregation

was coupled to spindle elongation in an *apc ipl1-321* cell. I was also interested in determining whether chromosome segregation was normal. Because *ipl1-321* mutants missegregate their chromosomes, I could not directly study chromosome segregation in an *apc ipl1-321* strain. I therefore used the same system as in Figure 5.2.1.C. Cells from the experiment in Figure 5.2.1.C were stained with DAPI to visualize DNA and analyzed by microscopy. Figure 5.2.3.A shows that DNA segregation occurs in a *pGAL-CDC20 ipl1-321* strain with an elongated spindle. In addition, segregation appears normal judging by the two masses of DNA of equal intensity at each pole, suggesting that sister chromatid separation has occurred. To confirm this result, I also analyzed DNA segregation in a *cdc26-100 mad1Δ* strain that was shifted for 4 hours to 37°C, fixed and processed for immunofluorescence and stained with DAPI to recognize DNA and with anti-Tub1 to recognize the spindle. Chromosome segregation also appears normal in cells with an elongated spindle (see Figure 5.2.3.B). These experiments suggests that sister chromatid separation and spindle elongation are initiated in an *apc ipl1-321* (or *apc mad1Δ*) strain even though the APC is inactive.

The Esp1p protease has been implicated both in sister chromatid separation and spindle elongation even though the latter is controversial as discussed in the introduction. I therefore wanted to determine whether Esp1p was required for spindle elongation in an *apc ipl1-321* mutant. To test this, I analyzed whether spindle elongation in an *apc ipl1-321* and *apc mad1Δ* strain was Esp1p dependent. *cdc26Δ, cdc26Δ ipl1-321, cdc26Δ esp1-1, cdc26Δ ipl1-321 esp1-1* and *cdc26Δ mad1Δ esp1-1* strain were shifted to 37°C for 4 hours and analyzed by microscopy as described above. As seen before, *cdc26Δ* cells show a majority of short spindles, whereas *cdc26Δ ipl1-321* cells elongate their spindle. *esp1-1* mutants are unable to segregate

their sister chromatids or elongate their spindle (Ciosk et al., 1998; Funabiki et al., 1996a). In agreement with that, 71% of *cdc26Δ esp1-1* cells arrest with a short spindle (see Figure 5.2.3.C). 82% of *cdc26Δ ipl1-321 esp1-1* cells and 80% of *cdc26Δ mad1Δ esp1-1* cells arrest with a short spindle, showing that spindle elongation in *apc ipl1-321* and *apc mad1Δ* cells is dependent on active Esp1p. In addition, this suggests that Esp1p may be activated in an *apc ipl1-321* mutant cell to lead to sister chromatid separation and spindle elongation.

### 5.2.4. Spindle elongation in an *apc ipl1-321* mutant cell is independent from Pds1p degradation

The evidence so far suggested that Esp1p may be getting activated in an *apc ipl1-321* mutant cell. Esp1p is normally kept inactive by binding Pds1p. One possible explanation for Esp1p activation is that Pds1p was getting inappropriately degraded in an *apc ipl1-321* mutant liberating an active Esp1p. To test this, I analyzed Pds1p levels in an *apc ipl1-321* mutant cell. *cdc26Δ ipl1-321* cells expressing Pds1-myc were shifted to the restrictive temperature (37°C) for 4 hours and were processed for immunofluorescence. The cells were stained with anti-Tub1 to recognize the spindle, with DAPI to recognize the DNA and with anti-myc antibodies to recognize Pds1-myc. I found that Pds1p levels stay high in *apc ipl1-321* cells with long spindles (Figure 5.2.4.A), indicating that the spindle is able to elongate without Pds1p being degraded.

To confirm that spindle elongation in an *apc ipl1-321* cell occurred without Pds1p degradation, I tested whether spindle elongation in an *apc ipl1-321* cell could occur when non-degradable Pds1p was overexpressed. To do this, *cdc26Δ* cells conditionally overexpressing non-degradable Pds1p, *pGAL-pds1mdb* (mutated in its

destruction box, (Cohen-Fix et al., 1996)) and *cdc26Δ ipl1-321 pGAL-pds1mdb* cells expressing Tub1-GFP were grown in raffinose and arrested in G1 using α-factor. 4% galactose was added to induce non-degradable Pds1p and 30 minutes later cells were released into the cell cycle in galactose at 37°C. Cells were harvested at t = 4 and 5 hours, fixed and analyzed by microscopy. At 4 and 5 hours, 95% and 80% respectively of *cdc26Δ* overexpressing non-degradable Pds1p cells arrested with a short spindle (Figure 5.2.4.B). This is comparable to *cdc26Δ* alone (see Figure 5.2.5.A), so overexpression of non-degradable Pds1p in a *cdc26Δ* background does not alter the terminal phenotype. At 4 hours, 48% of *cdc26Δ ipl1-321* cells overexpressing non-degradable Pds1p elongated their spindle (see Figure 5.2.4.B); this result is similar to *cdc26Δ ipl1-321* cells alone (see Figure 5.2.5.A). Also, at 5 hours *cdc26Δ ipl1-321* cells overexpressing non-degradable Pds1p were able to elongate their spindle (82% long spindles, see Figure 5.2.4.B), again comparable to *cdc26Δ ipl1-321* cells (68% long spindles, see Figure 5.2.5.A). Non-degradable Pds1p (*pds1-mdb*) was highly overexpressed to equivalent amounts in *cdc26Δ pGAL-pds1mdb* and *cdc26Δ ipl1-321 pGAL-pds1mdb* strains (data not shown). In conclusion, spindle elongation in an *apc ipl1-321* strain occurred normally in cells overexpressing non-degradable Pds1p. Therefore, Esp1p activation in an *apc ipl1-321* is independent from Pds1p degradation.

### 5.2.5. Spindle elongation in an *apc ipl1-321* mutant cell is not a direct consequence of sister chromatid separation

I next wanted to determine whether spindle elongation was a direct consequence of sister chromatid separation. Esp1p plays role in the FEAR network and in spindle elongation/stability even though the latter is controversial (see

introduction). I therefore wanted to determine whether these other functions of Esp1p were implicated in spindle elongation in an *apc ipl1-321* mutant. To address this, I decided to test whether I could uncouple spindle elongation from sister chromatid separation by overexpressing a non-cleavable version of the cohesin protein Scc1p that blocks sister chromatid separation. I expressed this in an *apc ipl1-321* cell to determine whether spindle elongation is blocked when sisters cannot separate. *cdc26Δ*, *cdc26Δ ipl1-321* and *cdc23-1 ipl1-321* cells conditionally overexpressing a non-cleavable version of Scc1p (*pGAL-scc1ndb,* mutated in its cleavage site, (Uhlmann et al., 1999)) expressing Tub1-GFP were grown in raffinose and arrested in G1 using α- factor. 4% galactose was added to induce non-cleavable Scc1p and 30 minutes later cells were released into the cell cycle in galactose at 37°C. Cells were harvested at t = 4 and 5 hours and fixed and analyzed by microscopy. Under these conditions, *cdc26Δ* cells arrested with a short spindle: 77% short spindles at 4 hours, 84% at 5 hours (see Figure 5.2.5.A). In this experiment, *cdc26Δ ipl1-321* cells elongated their spindle: 48% had long spindles at 4 hours and 67% at 5 hours. The spindle elongation at 4 hours may not be as dramatic as in other experiments because the cells are grown in galactose and therefore progression through the cell cycle is slowed down. Overexpression of non-cleavable Scc1p in *cdc23-1* cells gave rise to an arrest with short spindles as expected (data not shown). Spindle elongation is delayed but not abolished in an *apc ipl1-321* strain overexpressing non-cleavable Scc1p. At 4 hours, the majority of *cdc23-1 ipl1-321 pGAL-scc1ndb* cells have a short spindle (72%, see Figure 5.2.5.A), showing that the overexpression of non-cleavable Scc1p, which inhibits sister chromatid separation, affects spindle elongation. However at 5 hours, the spindle does elongate: 63% of *cdc23-1 ipl1-321 pGAL-scc1ndb* cells have

long spindles. This is similar to *cdc26Δ ipl1-321* cells, suggesting that spindle elongation can occur even when sister chromatids do not separate.

I wanted to make sure that spindle elongation was occurring without sister chromatid separation. First, I analyzed *scc1nb* levels throughout the experiment and found that it was highly overexpressed even at t = 5 hours (data not shown). Therefore, the inability of a *cdc23-1 ipl1-321 pGAL-scc1ndb* strain to maintain a short spindle cannot be explained by an inability to maintain high levels of non-cleavable Scc1p. Second, I analyzed DNA segregation in the experiment described above. The fixed cells were stained with DAPI to recognize DNA. Figure 5.2.5.B shows that even though spindle elongation occurs, DNA segregation does not. The DNA gets fractionated into several blobs of unequal size. This suggests that sister chromatid separation does not occur blocking proper DNA segregation. Therefore, spindle elongation can be uncoupled from sister chromatid separation and suggests that Esp1p function is required for spindle elongation in an *apc ipl1-321* mutant.

