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MITOTIC REGULATION BY POLO-LIKE KINASE 1 AND THE CHROMOSOMAL PASSENGER COMPLEX

by

Leena Ahonen

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From VTT Medical Biotechnology and University of Turku,
Department of Medical Biochemistry and Genetics, and
Turku Graduate School of Biomedical Sciences
Turku, Finland

Supervised by:
Docent Marko Kallio, PhD
VTT Medical Biotechnology and
Turku Centre for Biotechnology
University of Turku
Turku, Finland

Reviewed by:
Professor Klaus Elenius, MD, PhD
Department of Medical Biochemistry and Genetics
University of Turku
Turku, Finland

and

Professor Lea Sistonen, PhD
Turku Centre for Biotechnology
Department of Biology
Åbo Akademi University
Turku, Finland

Opponent:
Associate Professor Susanne Lens, PhD
Department of Medical Oncology
University Medical Center Utrecht
Utrecht, the Netherlands

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To my family

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VTT Medical Biotechnology and University of Turku,
Department of Medical Biochemistry and Genetics, and
Turku Graduate School of Biomedical Sciences
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ABSTRACT

During mitosis, the duplicated genome must be accurately divided between two daughter cells. Polo-like kinase 1 (Plk1) and Aurora B kinase, together with its binding partners Incenp, Survivin and Borealin (chromosomal passenger complex, CPC), have key roles in coordinating mitotic events. The accuracy of cell division is safeguarded by a signaling cascade termed the mitotic spindle checkpoint (SC), which ensures that chromosomes are not physically separated before correct bipolar attachments have been formed between kinetochores and spindle microtubules (MT). An inhibitory "wait anaphase" signal, which delays chromosome separation (anaphase onset), is created at individual kinetochores and broadcasted throughout the cell in response to lack of kinetochore-microtubule (kMT) attachment or proper interkinetochore tension. It is believed that the fast turnover of SC molecules at kinetochores contributes to the cell's ability to produce this signal and enables rapid responses to changing cellular conditions. Kinetochores that lack MT attachment and tension express a certain phosphoepitope called the 3F3/2 phosphoepitope, which has been linked to SC signaling. In the experimental part, we investigated the regulation of the 3F3/2 phosphoepitope, analyzed whether CPC molecules turn over at centromeres, and dissected the mitotic roles of the CPC using a microinjection technique that allowed precise temporal control over its function. We found that the kinetochore 3F3/2 phosphoepitope is created by Plk1, and that CPC proteins exhibit constant exchange at centromeres. Moreover, we found that CPC function is necessary in the regulation of chromatid movements and spindle morphology in anaphase. In summary, we identified new functions of key mitotic regulators Plk1 and CPC, and provided insighs into the coordination of mitotic events.

Keywords: Mitosis, SC, Plk1, Aurora B, CPC, 3F3/2 Phosphoepitope, Kinetochore

Leena Ahonen
Plk1:n ja Aurora B -kompleksin mitoottiset tehtävät

VTT Lääketieteen biotekniikka ja Turun yliopisto,
Lääketieteellinen biokemia ja genetiikka ja
Turun biolääketieteen tutkijakoulu
Turku
Annales Universitatis Turkuensis, Medica-Odontologica Series D
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TIIVISTELMÄ