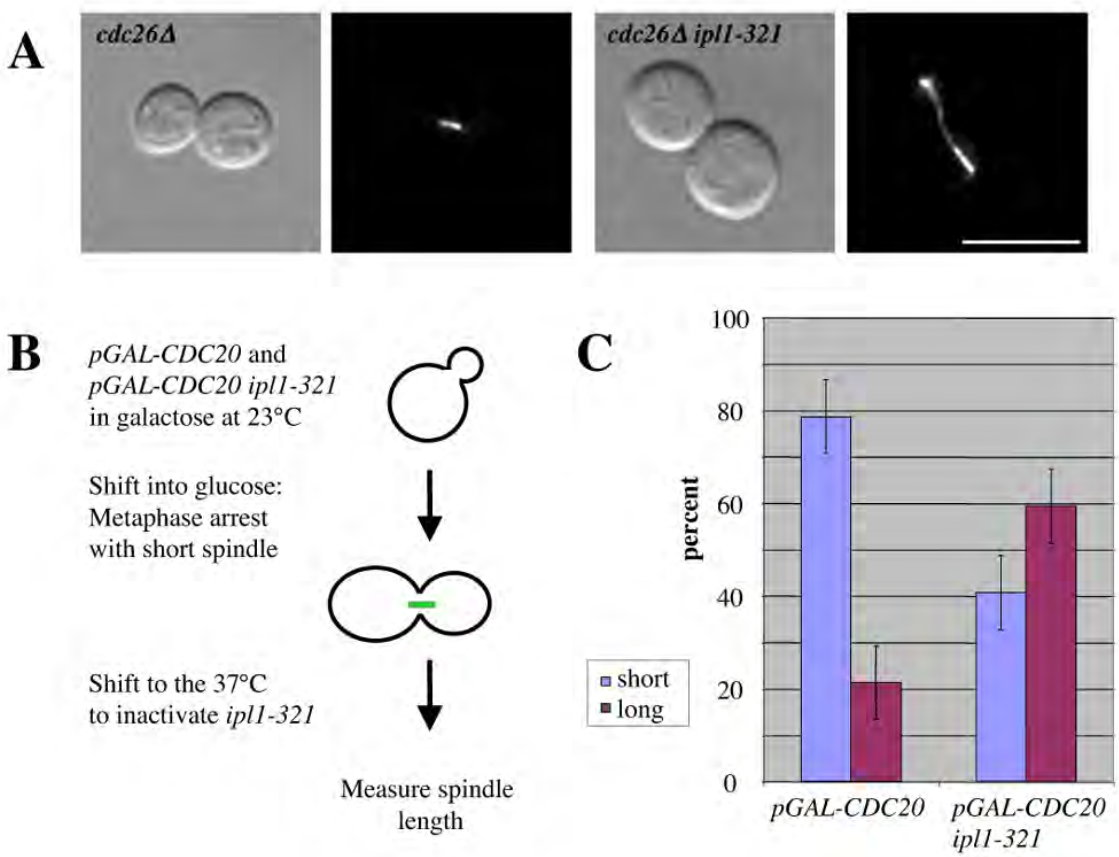


**Figure 5.2.1.** The spindle elongation in an *apc ipl1-321* mutant cell is independent from Ipl1's kinetochore function. (A) *cdc26Δ* (SBY912) and *cdc26Δ ipl1-321* (SBY913) cells expressing Tub1-GFP were grown to mid-exponential phase at permissive temperature and then shifted to the restrictive temperature (37°C) for 4 hours. The cells were then fixed and analyzed by microscopy. A representative example for each strain is shown. DIC is shown on the left and the corresponding fluorescence picture is shown on the right. *cdc26Δ* cells arrest with a short spindle, whereas *cdc26Δ ipl1-321* cells elongate their spindle. Bar: 10 μm. (B) Design of the experiment in (C) *pGAL-CDC20* (SBY952) and *pGAL-CDC20 ipl1-321* cells (SBY943) expressing Tub1-GFP were grown in galactose and then shifted to glucose for 3 hours to arrest cells in metaphase. Cells were then shifted to 37°C for 4 hours to inactivate Ipl1-321p, fixed and analyzed by microscopy. C. Quantification of the cells as described in B. Pole-to-pole distance was measured. 1-3 μm corresponds to a short spindle, 4 μm and higher to a long spindle. 79% of *pGAL-CDC20* cells stay arrested in metaphase with a short spindle, whereas 59% of *pGAL-CDC20 ipl1-321* cells elongate their spindle. Bars represents the 95% confidence interval.

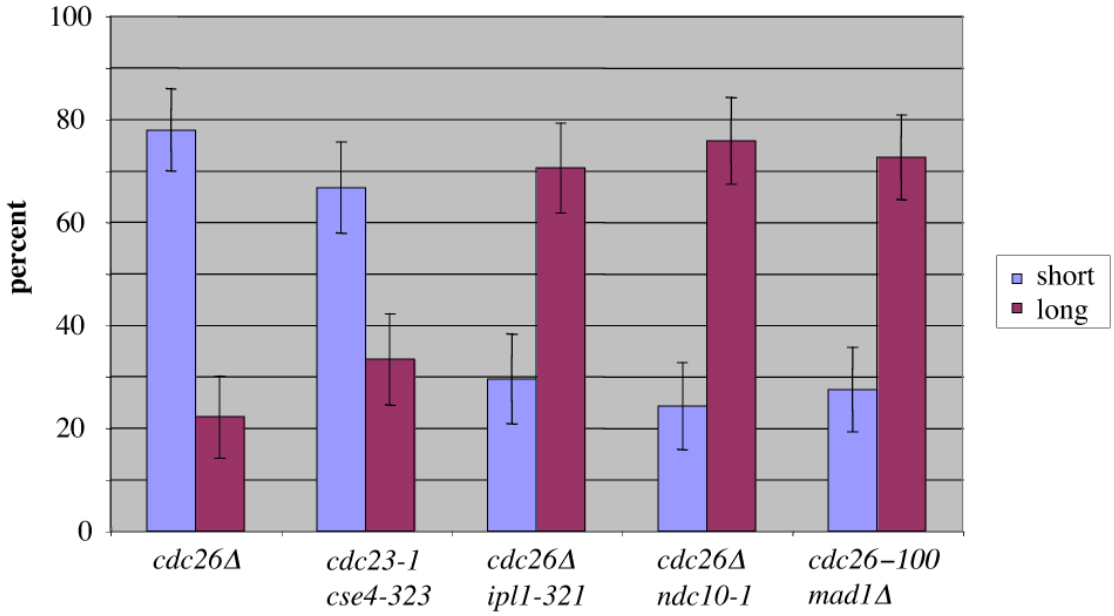


**Figure 5.2.2.** Spindle elongation in an *apc ipl1-321* mutant cell is a general function of the spindle checkpoint. *cdc26Δ* (SBY912), *cdc23-1 cse4-323* (SBY815), *cdc26Δ ipl1-321* (SBY913), *cdc26Δ ndc10-1* (SBY916) and *cdc26-100 mad1Δ* (SBY953) cells expressing Tub1-GFP were grown to mid-exponential phase at permissive temperature and then shifted to the restrictive temperature (37°C) for 4 hours. The cells were then fixed and the pole-to-pole distance was measured as described in Figure 5.2.1. *cdc26Δ* (78%) and *cdc23-1 cse4-1* (66%) cells arrest with a short spindle, whereas *cdc26Δ ipl1-321* (70%), *cdc26Δ ndc10-1* (76%) and *cdc26- mad1Δ* (73%) cells elongate their spindle. Bars represents the 95% confidence interval.