Solujaossa (mitoosi) solusyklin aikana kahdentunut perintöaines jakautuu tasan kahden tytärsolun kesken. Plk1 (Polon kaltainen kinaasi 1) ja Aurora B -kinaasi yhdessä Incenp-, Survivin- ja Borealin-proteiinien kanssa (chromosomal passenger complex, CPC) ovat keskeisiä mitoottisten tapahtumien säätelijöitä. Mitoottinen tarkastuspiste (spindle checkpoint, SC) varmistaa, että kromosomien jakautuminen (anafaasi) käynnistyy vasta sitten, kun niiden sisarkinetokorien ja tumasukkulan mikrotubulusten välille on muodostunut oikeaoppinen kaksi-polaarinen kytkentä. Kinetokorit säätelevät kromosomien jakautumisen ajankohtaa. Jos kinetokorien mikrotubulus-kytkennät ovat virheellisiä tai ne puuttuvat kokonaan, on sisarkinetokorien välinen fyysinen jännite alentunut. Tässä tilanteessa kinetokoreissa muodostuu kromosomien eroamista estävä “odota anafaasi” -signaali. Signaalin muodostavien proteiinien tiedetään sitoutuvan kinetokoreihin lyhytaikaisesti, minkä uskotaan vaikuttavan solun kykyyn reagoida nopeasti muuttuviin olosuhteisiin. Kinetokorit, joista puuttuu mikrotubuluskytkennät tai niiden aikaansaama jännite, ilmentävät 3F3/2-fosfoepitooppia, jonka arvellaan liittyvän SC-signalointiin. Kokeellisessa osassa analysoimme 3F3/2-fosfoepitoopin säätelyä ja CPC-molekyylien liikkuvuutta sentromeereissä. Lisäksi tutkimme CPC:n mitoottisia tehtäviä mikroinjektiotekniikalla, joka mahdollistaa kompleksin toiminnan estämisen halutussa mitoosin vaiheessa. Tulokset osoittivat, että Plk1 luo kinetokoreihin 3F3/2-fosfoepitoopin, CPC-molekyylit sitoutuvat dynaamisesti sentromeereihin riippuen Aurora B -kinaasin aktiivisuudesta ja CPC toimii kromosomien ja tumasukkulan liikkeiden säätelijänä solujaon loppuvaiheessa. Tutkimus johti uusien Plk1:n ja CPC:n tehtävien tunnistamiseen ja lisäsi ymmärtämystämme mitoottisten tapahtumien säätelystä.

Avainsanat: mitoosi, mitoottinen tarkastuspiste, Plk1, Aurora B, CPC, 3F3/2-fosfoepitooppi, kinetokori

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ABBREVIATIONS

APC/C	Anaphase promoting complex/cyclosome
Bub	Budding uninhibited by benzimidazole
BuBR1	Bub1-related protein 1
Cdc20	Cell division cycle 20
Cdc27	Cell division cycle 27
Cdk1	Cyclin-dependent kinase 1
Cenp	Centromere protein
CPC	Chromosomal passenger complex
FRAP	Fluorescence recovery after photo-bleaching
GFP	Green fluorescent protein
Hec1	Highly enhanced in cancer
IAP	Inhibitor of apoptosis protein
Incenp^{-ab}	Anti-Incenp antibody
kMT	Kinetochore-microtubule
Mad	Mitotic arrest deficient
MCAK	Mitotic centromere-associated kinesin
MCC	Mitotic checkpoint complex
Mklp	Mitotic kinesin like protein
MT	Microtubule
Ndc80	Non disjunction of chromosomes 80
NEB	Nuclear envelope breakdown
PBD	Polo-box domain
Plk1	Polo-like kinase 1
PP1	Protein phosphatase 1
Recf	Recovery of fluorescence
RNAi	RNA interference
SC	Spindle checkpoint
siRNA	Small interfering RNA
$T_{1/2}$	Half-time of recovery

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-III. The original communications have been reproduced with the permission of the copyright holders. Unpublished data is also included.

I Ahonen LJ*, Kallio MJ*, Daum JR*, Bolton M, Manke IA, Yaffe MB, Stukenberg PT and Gorbsky GJ. 2005. Polo-like kinase 1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of spindle-checkpoint proteins at kinetochores. Curr. Biol. 15: 1078-89.

II Beardmore VA*, Ahonen LJ*, Gorbsky GJ and Kallio MJ. 2004. Survivin dynamics increases at centromeres during G2/M-phase transition and is regulated by MT-attachment and Aurora B kinase activity. J. Cell Sci. 117: 4033-42.