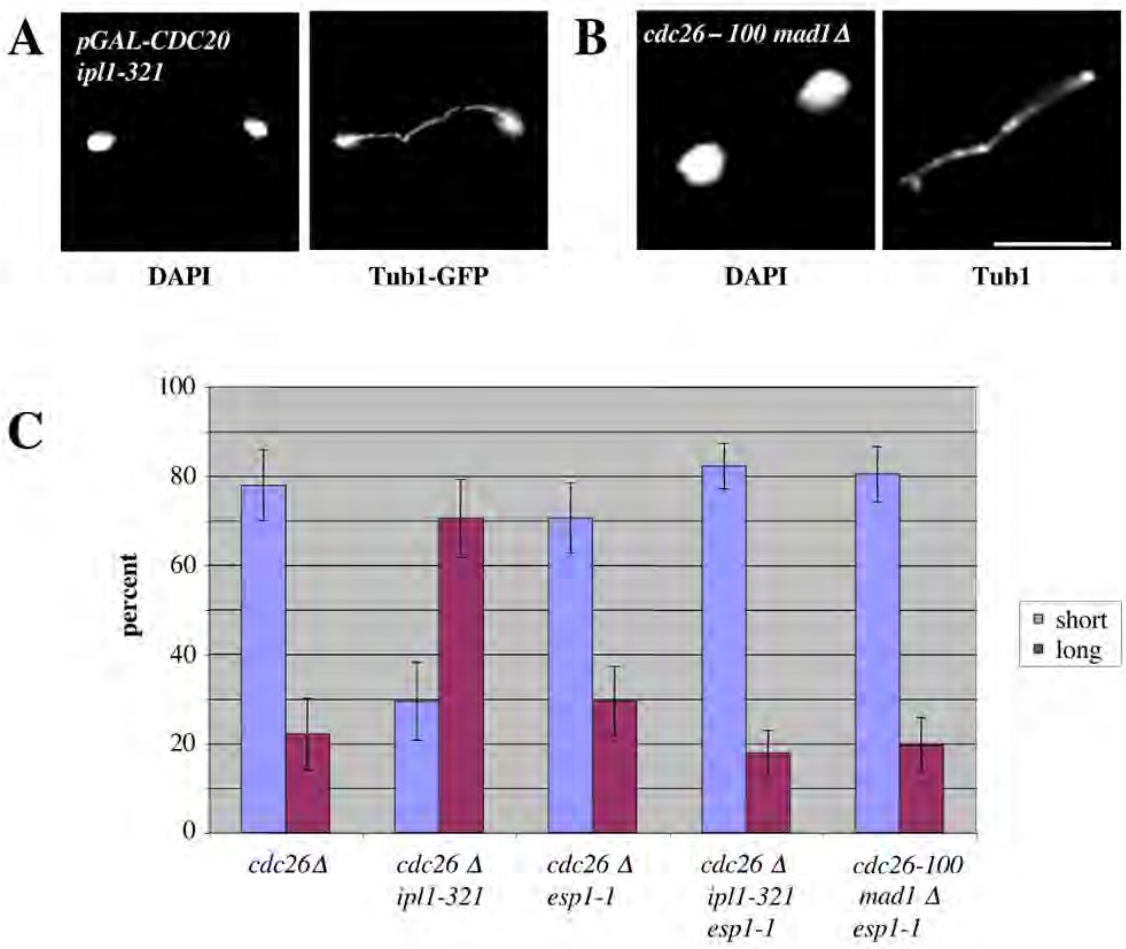


**Figure 5.2.3.** Spindle elongation in *apc ipl1-321* cells is due to Esp1p activity. (A) DNA segregation occurs normally in an *apc ipl1-321* cell when Ipl1-321p is inactivated after metaphase. *pGAL-CDC20 ipl1-321* cells (SBY952) expressing Tub1-GFP from experiment in Figure 5.2.1.C. were stained with DAPI to recognize DNA and analyzed by microscopy. DAPI staining is shown on the left and GFP-Tub1 on the right. The DAPI staining shows that DNA segregates equally under these conditions. (B) DNA segregation occurs normally in *apc mad1Δ* cells. *cdc26-100 mad1Δ* cells (SBY953) shifted for 4 hours to 37°C, were processed for immunofluorescence and stained with DAPI (left) to recognize DNA and with anti-Tub1p to recognize the spindle (right). The DAPI staining shows that DNA segregates normally. Bar: 10μm. (C) Spindle elongation is blocked in *apc ipl1-321* cells and *apc mad1Δ* mutant cells when Esp1p is inactivated. *cdc26Δ* (SBY912), *cdc26Δ ipl1-321* (SBY913), *cdc26Δ esp1-1*(SBY914), *cdc26Δ ipl1-321 esp1-1* (SBY915) and *cdc26-100 mad1Δ esp1-1* (SBY983) cells expressing Tub1-GFP were shifted to 37°C for 4 hours and analyzed as described in Figure 5.2.1. Spindle elongation does not occur in *cdc26Δ ipl1-321* and *cdc26-100 mad1Δ* strains when Esp1p is inactive. Bars represent the 95% confidence interval.

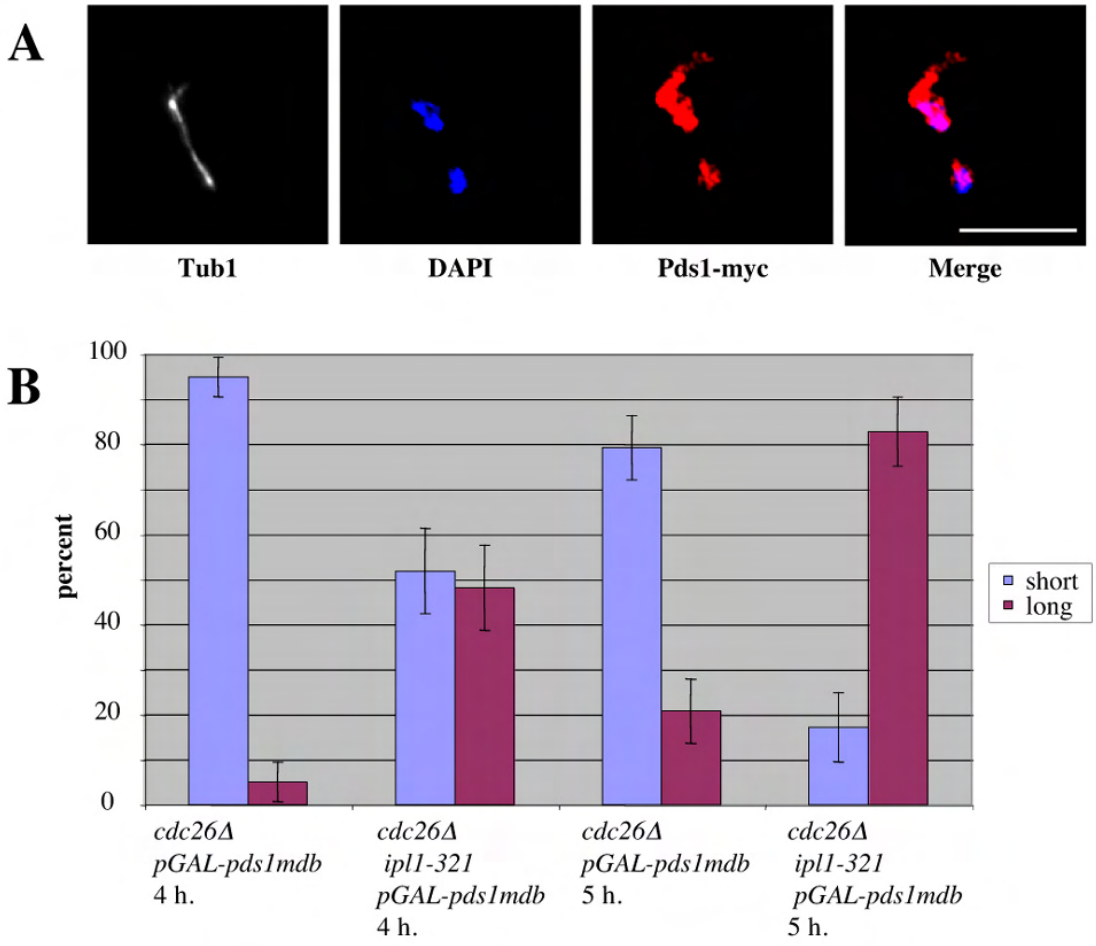


**Figure 5.2.4.** Spindle elongation in an *apc ipl1-321* mutant cell does not depend on Pds1p degradation. (A) Pds1 is still present in an *apc ipl1-321* mutant cell with a long spindle. *cdc26Δ ipl1-321* cells expressing Tub1-GFP and Pds1-18myc (SBY938) were shifted to 37°C for 4 hours and indirect immunofluorescence was performed. The cells were stained with anti-Tub1 to recognize the spinde (left pannel), DAPI to recognize DNA and with anti-myc to recognize Pds1-18myc. The merge (on the right) shows that Pds1p levels are still high in a *cdc26Δ ipl1-321* mutant cell with a long spindle. Bar: 10μm. (B) The overexpression of non-degradable Pds1p does not prevent spindle elongation in an *apc ipl1-321* mutant cell. *cdc26Δ pGAL-pds1mdb* (SBY1031) and *cdc26Δ ipl1-321 pGAL-pds1mdb* (SBY1032) expressing Tub1-GFP were grown in raffinose and then arrested in G1 using α- factor. 4% galactose was added to induce non-degradable Pds1p and 30 min later cells were released into the cell cycle in galactose at 37°C. Cells were harvested at t = 4 and 5 hours, fixed and analyzed as described in Figure 5.2.1. Spindle elongation still occurs in *cdc26Δ ipl1-321* cells when non-degradable Pds1p is overexpressed. Bars represent the 95% confidence interval.

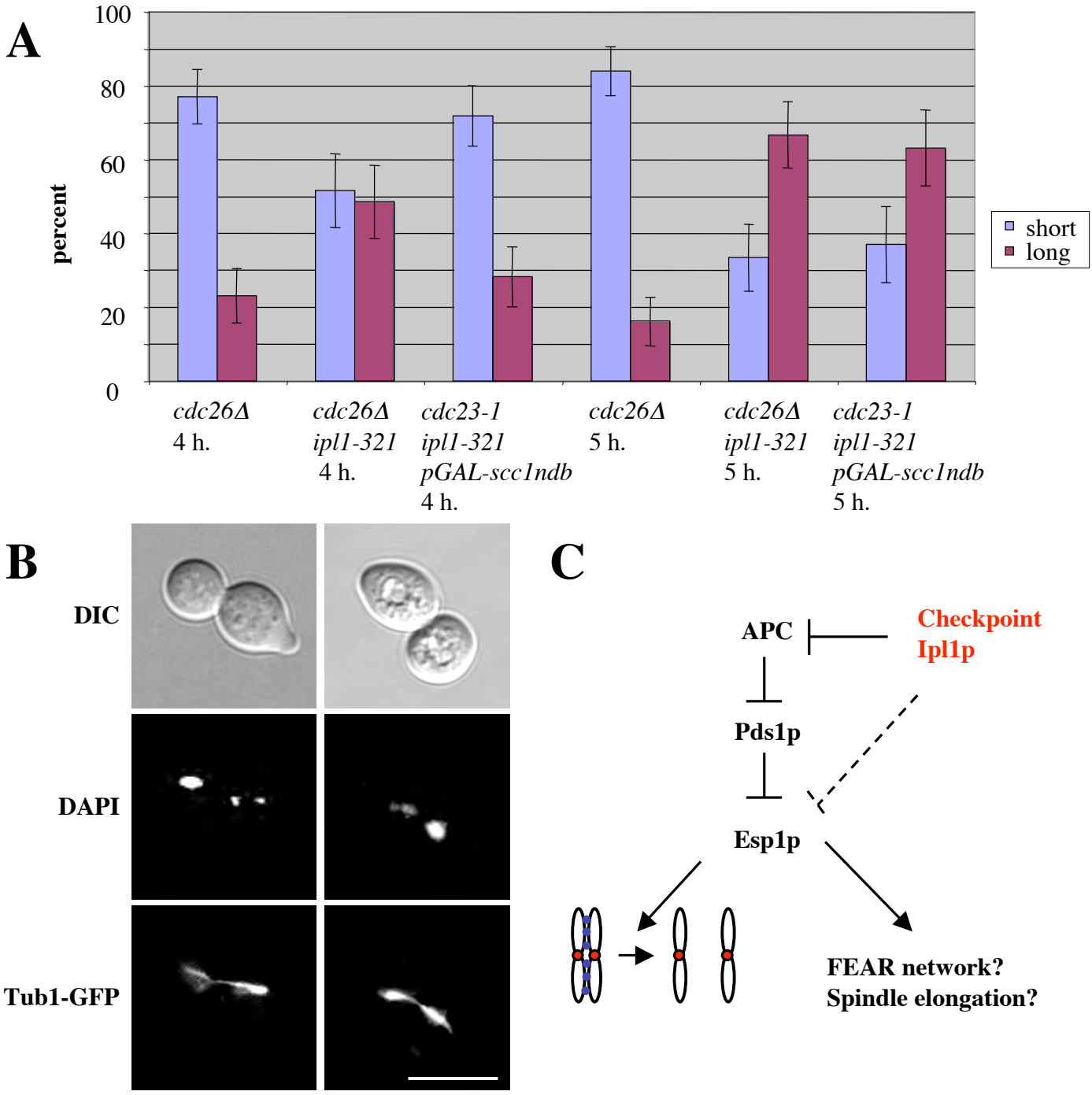


**Figure 5.2.5.** Spindle elongation in an *apc ipl1-321* mutant cell is not a direct consequence of sister separation. (A) Overexpression of a non-cleavable Scc1p delays but does not prevent spindle elongation. *cdc26Δ* (SBY912), *cdc26Δ ipl1-321* (SBY913) and *cdc23-1 ipl1-321 pGAL-scc1ndb* (SBY1058) cells expressing Tub1-GFP were grown in raffinose and then arrested in G1 using α- factor. 4% galactose was added to induce non-cleavable Scc1p and 30 min later cells were released into the cell cycle in galacose at 37°C. Cells were harvested at t = 4 and 5 hours and fixed and analyzed as described in Figure 5.2.1. Bars represent the 95% confidence interval. (B) Spindle elongation occurs without proper DNA segregation. *cdc23-1 ipl1-321 pGAL-scc1ndb* (SBY1058) cells from A were stained with DAPI to recognize DNA and analyzed microscopy. Two examples are shown. DIC is shown on the top, DAPI and Tub1-GFP on the bottom. The spindle elongates even though the DNA did not segregate properly. Bar: 10μm. (C) Model. The spindle checkpoint inhibits the APC, which is responsible for the degradation of Pds1p. Pds1p is an inhibitor of the protease Esp1p. Esp1p plays a role in both sister chromatid separation and in the FEAR network promoting anaphase. In addition Esp1p might play a role in spindle elongation. We propose that the spindle checkpoint also acts by directly inhibiting Esp1p by an unknown mechanism.

## 5.3. DISCUSSION

### 5.3.1. Spindle elongation in an *apc ipl1-321* likely represents a new function of the spindle checkpoint

As previously published, I show here that the spindle elongates in an *apc ipl1* double mutant cell over time (Biggins et al., 1999). This phenomenon can be observed with mutations in three different subunits of the APC complex: Cdc23p, Cdc26p and Cdc20p. Thus it is not allele specific and most likely represents a general property of *apc ipl1* mutants. In contrast to what had been previously published (Biggins et al., 1999), I show that this does not represent Ipl1p's function in promoting bi-orientation, as demonstrated by the fact that the spindle elongates in an *apc ipl1* double mutant even when Ipl1p's function in bi-orientation is fulfilled. Spindle elongation in an *apc ipl1* double mutant cell likely reveals a new function of the spindle checkpoint since checkpoint mutant or mutants that are checkpoint deficient also elongate their spindle in an *apc* mutant cell. This result is in disagreement with the work of Tavormina and Burke (Tavormina and Burke, 1998), who found that a *cdc20-1 mad1Δ* double mutant cell arrested in metaphase with a short spindle. This likely represents an allele specific difference since *cdc20-1* is not a null mutation. However, since I observed spindle elongation in *apc* checkpoint deficient cells with several alleles, I believe that spindle elongation is a general property of cells defective in both APC and checkpoint function.

The only other mutant that has been shown to elongate its spindle in combination with an *apc* mutation is *dam1*. However, this seems to be allele specific. As alleles of Dam1p affect spindle function and stability I cannot interpret those results. I propose that spindle elongation in an *apc ipl1* mutant cell represent a new function of the spindle checkpoint.

An alternative hypothesis would be that the spindle elongation in the *apc ipl1* double mutant is a bypass of a weak metaphase arrest. I do not think this is the case for two reasons. First, this phenomenon is observed with several APC mutants, some of which give an extremely tight metaphase arrest. Second, the APC complex does not get activated in an *apc ipl1* mutant cell since Pds1p destruction does not occur. It is therefore not allele specific and does not require APC activity, suggesting that there is another mechanism that allows spindle elongation.

### 5.3.2. Esp1p may be activated in an *apc ipl1-321* strain

The data presented here suggest that the spindle checkpoint negatively regulates Esp1p in an *apc* mutant cell. I found that spindle elongation in an *apc ipl1* mutant cell is independent from Pds1p degradation by two means. First, Pds1p is still present in *apc ipl1* cells when spindle elongation has occurred. Second, spindle elongation is not stopped by overexpression of non-degradable Pds1p. This also suggests that the APC complex does not get activated in an *apc ipl1* mutant and therefore that the APC is not the target of the spindle checkpoint in this situation. Scc1p does not appear to be the target either, as overexpression of non-cleavable Scc1p does not abolish spindle elongation. Taken together, neither the APC, nor Pds1p nor Scc1p seem to be affected in an *apc ipl1* mutant strain. However, Esp1p may be activated in the double mutant for the following reasons. First, spindle elongation in an *apc ipl1* mutant cell is Esp1p dependent. Second, DNA segregation occurs normally in an *apc ipl1* mutant cell. Although I did not show that sister chromatids separate normally, it is likely since the DNA segregates into two equally intense spots (see figure 5.2.5.B). Since sister chromatid separation is initiated by cleavage of Scc1 by Esp1p the normal sister chromatid segregation suggests that

Esp1p has cleaved Scc1p. Taken together, these results suggest that Esp1p is being activated in an *apc ipl1* mutant cell. Although I cannot exclude that an unknown mechanism is being regulated in an *apc ipl1* cell, it is likely that Esp1p is activated even though I have not yet formally demonstrated this. I propose the following model (see Figure 5.2.5.C): the spindle checkpoint inhibits the APC, which regulates Pds1p destruction. Pds1p normally inhibits the Esp1p protease. Esp1p activation leads to sister chromatid separation and spindle elongation. In addition, I propose that the spindle checkpoint inhibits Esp1p directly, at least in an *apc* mutant. When the checkpoint is inactivated by mutations in Ipl1p or checkpoint proteins, Esp1p is activated, leading to sister chromatid separation and spindle elongation.