III Ahonen LJ, Kukkonen AM, Pouwels J, Bolton M, Jingle CD, Stukenberg PT, and Kallio MJ. 2008. Perturbation of Incenp function impedes anaphase chromatid movements and chromosomal passenger protein flux at centromeres. Chromosoma 118, in press.

* Equal contribution

1. INTRODUCTION

During the cell cycle, the cell's genome is first duplicated at the replication-phase (S-phase), and later segregated into two newly formed daughter cells at mitosis (M-phase). To maintain genomic integrity, both progeny cells recieve one copy of the genome. The fidelity of chromosome segregation is guarded by a mitosis-controlling signaling network termed the spindle checkpoint (SC), which monitors kinetochore-microtubule (kMT) attachments and prevents mitotic progression to anaphase in the presence of incorrect attachments. The SC allows time for processes that convert erroneous connections into correct bipolar attachments. It therefore aims to guard cells from the gain or loss of chromosomes (aneuploidy), which is a hallmark of cancer, and the SC signaling pathway has recently been shown to drive tumorigenesis in animal models.

The kinetochore, a central organelle in SC signaling, is a multiprotein structure that is formed on the centromeres of mitotic chromosomes. It is responsible for attaching chromosomes to spindle microtubules (MTs), which facilitate chromosome movements during all phases of cell division. In addition to functioning as a platform for MT attachment, the kinetochore monitors the correctness of attachments. Kinetochores must attain a full set of MTs, which is about 20 MTs per kinetochore in vertebrate cells, and become under tension by pulling MTs to switch off the SC.

How SC signaling functions is not completely understood. Based on current knowledge, an SC signal that is composed of multiple proteins, also called "wait anaphase" signal, is generated at kinetochores that are unattached or lack tension. This signal is spread throughout the cell to inhibit the activity of the anaphase promoting complex (APC/C), the mitotic ubiquitin ligase that promotes sister chromatid separation and exit from M-phase.

Proteins that are involved in SC signaling and other mitotic processes are regulated by post-translational modifications, such as reversible phosphorylation events. The kinetochores that create the inhibitory "wait anaphase" signal express a certain phosphoepitope, named the 3F3/2 phosphoepitope, which undergoes reversible phosphorylation changes in response to kMT attachment and tension.

Two important kinases that have been implicated in mitotic progression are Polo-like kinase 1 (Plk1) and Aurora B kinase. Aurora B forms, together with Incenp, Survivin and Borealin, the chromosomal passenger complex (CPC). Plk1 and Aurora B, the enzymatic core of the CPC, phosphorylate a number of mitotic substrates at centromeres, kinetochores, and other mitotic cell organelles to regulate their activities. Aurora B localization and function are highly dependent on the other CPC members.

The purpose of this thesis project was to uncover new regulatory proteins within the 3F3/2 phosphoepitope pathway. In addition to this, the aim was to determine the mitotic functions of the CPC at different mitotic phases. Unlike previous studies, which

used siRNA or expression of dominant negative mutants, microinjection of an antibody allowed us to block CPC function in a temporally controlled manner. To uncover details about the regulation of CPC activity, the turnover of CPC molecules was analyzed at centromeres and other structures in cells.

2. REVIEW OF THE LITERATURE

2.1. Mitosis

2.1.1. Mitotic phases

The cell cycle consists of alternating phases termed interphase and mitosis. Interphase is the period during which cells replicate their genome (synthesis or S-phase) and accumulate cellular material (gap-phases G1 and G2). During mitosis, the replicated genome is segregated into two daughter cells in stages classified as prophase, prometaphase, metaphase, anaphase and telophase. These mitotic phases describing vertebrate mitosis in a general manner are shown as schematic drawings (Fig. 1A), and as images acquired with the fluorescent microscope after fixing and staining cells with antibodies to visualize important mitotic structures (Fig. 1B).