Alternatively, it is possible that Esp1p is not directly regulated by the spindle checkpoint and that its activation in an *apc* checkpoint deficient cell is triggered by an indirect mechanism. One possibility is that another component of the FEAR network is activated in an *apc ipl1* mutant cell and that this results in Esp1p activation. The order of action of the different components of the FEAR network is not known and it is therefore possible that Esp1p is not the first actor in the network. It is interesting to note that the polo kinase, Cdc5p (a member of the FEAR network) has been shown to be regulated by the spindle checkpoint for its role in regulating the MEN (Hu et al., 2001; Stegmeier et al., 2002). In addition, Cdc5p has six potential Ipl1p phosphorylation sites. It may therefore be another candidate for the substrate of the spindle checkpoint in an *apc* mutant. Slk19p, another component of the FEAR network also possesses three potential Ipl1p phosphorylation sites. However, Slk19p is thought to act after Esp1p in the FEAR network as it is cleaved by it (Sullivan et al., 2001). Nevertheless, it is conceivable that some feedback loop exists in the FEAR

network and that activation of Slk19p results in activation of Esp1p. Other possible mechanisms will require more knowledge of the regulation of Esp1p.

### 5.3.3. How is Esp1p regulated in an *apc ipl1* cells?

Esp1p seems to be activated in an *apc ipl1* mutant cell in the presence of high levels of Pds1p. Either the Pds1p/Esp1p complex is disrupted in an *apc ipl1-321* leading to the liberation and activation of Esp1p, or Esp1p is activated by a new mechanism even when bound to Pds1p. It has recently been shown that phosphorylation regulates the stability of the Esp1p/Pds1p complex (Agarwal and Cohen-Fix, 2002). Phosphorylation of Pds1p by the cyclin-dependent kinase Cdc28p is important for efficient binding of Pds1p to Esp1p (Agarwal and Cohen-Fix, 2002). I reasoned that if phosphorylation of Pds1p was altered in an *apc ipl1-321* cell, this might change the stability of the Esp1p/Pds1p complex leading to Esp1p activation. In collaboration with O. Cohen-Fix's lab, we analyzed Pds1p phosphorylation in an *apc ipl1-321* as compared to *apc* cells. We did not find a difference in Pds1p phosphorylation (data not shown). This suggests that Esp1p is activated by a new mechanism that does not seem to implicate Pds1p unless the Esp1p/Pds1p complex is affected in a manner we have not yet detected. Unfortunately, I was not able to determine whether Esp1p phosphorylation status was affected in an *apc ipl1* mutant. It is therefore unclear how the spindle checkpoint regulates Esp1p (see future experiments). It is noteworthy that no evidence exists that the inhibition of Esp1p by the spindle checkpoint occurs in a normal cell cycle. However, the spindle checkpoint gets activated every cell cycle, making it possible that this mechanism plays a role in a normal cell cycle but would not normally be required.

Alternatively, it cannot be excluded that Esp1p exists in different pools independent of Pds1p. There is some precedent for nuclear division independent of Pds1p degradation in budding yeast. Lack of S-phase cyclins *CLB5* and *CLB6* bypasses the requirement of Pds1p destruction for sister chromatid separation in the presence of DNA damage (Meyn and Holloway, 2000). A critical DNA damage checkpoint is an arrest at metaphase. This arrest requires the S-phase cyclins Clb5p and Clb6p. There are some striking similarities between the bypass of the DNA damage checkpoint arrest in a *CLB5 CLB6* double mutant and the bypass of the *apc* arrest in a spindle checkpoint deficient cell (Meyn and Holloway, 2000). In both cases, Pds1p destruction is not required for the cells to proceed to anaphase. In addition, nuclear division is not inhibited by overexpressing non-degradable Pds1p. No evidence for a direct action on Esp1p was found. However it is unlikely that the two checkpoints act in a single pathway as a deletion of *CLB5* does not bypass a metaphase arrest due to mutation in the APC complex (Shirayama et al., 1999). It is possible that deletion of both S-phase cyclins, Clb5p and Clb6p, is necessary to bypass this arrest, however this is unlikely as deletion of Clb5p shows a stronger phenotype than Clb6p (Shirayama et al., 1999). It is possible that the two checkpoints act in parallel pathways to inhibit Esp1p by a Pds1p independent mechanism. Two different checkpoints have already been shown to act in parallel pathway to regulate mitotic exit (Hu et al., 2001).

### 5.3.4. Sister separation and spindle elongation can be uncoupled

In the literature, conflicting results exist concerning whether spindle elongation is a direct consequence of sister chromatid separation. Here I show that spindle elongation can be uncoupled from sister chromatid separation. Indeed, spindle

elongation can occur even though sister chromatid separation is inhibited (Figure 5.2.5.A), leading to nuclear fractionation. This suggests that spindle elongation is regulated independently of sister chromatid separation, even though the two events are normally coupled. As both sister chromatid separation and spindle elongation are inhibited in an *apc ipl1 esp1* mutant cell and as spindle elongation is not inhibited in the absence of sister chromatid separation, this suggests that spindle elongation in an *apc ipl1* mutant cell is dependent on another of Esp1p's function. However, I cannot determine whether Esp1p's role is to directly promote spindle elongation or whether this is a result of Esp1p's function in the FEAR network. As stated in the introduction, it is controversial whether Esp1p plays a role in spindle elongation. One study found that *esp1* mutant cells were unable to elongate their spindle in the absence of the cohesin complex (*scc1* mutant) suggesting that Esp1p played a role in spindle elongation (Jensen et al., 2001). However, two other studies found no defect in elongation in *esp1* mutant cells in the absence of the cohesin complex and the spindle checkpoint (Severin et al., 2001b; Stegmeier et al., 2002). The authors came to the conclusion that Esp1p did not play a role in spindle elongation. It is possible that the difference is due to the presence or absence of the checkpoint. In an *esp1 scc1* double mutant cell, the spindle checkpoint is activated, which would explain why spindle elongation would be inhibited in this case. Alternatively, it is possible that spindle elongation is a consequence of activation of the FEAR network, as Esp1p is known to play a role in this network, which is activated at about the time of the initiation of spindle elongation (Stegmeier et al., 2002). Several proteins implicated in the FEAR network have been shown to regulate spindle stability (Sullivan et al., 2001; Uhlmann et al., 2000; Zeng et al., 1999). More work needs to be done to

elucidate the various Esp1p functions and their consequence on spindle elongation and/or stability.

## 5.4. FUTURE PLANS

### 5.4.1. Esp1p activation in an *apc ipl1-321* mutant cell

All the data accumulated here suggest that Esp1p is activated in an *apc* checkpoint deficient cell. However, the mechanism for this activation is not clear. One likely possibility is that the spindle checkpoint is regulating Esp1p by phosphorylating it, as several spindle checkpoint proteins are kinases, including Ipl1p. In this regard, it is interesting to note that Esp1p contains one potential Ipl1p phosphorylation site. I will therefore analyze Esp1p phosphorylation status in *apc* versus *apc* checkpoint deficient cells to determine whether Esp1p is phosphorylated in a checkpoint dependent manner. *In vivo* phosphate labeling will be performed on *apc* versus *apc ipl1* or *apc mad* mutant cells shifted to the restrictive temperature (37°C) for 4 hours to inactivate the mutations. Esp1p phosphorylation status will then be analyzed by autoradiography. This will determine whether the checkpoint is regulating Esp1p by phosphorylating the protein.

If Esp1p is phosphorylated by the checkpoint, I will determine the phosphorylation site by mass spectrometry (Shou et al., 2002). One likely candidate is the Ipl1p potential phosphorylation site. The site(s) will then be mutated and I will determine whether the mutation is able to bypass an *apc* arrest suggesting that this site is responsible for the inhibition by the checkpoint. I will also analyze the phenotype of the mutated Esp1p during a normal cell cycle to determine whether the inhibition of Esp1p by the checkpoint plays a role during an unperturbed cell cycle.

If Esp1p is not phosphorylated in a checkpoint dependent manner, it would suggest that either the checkpoint activates Esp1p by an indirect mechanism, or that the checkpoint acts directly on Esp1p but not by changing its phosphorylation state. To test the first possibility and if the FEAR network is implicated in spindle elongation in an *apc* checkpoint deficient cell (see below), I will test other components of the FEAR network to see if they are phosphorylated in a checkpoint dependent manner. I will start with Cdc5p because of the precedents in the literature for regulation of Cdc5p by the spindle checkpoint (Hu et al., 2001). If none of these proteins are regulated by phosphorylation, it will be difficult to determine how checkpoint mutants are bypassing an *apc* arrest before more is known about Esp1p function.

### 5.4.2. Is the FEAR network implicated?

Spindle elongation in an *apc ipl1* mutant cell does not seem to be a direct consequence of sister chromatid separation, thus implying that Esp1p is activated for a second function. One possibility is that Esp1p's role in the FEAR network is required to initiate spindle elongation. I will therefore test another FEAR mutant in our assay to see whether it prevents spindle elongation in an *apc ipl1* background as an *esp1* mutation does. The FEAR network is composed of Slk19p, Spo12p and Cdc5p in addition to Esp1p (Stegmeier et al., 2002). I will test the effect on spindle elongation by mutating them in an *apc ipl1* cell. *slk19* and *spo12* mutant cells have weaker phenotypes in inhibiting Cdc14p release than *esp1* mutant cells, so they might not arrest spindle elongation in an *apc ipl1* double mutant cells as well as *esp1*. Cdc5p plays several roles in mitotic exit and the triple mutant might result in a phenotype that is not easy to interpret. By testing all three of them I should be able to determine

whether the FEAR network is implicated in spindle elongation in an *apc ipl1* mutant cell. If the FEAR network is not implicated it would imply that it requires another function of Esp1p, probably in spindle elongation/stability. More work will be needed to define more precisely Esp1p function on spindle dynamics as well as whether other proteins act in concert with Esp1p to achieve those functions before I can determine whether this function is implicated in spindle elongation in *apc* checkpoint deficient cells.

# CHAPTER 6. GENERAL CONCLUSION

The Ipl1/Aurora protein kinase is an important regulator of chromosome segregation and cytokinesis. In addition, it plays a role in the spindle checkpoint. Human homologs of the Ipl1p/Aurora family are oncogenes and several lines of evidence suggest that the regulation of this protein is important. Here I present new information on the regulation of the localization of this protein as well as discover two previously unidentified functions for Ipl1p in spindle disassembly and spindle orientation.

By analyzing Ipl1p localization throughout the cell cycle, I found that it localizes to the kinetochores from G1 to metaphase consistent with its known function in kinetochore bi-orientation. Ipl1p then relocalizes to the spindle. This relocalization is independent from tension establishment, the unloading of cohesin and spindle checkpoint inactivation. However, it requires the presence of microtubules. I therefore propose that Ipl1p is being transported away from the kinetochores by an active mechanism, a likely candidate being a motor protein. In addition, I found that complex formation with the Bir1p/Survivin protein was required for optimal relocalization.

In late anaphase, Ipl1p accumulates at the spindle midzone where is plays a role in promoting spindle disassembly. I show that this represents a previously unidentified function for Ipl1p that is independent from its role in chromosome segregation and the spindle checkpoint. Ipl1p tracks the plus end of the depolymerizing spindle back to the poles, suggesting that it might act on the plus ends either directly or by the intermediary of a protein. I found that a number of Ip1lp substrates localize to the midzone. I propose that the spindle midzone is a kinetochore-like structure and that the regulation of the spindle midzone in anaphase

is similar to the regulation of kinetochore-microtubule attachment in metaphase. I also found that Ipl1p plays a role in spindle orientation, likely acting on cytoplasmic microtubules. I therefore propose that Ipl1p is a general microtubule plus end regulator.

In another study, I found that inactivation of the spindle checkpoint is able to bypass a metaphase arrest caused by mutation in subunits of the APC complex. I found that this bypass is independent of APC activation and Pds1p destruction and is likely due to activation of the Esp1p protease. Esp1p is activated for both sister chromatid separation and another less well defined function in spindle elongation. I propose that the spindle checkpoint acts as an inhibitor of the Esp1p protease.

# CHAPTER 7. MATERIALS AND METHODS

## 7.1. Microbial techniques

Media and genetic and microbial techniques were essentially as described (Rose et al., 1990; Sherman et al., 1974). All experiments where cells were released from G1 arrest were carried out by adding 1 μg/ml α-factor (stock: 10 mg/ml in DMSO, United Biochemical Research Inc., Seattle, WA) at the permissive temperature (23°C) for 3 h., washing the cells twice in α-factor-free media, and resuspending them in fresh media. α-factor was added back to 1 μg/ml after cells had budded to prevent cells from entering the next cell cycle. Galactose induction was performed by growing cells in 2% raffinose and adding galactose to a final concentration of 4%. Galactose repression was performed by washing the cells twice with glucose media and resuspending in glucose media. All experiments were repeated at least twice with similar results.

## 7.2. Yeast strain construction

Yeast strains are listed in Table 1 and were constructed by standard genetic techniques. Diploids were isolated on selective media at 23°C and subsequently sporulated at 23°C. All strains containing the galactose repressible *pGAL-CDC20* construct were obtained through crosses with the strain SLJ577, a gift of S. Jaspersen and M. Winey, UC-Boulder. Strains containing *TUB1-GFP:URA3* were obtained by integrating pMAS27 (a gift from M. Shonn) cut with *Stu1*. SBY938 was created by integration of a plasmid that was a gift from K. Nasmyth (*pds1-myc18:LEU2*; (Shirayama et al., 1998)). SBY1036 was created by integration of a plasmid (*TUB1-CFP:URA3*; gift of K. Bloom) with *Stu1* at the *URA3* locus. Strains containing *CSE4-myc12* were obtained by integrating pSB246 (*CSE4-myc12:URA3,* myc internal to the

gene) with *Cla1* at the *CSE4* locus. Strains containing Clb2-LacZ were obtained as previously described (Hwang and Murray, 1997). Strains containing *TUB1-GFP:LEU2* were obtained by integrating plasmid pSB340 cut with *Age1* at the *LEU2* locus. SBY554 was obtained by integrating pSB164 (*pGAL-IPL1:URA3,* Biggins et al., 1999) cut with *Stu1* at the *URA3* locus. SBY736 was obtained by integrating pSB257 (*pGAL-myc12-IPL1:URA3*) cut with *Stu1* at the *URA3* locus. SBY676 was obtained by integrating pSB252 (*pGAL-myc12-IPL1-catalytic domain:URA3*) cut with *Stu1* to integrate at the *URA3* locus. SBY735 was obtained by integrating pSB256 (*pGAL-myc12-IPL1-N-terminus:URA3*) cut with *Stu1* to integrate it at the *URA3* locus. SBY1031 and SBY1032 were obtained by integrating pSB369 (*pGAL-pds1mdb:LEU2* a gift from O. Cohen-Fix) cut with *EcoRI* to integrate is at the *LEU2* locus. SBY1058 was obtained by integrating pSB373 (*pGAL-scc1ndb:URA3* a gift from A. Murray) cut with *Stu1* to integrate it at the *URA3* locus. Strains containing *pGAL-Δ176-CLB2:LYS2* were generated as described (Biggins et al., 2001). To make *ipl1-as5* mutant, pSB428 (*ipl1-as5:LEU2*) was integrated at the *LEU2* locus. Deletions in yeast gene as well as GFP, CFP and Myc epitope tags were made using the PCR based integration system described in (Longtine et al., 1998). YFP epitope tags were made by PCR integration using pDH5, a gift from T. Davis. We generated the following gene deletions and epitope tags using the primers listed in parentheses after each gene: *mad1Δ* (primers SB210 and SB211), *mad2Δ* (primers SB194 and SB195), *IPL1-GFP* and *IPL1-YFP* (primers SB62 and SB44) *NDC10-CFP* and *NDC10-GFP* (primers SB51 and SB52), *cdc26Δ* (primers SB169 and SB170), *kip3Δ* (primers SB287 and SB275), *SLI15-GFP* (primers SB153 and SB154), *bir1ΔN-terminus* (primers SB147 and SB162), *ASE1-GFP* (primers SB264 and SB265), *KIP3-GFP* (primers SB274 and SB275), *STU2-GFP* (primers SB345 and SB346),

*pGAL-HA-SCC1* (primers SB258 and SB259) and *DAM1-GFP* (primers SB261 and SB262). All deletions and epitope tags were confirmed by PCR. The bacterial strain XL1-Blue was used for all plasmid amplifications.

Table I. Yeast strains used in this study.

| | |
|---|---|
| SBY3 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ* |
| SBY97 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 ipl1-321* |
| SBY130 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100* |
| SBY214 | *MAT**a** ura3-1 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100* |
| SBY322 | *MAT**a** ura3-1 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 ipl1-321* |
| SBY539 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ NDC10-GFP:KAN* |
| SBY554 | *MAT**a** ura3-1:pGAL-IPL1:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ* |
| SBY556 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ can1-100 IPL1-GFP:KAN* |
| SBY617 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ can1-100 CSE4-myc12:URA3* |
| SBY676 | *MAT**a** ura3-1:pGAL-myc12-IPL1-catalytic domain:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ* |

SBY735 *MAT**a** ura3-1:pGAL-myc12-IPL1-N-terminus:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ*

SBY736 *MAT**a** ura3-1:pGAL-myc12-IPL1:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ*

SBY815 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ bar1Δ cdc23-1 cse4-323*

SBY875 *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ SLI15-GFP:HIS3*

SBY896 *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ IPL1-GFP:TRP1 bir1ΔN-terminus:HIS3*

SBY912 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ cdc26::KAN*

SBY913 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ bar1Δ can1-100 ade2-1 ipl1-321 cdc26::KAN*

SBY914 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ:pGAL-176-Clb2:LYS2 bar1Δ can1-100 ade2-1 esp1-1 cdc26::KAN*

SBY915 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1 lys2Δ bar1Δ can1-100 ade2-1 esp1-1 ipl1-321 cdc26::KAN*