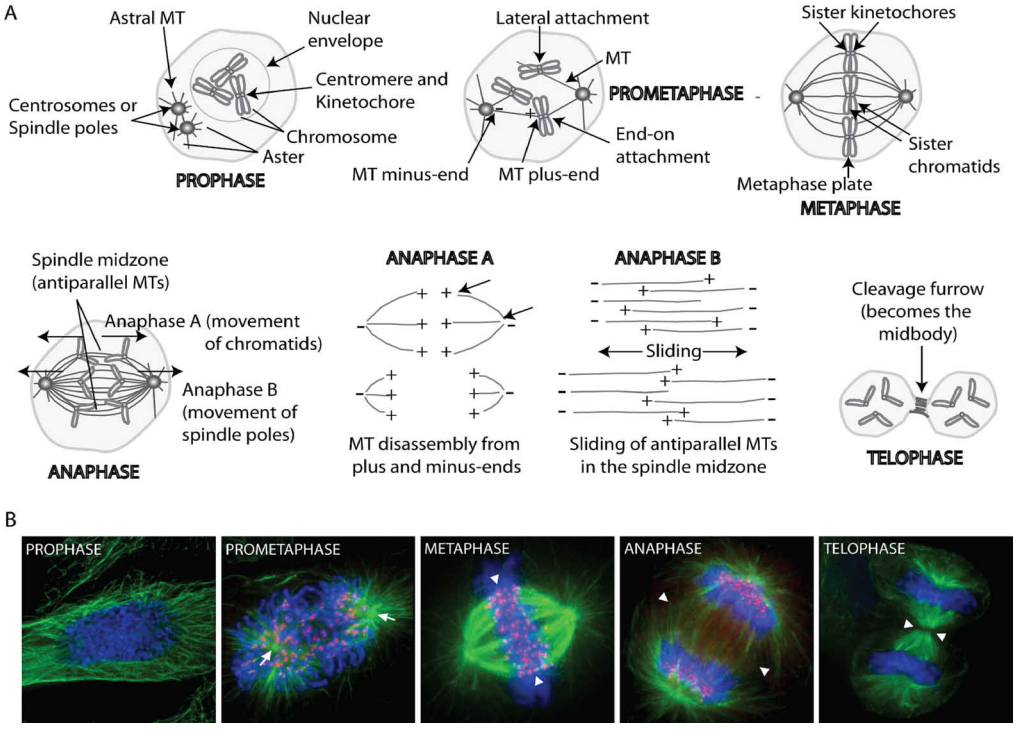

Figure 1. Mitotic phases. Drawings of the mitotic phases and central structures (A). Immunofluorescent images of HeLa cells (B) that were fixed and stained with antibodies against MTs (green), kinetochores (red) and DNA (blue). (B) Arrows show the location of centrosomes, and arrow heads the location of the metaphase plate (metaphase), spindle midzone (anaphase) and midbody (telophase).

In prophase, nuclear chromatin is compacted tightly in a process termed condensation through the action of condensins (Strunnikov 2003). It now becomes visible in the light microscope as chromosomes. Chromosomes, consisting of a pair of identical sister chromatids, are held together by cohesion. This “glue” between sister

chromatids is formed through the action of cohesins (Michaelis et al. 1997). Cohesion is established in S-phase, and resolved from chromosomes in two steps during mitosis (Waizenegger et al. 2000). It is removed from chromosome arms during prophase and from centromeres at metaphase-to-anaphase transition. For this reason, mitotic chromosomes appear as X-shaped structures until the sister chromatids are separated at anaphase onset.

During prophase, the mitotic spindle starts to form as asters in the cytoplasm as the pair of centrosomes, also referred to as spindle poles, begin movement to the opposite ends of the cell and radiate MTs. This is accompanied by nuclear envelope breakdown (NEB), which marks the beginning of prometaphase.