SBY916 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 ndc10-1 cdc26::KAN*

| | |
|---|---|
| SBY938 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112:PDS1-myc18:LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ bar1Δ can1-100 ade2-1 ipl1-321 cdc26::KAN* |
| SBY943 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 cdc20::LEU2 ipl1-321 [pGAL(low)-CDC20-HIS3(CEN)]* |
| SBY952 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 cdc20::LEU2 [pGAL(low)-CDC20-HIS3(CEN)]* |
| SBY953 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ cdc26-100 Clb2-LacZ mad1::HIS3* |
| SBY964 | *MAT**a** ura3-1 leu2-3,112:TUB1-GFP:LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:URA3:TRP1 lys2Δ ade2-1 bar1Δ ipl1-321 mdc1-1 cdc26::KAN* |
| SBY965 | *MAT**a** ura3-1 leu2-3,112:TUB1-GFP:LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:URA3:TRP1 lys2Δ ade2-1 mdc1-1 cdc26::KAN* |
| SBY983 | *MAT**x** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1:lacO:TRP1 ade2-1 bar1Δ cdc26-100 Clb2-LacZ lys2Δ:pGAL-176-Clb2:LYS2 esp1-1 mad1::HIS3* |
| SBY1031 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112:pGAL-pds1mdb:LEU2 his3-11 trp1-1 can1-100 ade2-1 bar1Δ cdc26::KAN* |
| SBY1032 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112:pGAL-pds1mdb:LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ bar1Δ can1-100 ade2-1 ipl1-321 cdc26::KAN* |
| SBY1036 | *MAT**a** ura3-1:TUB1-CFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ can1-100 IPL1-GFP:KAN* |

SBY1058 *MAT**a** ura3-1:pGAL-scc1ndb:URA3 leu2-3,112:TUB1-GFP:LEU2 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 bar1Δ cdc23-1 ipl1-321*

SBY1115 *MAT**α** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ DAM1-GFP:TRP1*

SBY1162 *MAT**x** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ ASE1-GFP:TRP1*

SBY1246 *MAT**α** ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 cdc20::LEU2 IPL1-YFP:HIS3 NDC10-CFP:KAN [pGAL(low)-CDC20-HIS3(CEN)]*

SBY1276 *MAT**a** ura3-1 leu2-3,112 his3-1 trp1-1 can1-100 ade2-1 ipl1-321 KIP3-GFP:TRP1*

SBY1323 *MAT**a** ura3-1:CSE4-myc12:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1*

SBY1326 *MAT**a** ura3-1:CSE4-myc12:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 mad1::HIS3*

SBY1355 *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ KIP3-GFP:TRP1*

SBY1356 *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ ipl1::KAN:ipl1-as5-GFP:TRP1:LEU2*

SBY1422 *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 mad2::KAN*

| | |
|---|---|
| SBY1423 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11:pCUP1-GFP12-lacI12:HIS3 trp1-1:lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 mad1::KAN* |
| SBY1447 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ STU2-GFP:TRP1* |
| SBY1448 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 ipl1-321 STU2-GFP:TRP1* |
| SBY1475 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ IPL1-GFP:KAN SCC1:pGAL-HA-SCC1:HIS3* |
| SBY1538 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1:lacO:TRP1 can1-100 ade2-1 bar1Δ kip3::KAN* |
| SBY1539 | *MAT**a** ura3-1:TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1:lacO:TRP1 can1-100 ade2-1 bar1Δ kip3::KAN lys2Δ ipl1-321* |
| SBY1543 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ kip3::KAN IPL1-GFP:TRP1* |
| SBY1802 | *MAT**a** ura3-1 leu2-3,112 his3-11 trp1-1:lacO:TRP1 can1-100 ade2-1 bar1Δ ASE1-GFP:TRP1 ipl1-321* |

All strains are isogenic with the W303 strain background. Plasmids are indicated in brackets.

### 7.3. Plasmid constructions

A clone that encoded Cse4p internally tagged with 12 copies of the myc epitope (pSB246) was constructed by PCR amplification of the myc tag from pSB162 (Biggins et al., 1999) using primers SB84 and SB85. The PCR product was digested with *SpeI* and ligated into the *XbaI* site of pSB241, a *CSE4*, *URA3* integrating vector.

pSB241 was created by digesting a genomic clone containing *CSE4* with *EcoRI* and *PstI* and the resulting 1.7 kb fragment was ligated into yiplac211 (Gietz and Sugino, 1988) digested with *EcoRI* and *PstI*. To create *TUB1-GFP: LEU2*, pMAS27 was digested with *Eag1* and the *TUB1-GFP* fragment was ligated into the *EagI* site of pRS305 to create pSB340. To create a *pGAL-myc12-IPL1* clone, *IPL1* was PCR amplified from genomic DNA, digested with *Spe1* and *Sac1* and then ligated into pSB209 digested with the same enzymes to create pSB257. pSB209 is a *pGAL-myc13:URA3* integrating vector. To create pSB252, *pGAL-myc12-IPL1catalytic domain:URA3,* (amino acid 97 to 367), pSB209 was cut with *SpeI* and *SacI* and ligated to the C-terminal catalytic domain of Ipl1p that was PCR amplified using primers SB109 (containing a *SpeI* site) and SB110 (containing a *SacI* site). To create pSB256, *pGAL-myc12-IPL1N-terminus:URA3* (amino acid 1 to 96), pSB209 was cut with *SpeI* and *SacI* and ligated to the N-terminus of Ipl1p that was PCR amplified using primers SB107 and SB108. To create pSB428, *ipl1-as5-SSA(M181G, T244A):LEU2*, pSB338 (*ipl1-as5-SSA(M181G,T244A):URA3:CENI* was digested with *BamHI* and *HindIII* and ligated into pSB425 (*IPL1endogenous promoter:LEU2*) digested with the same enzymes. pSB338 was made by engineering a second mutation site on a plasmid containing *ipl1-as1* on pSB316 ( a gift from Charles Kung in the Shokat lab).

### 7.4. Statistics

The Bernoulli trial was used to calculate the confidence interval. For each experiment, the 95% confidence is shown (bars) but all experiments were significant with 99% confidence. The formula used is: $p = x \pm 1.96\sqrt{\frac{x \times (1-x)}{n}}$ where x is the

percentage of spindle breakdown and n is the number of cells counted. At least 100 cells were counted in each experiment.

### 7.5. Protein and immunological techniques

Protein extracts were made and immunoblotted as described (Minshull et al., 1996). Anti-Tub1p antibodies were obtained from Accurate Chemical and Scientific (Westbury, NY) and used at a 1:1,000 dilution, anti-Clb2p antibodies (a gift from A. Rudner) were used as described (Rudner et al., 2000), and anti-Ipl1p antibodies were used 1:1,000. To generate anti-Ipl1p antibodies, 2.5 mg of GST-Ipl1p was purified as previously described (Biggins et al., 1999) and 1.5 mg was injected into rabbits at Cocalico Biological Inc (Reamtown, PA) according to their protocol. For the $4^{th}$ and $5^{th}$ boosts, 1 mg of boiled protein total was injected. The antibodies were affinity purified using a column that was made by coupling GST-Ipl1p to SulfoLink Coupling gel as directed by the manufacturer (Pierce Chemicals, Rockford, IL). The column was equilibrated in 10 mM Tris, pH 7.5, and serum (previously spun for 10 min to eliminate insoluble material) was loaded on the column. After the column was washed once with 25 column volume of 10 mM Tris, pH7.5, and then once with 50 column volume of 10 mM Tris, pH 7.5 containing 0.5 M NaCl, the antibodies were eluted with 100 mM glycine, pH 2.5. 1.5 M Tris pH 8.8 was immediately added to neutralize the solution. Fractions containing the antibodies were pooled and dialyzed into 1X PBS containing 0.02% $NaN_3$ and 30% glycerol.

### 7.6. Microscopy

For live microscopy to analyze GFP fusion proteins, cells were grown to mid-logarithmic phase in liquid YM media, washed and resuspended in 1/10 volume

minimal media with casamino acids. For live microscopy at room temperature, the protocol described in (Maddox et al., 2000) was followed with modifications. Agarose (Fisher Scientific, US) was added to minimal media with casamino acids to 2% (w/v) and then heated to liquefy the preparation. 0.5 ml of this solution was then placed between two cleaned microscope slides. When solidified, the slides were pried apart, leaving a slab of about 0.5 μm on one of the slides and 1.5 μl of the cell preparation was pipetted onto the pad and covered with a coverglass. The slide was then sealed with VALAP (1:1:1 vaseline: lanolin: paraffin) and imaged. For live microscopy at 35°C, prepared cells were mounted directly onto a heated stage (Bioptechs, Butler, PA). Images were collected through an Olympus 1X17 60x objective with a CH350 CCD camera (Roper Scientific, AZ) using the softwox 2.5 (Applied Precision, WA) software. The same software was used for deconvolution. At least 10 cells were analyzed for all reported experiments.