In prometaphase, MTs emanating from the centrosomes reach the chromosomes, which are now highly condensed, and begin their movement toward the center of the cell. MTs are highly dynamic polymers and they continuously probe the cytoplasm with their plus-ends to search and capture chromosomes (Hayden et al. 1990; Holy and Leibler 1994). Chromosomes first bind along the MTs, rather than the tip, via kinetochores in a transient manner by forming "lateral attachments" (Hayden et al. 1990; Merdes and De Mey 1990; Rieder and Alexander 1990). These are later converted to stable "end-on attachments", in which MT plus-ends become inserted into kinetochores.

MTs that grow from opposite centrosomes become attached to sister kinetochores (kinetochores on a pair of sister chromatids) in a bipolar fashion. In this attachment, also referred to as amphitelic attachment (Fig. 5), one sister kinetochore is bound through MTs to the spindle pole facing that kinetochore, whereas the other sister kinetochore is attached to the opposite spindle pole. Usually many capture and release events occur before correct amphitelic attachments are made. In the course of normal chromosome congression, sister kinetochores capture MTs first from one pole and then from the other pole. Therefore, amphitelic attachments are often preceded by monotelic, syntelic or merotelic attachments (Fig. 5). In these attachments only one sister kinetochore is bound, both sister kinetochores are bound to the same pole, or one of the sister kinetochores is bound to both poles. Incorrect attachments need to be destabilized to allow the establishment of amphitelic attachments (Tanaka 2008).

Before metaphase alignment, chromosomes undergo poleward and anti-poleward movements called oscillation. They finally line up at the center of the cell to form the metaphase plate. This alignment is due to the counterbalance between the pulling forces generated by spindle MTs from opposite spindle poles, and the forces generated by kinetochores (Inoue and Salmon 1995). The alignment of chromosomes at the metaphase plate is a prerequisite for the accurate segregation of sister chromatids into daughter cells in the following stage, which is termed anaphase.

In anaphase, sister chromatids separate simultaneously from each other after the removal of centromeric cohesion (de Gramont and Cohen-Fix 2005). During the first

stage of anaphase, called anaphase A, the separated sister chromatids are pulled towards the opposite poles of the mitotic spindle. This movement is a cooperative force generated by MT depolymerization at the kinetochores and at the spindle poles. During the latter stage of anaphase, called anaphase B, centrosomes move apart causing spindle elongation and further separation of the sister chromatids. This movement is mediated by anti-parallel MTs that form the spindle midzone at the same position where the metaphase plate used to lie. Motor proteins that localize to the spindle midzone slide MTs in relation to one another and push the poles away from the midzone and each other.

In telophase, the cleavage furrow ingresses at the spindle midzone and cleaves the separated chromatids and cytoplasm into two new daughter cells in a process called cytokinesis (Barr and Gruneberg 2007). This is accomplished through recruitment of myosin, actin and certain regulatory proteins to the spindle midzone. Myosin and actin filaments form a contractile ring, which assembles at the cell cortex (adjacent to the cell membrane), and undergoes contraction after signals from regulatory proteins. Cytokinesis is finalized by the abscission of the midbody, a remnant of the cytokinetic furrow, in a process involving membrane trafficking and membrane fusion. Now each daughter cell has a complete copy of the genome of its parent cell, and mitosis is complete.

2.1.2. Centromere and kinetochore structure

Chromatin and associated proteins that are located at the primary constriction site of a chromosome are referred to as the centromere (Fig. 2). This structure is characterized by three unique features. Firstly, the centromere is formed of repetitive DNA sequences, called α-satellite DNA, which bear little or no sequence homology among species. Secondly, in the centromeric chromatin a specific histone, CenpA, replaces the normal nucleosomal subunit histone H3 at certain intervals (Blower et al. 2002). Thirdly, the centromere region is flanked by heterochromatin, which is tightly packed and not transcribed (Pidoux and Allshire 2005). The repetitive nature of the centromere chromatin is important in specifying the site of kinetochore assembly (Zinkowski et al. 1991; Blower et al. 2002). The repeating units come together in a 3-dimensional array at one face of the chromatin that points toward the pole, and function as the basis for the recruitment of centromere proteins (Cenps) and kinetochore proteins. The site of kinetochore assembly is thought to be controlled primarily by epigenetic mechanisms, such as histone modifications and the formation of heterochomatin, and not by DNA sequence-specific information (Karpen and Allshire 1997). Mechanisms that target CenpA to specific nucleosomes and remove it from other sites, ensure that centromere identity is maintained (Collins et al. 2004; Hayashi et al. 2004). It has been suggested that the centromere-specific CenpA nucleosomes confer rigidity that is needed for kinetochore function (Black et al. 2004).

The vertebrate kinetochore is a complex trilayered structure consisting of an inner, middle and outer kinetochore (Brinkley and Stubblefield 1966) (Fig. 2). A dense array

of fibers, called the fibrous corona, extends away from the outer kinetochore. Over 80 kinetochore proteins have been described, and newly identified proteins emerge every year (Cheeseman and Desai 2008). The complexity of the kinetochore is therefore not fully understood. Kinetochores have two central functions in mitosis: they mediate MT attachment to chromosomes and monitor errors in these attachments.

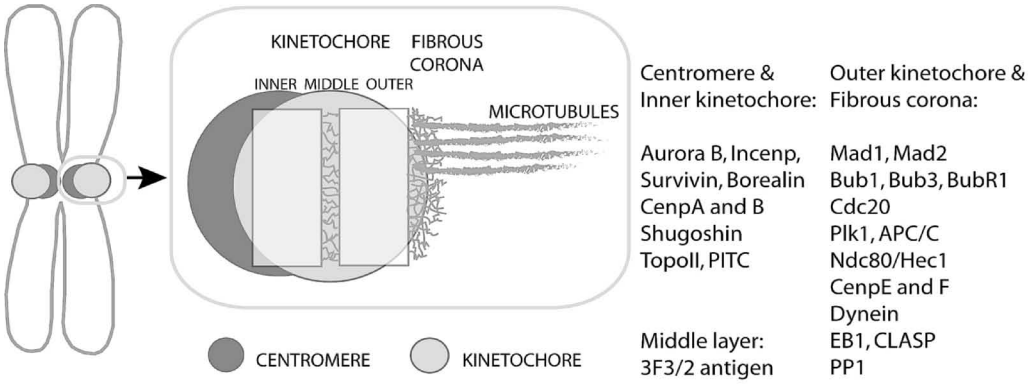

Figure 2. Organization of the centromere-kinetochore region, kinetochore layers and their constituent proteins. Modified from (Maiato et al. 2004a).

The centromere and inner kinetochore regions (Fig. 2) are composed of proteins that are involved in mediating the binding of the kinetochore to DNA, and in kinetochore assembly (Maiato et al. 2004a; Cheeseman and Desai 2008). The CPC molecules, which are involved in many aspects of centromere/kinetochore functions, reside in the inner centromere. The middle kinetochore contains molecules that are believed to play a role in sensing tension across the kinetochore as a result of MT pulling forces (3F3/2 antigen). The outer kinetochore and fibrous corona consist of various proteins that generate SC signals (Bub1, Bub3, BubR1, Cdc20 and Mad2), are involved in MT capture (CenpE and F, Ndc80/Hec1 and MT-associated proteins EB1 and CLASPs), or power movements of chromosomes along MTs (motor proteins CenpE and dynein). It should be noted that the exact localization of many proteins to different kinetochore subregions is not known at electronmicroscopic level; therefore the shown localization does not exlude localization to other subregions. In vertebrates, the outer plate of each kinetochore has about 20 attachment sites for kMTs, whereas the kinetochores of budding yeast bind only a single MT.