Chromosome spreads were performed as described (Loidl et al., 1991; Michaelis et al., 1997). Lipsol was obtained from Lip Ltd. (Shipley, England). 4',6'-diamidino-2-phenylindole (DAPI) was obtained from Molecular probes (Eugene, OR) and used at 1 μg/ml final concentration. 9E10 antibodies that recognize myc tag were used at a 1:500 dilution and obtained from Covance (Princetown, NJ) Anti-Tub1p antibodies (Accurate Chemical and Scientific, Westbury, NY) were used at a 1:500 dilution. Anti-Ipl1p antibodies were used at a 1:250 dilution. Alexaflour-594 and Alexaflour-488 secondary antibodies were obtained from Molecular Probes (Eugene, OR) and used at a 1:250 dilution.

Indirect immunofluorescence was carried out as described (Rose et al., 1990). 4',6'-diamidino-2-phenylindole (DAPI) was obtained from Molecular probes (Eugene, OR) and used at 1 μg/ml final concentration. C-myc A-14 (Santa Crux

biotechnology, CA) that recognize the myc tag were used at 1:1000 dilution and anti-Tub1p antibodies (Accurate Chemical and Scientific, Westbury, NY) were used at 1:200. Cy3 secondary antibodies were obtained from Jackson immunoresearch (West Grove, PA) and used at a 1:2000 dilution. FITC secondary antibodies were obtained from Jackson immunoreseach and used at a 1:500 dilution.

### 7.7. Cse4 histone fold domain purification

The histone fold domain of *CSE4* (encoding amino acids 121 to 229) was PCR amplified from *S. cerevisiae* genomic DNA and cloned into a T7 expression vector of the pCRT7/CT TOPO TA cloning kit (Invitrogen, CA). The resulting expression plasmid (pT7Cse4c) was transformed into BL21-CodonPlus (DE3)-RIL competent cells (Stratagene, CA) and grown overnight. This culture was used to inoculate 2 liters of 2xTY media. The culture was induced with ITPG to 0.2 mM for 2.5 hours at 37°C and Cse4 was purified under denaturing conditions as described (Gelbart et al., 2001). The final preparation of Cse4 histone fold domain was verified for purity by SDS-PAGE and Coomassie staining, extensively dialyzed against water and stored in 1 ml aliquots at -80°C. A substantial portion of the protein was not soluble in water, but solubility was adequate for kinase assays *in vitro*.

### 7.8. Ipl1p kinase assays

Cells from 40 ml cultures of mid-log cells were collected and resuspended in 500 µl lysis buffer (100 mM NaCl, 50 mM Tris pH 7.5, 50 mM NaF, 50 mM β-glycerophosphate pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.1% Triton-X-100). The following reagents were added fresh: 2 mM NaVO4, 2 mM PMSF, 10 µg/ml LPC (leupeptin, pepstatin and chymostatin; Chemicon, CA), 1 mM DTT, 0.1 µg/ml

microcystin (Calbiochem; 100 μM stock in 100% EtOH and stored at -80°C). All subsequent steps were performed at 4°C. Cells were lysed with glass beads in a beater (Biospec Products Inc, OK) for 30 s and then centrifuged for 10 min. 400 μl supernatant was added to 5 μl magnetic Protein G beads (Dynal Biotech Inc., NY) and 4 μl anti-Ipl1p antibodies. Samples were rotated for 2 hours, and beads were washed three times with 400 μl lysis buffer and once with 100 μl kinase buffer without ATP (50 mM Tris pH 7.4, 1 mM DTT, 25 mM β-glycerphosphate, 5 mM $MgCl_2$). Beads were resuspended in kinase buffer with 10 μM ATP, 5 μCi $^{32}$P-ATP and 5 μg Cse4p substrate and incubated at 30°C for 30 min. 2x sample buffer was added to stop the reaction. The kinase assays were separated on SDS-PAGE and subjected to either autoradiography using a Phosphorimager Screen (Molecular Dynamics, NJ) or immuoblotting with anti-Ipl1p antibodies. Quantitative immuoblots were performed as described using the LI-COR Biosciences Odyssey infrared imaging system (Reeves and Hahn, In Press). Kinase assays were quantified using ImageQuant (Molecular Dynamics, NJ) software.

# CHAPTER 8. ABBREVIATIONS

| | |
|---|---|
| 1-NA | 1-naphtyl-PP1 |
| ATP | Adenosine triphosphate |
| APC | Anaphase promoting complex |
| CEN | Centromere |
| Cdk | Cyclin dependent kinase |
| CFP | Cyan fluorescent protein |
| Cy3 | Cyan 3 |
| DAPI | 4,6-diamidino-2-phenylindole |
| DIC | Differential interference contrast |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Ditiothreitol |
| EDTA | Ethylene diamine tetraactetate |
| EGTA | Ethylenebis(Oxyethylenitriol) Tetraacetic acid |
| FEAR | Cdc Fourteen early anaphase release |
| FITC | Fluorescein isothiocyanate |
| FRAP | Fluorescence redistribution after photobleaching |
| Gal | Galactose |
| GDP | Guanosine diphosphate |
| GFP | Green fluorescent protein |
| GST | Glutathione S transferase |
| GTP | Guanosine triphosphate |
| h | Hour |
| IP | Immunoprecipitation |

| | |
|---|---|
| IPTG | Isopropyl β-D-thiogalactopyranoside |
| kb | Kilo base |
| kD | Kilo Dalton |
| MAP | Microtubule associated protein |
| MEN | Mitosis exit network |
| min | Minute |
| mRNA | Messenger ribonucleic acid |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PMSF | Phenylmethylsulfonyl fluoride |
| RFP | Red fluorescent protein |
| s | Second |
| SDS-PAGE | Sodium dodecylsulfate polyacrylamide gel electrophoresis |
| SPB | Spindle pole body |
| ts | Thermosensitive |
| WT | Wild type |
| YFP | Yellow fluorescent protein |

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# CHAPITRE 11: CURRICULUM VITAE

## PERSONAL DATA

Stéphanie Buvelot

Born on April 29th, 1977 in Rolle, Switzerland

Swiss citizen, Single

Private address: ch. Veyrassat 12, 1180 Rolle

Tel.: +41-21-825-2497

E-mail: sbuvelot@hotmail.com

## EDUCATION

**2000 - 2003** Ph.D. thesis in Biochemistry, under the supervision of Prof. A Conzelmann. Institute of Biochemistry, University of Fribourg, Switzerland.

Studies undergone at the Fred Hutchinson Cancer Research Center (FHCRC) at Seattle, under the supervision of Dr. Sue Biggins

Title: The Dynamic Localization and Novel Functions of the Ipl1/Aurora Protein Kinase

**2000** Diploma in Biology, University of Lausanne

Under the supervision of Dr. Viesturs Simanis. ISREC, University of Lausanne, Switzerland

Title: "Characterization of *pab2*, a second regulatory subunit of the protein phosphatase 2A involved in the formation of the septum."

**1995-2000** Biology studies at the University of Lausanne, Switzerland

**1995** Maturity certificate

**1992-1995** Cessouest High school, scientific section, Nyon, Switzerland

## LANGUAGES

French, mother language

English, fluent

German, maturity level

## CONFERENCES

**14-19 August 2001,** "Yeast Cell Biology Meeting" in Cold Spring Harbor, NY

Presented a poster: "Localization and Spindle Function of the Ipl1/Aurora kinase"

**25-30 July 2000,** "Yeast Genetics and Molecular Biology Meeting" in Seattle, WA

Presented a poster and a talk on my diploma work.

## COURSES

**Spring 2001** "The cell cycle"

By Linda Breeden, FHCRC

**Fall 2000** "The dynamic chromosome"

By Dan Gottschling, FHCRC

**Fall 2000** "Gene Transcription and RNA processing"

By Toshio Tsukiyama, FHCRC

**Fall 2000** Literature Review

By Barry Stoddard, FHCRC

## LIST OF PUBLICATIONS

Stéphanie Buvelot, Sean Y. Tatsutani, Danielle Vermaak and Sue Biggins. (2003).

The Budding Yeast Ipl1/Aurora Protein Kinase Regulates Mitotic Spindle Disassembly. Journal of Cell Biology, 160: 329-339.

Le Goff, X., Buvelot, S., Salimova, E., Guerry, F., Schmidt, S., Cueille, N., Cano, E., and Simanis, V. (2001). The protein phosphatase 2A B'-regulatory subunit par1p is implicated in regulation of the *Schizosaccharomyces pombe* septation intitiation network, FEBS lett *508*, 136-42.

**ORAL PRESENTATION**

**February 2002,** at the Seattle area yeast group

**January 2002,** at the Mitosis group of Seattle

**March 2001,** at the Departmental seminars of the FHCRC

**July 2000,** at "Yeast Genetics and Molecular Biology Meeting", Seattle

**FELLOWSHIP**

Department of Defense Breast Cancer Research Program Fellowship for 3 years starting in Spring 2002.

Roche Research Foundation Fellowship for one year (September 2000-August 2001).

Rolle, le 27 janvier 2003