Some proteins, such as Cenp-proteins that bind to chromatin, remain associated with the centromere region throughout the cell cycle. Other components become concentrated to the kinetochore region in late G2 or prophase. Some of these M-phase specific proteins, such as the outer kinetochore component Ndc80/Hec1, remain relatively constant at kinetochores through mitosis (Hori et al. 2003). Other proteins associate with the kinetochore to a variable extent depending on the mitotic stage and the attachment status of kinetochores to MTs. Components that change in concentration at kinetochores during mitosis include MT motor proteins CenpE and

dyneins and SC proteins (Mad1, Mad2, BubR1 and Cdc20). They assemble at high concentrations to kinetochores in the absence of MTs during prophase and early prometaphase, and are reduced in concentration by interactions with spindle MTs in late prometaphase and metaphase (King et al. 2000; Hoffman et al. 2001; Howell et al. 2004; Shah et al. 2004).

In addition to varying local concentrations during mitotic progression, kinetochore proteins have variable residence times at the kinetochore. Some proteins, such as Ndc80/Hec1 (Hori et al. 2003), are stably associated with the kinetochore. Other proteins, such as Cdc20 and Mad2 (Kallio et al. 2002a; Howell et al. 2004; Shah et al. 2004), bind to the kinetochore with a high turnover indicating that they are constantly released from the kinetochore so that new molecules can bind from the cytoplasm. The residence time can vary from a few seconds to tens of minutes and may provide a means to rapidly alter the protein content of kinetochores in response to changing cellular conditions. Centromere and kinetochore located proteins also undergo complex post-translational modifications, such as phosphorylation, sumoylation, farnesylation and ubiquitination (Hussein and Taylor 2002; Vong et al. 2005; Zhang et al. 2008). These post-translational modifications may alter individual kinetochores and contribute to the cells' responses.

2.1.3. Microtubules and the mitotic spindle

MTs provide mechanical support for the shape of cells and function as tracks along which cell organelles move. They are polar filaments that are assembled from αβ-tubulin heterodimers through their head-to-tail and lateral associations (Fig. 3) (Inoue and Salmon 1995). A single MT is a hollow cylindrical structure with a 25 nm diameter. It is composed of 13 protofilaments that are each assembled from αβ-tubulin dimers through their head-to-tail assembly. α- and β-tubulins are very similar in mass, about 55 000 Daltons, and share about 40% sequence similarity (Little and Seehaus 1988). Different isoforms of these tubulins have been described in the literature and they may be involved in regulating specific MT-based functions (Luduena 1998). Furthermore, tubulins undergo a series of post-translational modifications, and these are thought to contribute to specific functions of MTs (Hammond et al. 2008). In addition to α- and β-tubulins, other tubulins with mostly unknown fuctions have been discovered (Oakley 2000). An exception is gamma-tubulin, which has a well established role at the centrosome in MT nucleation (Joshi et al. 1992).

MTs have intrinsic structural polarity due to the head-to-tail association of αβ-heterodimers. They have a plus-end that points outward from the centrosome and attaches to cell organelles, such as the chromosomes, and a minus-end at the centrosome (Fig. 1A, prometaphase) (Howard and Hyman 2003). MTs experience dynamic turnover due to polymerization (addition of tubulin subunits) at plus-ends and depolymerization (removal of tubulin subunits) at minus-ends (Fig. 3A). Incorporation of new tubulin into the plus-end requires the GTP-bound form of tubulin, while most tubulin in the spindle is in the hydrolyzed GDP-bound form (Fig. 3A). The plus-end is

terminated by the β-subunit, while the minus-end is terminated by the α-subunit due to the polar head-to-tail association. In addition to the dynamic turnover, which leads to net growth at the plus-end, plus-ends of MTs exhibit dynamic instability (Fig. 3B), which refers to transitions between periods of polymerization (rescue) and depolymerization (catastrophe) (Mitchison and Kirschner 1984). During periods of rescue, MT ends are bound by GTP and straight (Fig. 3A), and during periods of catastrophe, the ends are curved and bound to GDP, which promotes disassembly (Fig. 3B) (Hyman et al. 1995; Muller-Reichert et al. 1998). The discovery of dynamic instability provided for the first time a mechanism by which MTs could reassemble into different structures during the cell cycle.

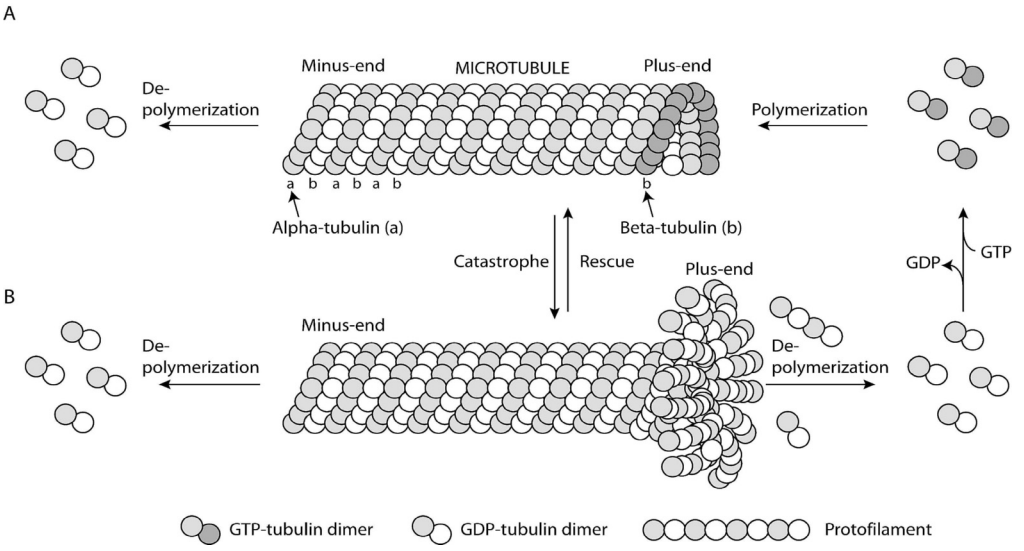

Figure 3. MT assembly (A) and dynamic instability (B). Modified from (Cheeseman and Desai 2008).

The dynamic properties of MTs are essential for spindle morphogenesis and chromosome movements during mitosis (Inoue and Salmon 1995). The kMTs can undergo rescue and catastrophe without loosing their attachment to the kinetochore. This allows prometaphase congression and anaphase movements of chromosomes. Depolymerization at the minus-ends (at poles) contributes to a lesser extent to chromosome movements in prometaphase. However, depolymerization at both plus- and minus-ends contributes to the movements of chromatids in anaphase (Fig. 1A, anaphase A). Although mitotic spindle formation is mainly driven by MT nucleation at spindle poles, MT nucleation at kinetochores may contribute to this process (Maiato et al. 2004b).

2.1.3.1. Motor proteins

Polarized arrays of MTs provide tracks for the movements of chromosomes but this transport is mainly driven by motor proteins that interact with MTs and move along

their lateral surface. A delicate balance between the activities of various motor proteins is required during mitosis to assemble the mitotic spindle, to align chromosomes on the spindle, and to segregate sister chromatids into daughter cells. MT-based motor proteins are divided into two families of enzymes that use ATP to generate force: the kinesins that are plus-end directed (move towards plus-ends of MTs) and the dyneins that are minus-end directed motors (move towards minus-ends of MTs).

Motor proteins are needed in many processes during mitosis. Firstly, they are involved in the crosslinking and sliding of MTs in relation to one another (Sharp et al. 1999). This force is responsible for sliding antiparallel MTs in the spindle midzone to power anaphase B movements (Fig. 1A, anaphase B), and for sliding astral MTs in relation to the cell cortex to position spindle poles. Secondly, motor proteins are involved in the transport of chromosomes along spindle MTs (Wood et al. 1997). Plus-end directed motors move chromosomes toward the metaphase plate and minus-end directed motors move them toward the spindle poles. Two important mitotic motor proteins, CenpE, a plus-end directed motor, and dynein, a minus-end directed motor, localize to kinetochores (Steuer et al. 1990; Yen et al. 1992). In addition to moving chromosomes, these kinetochore located motor proteins could maintain attachment to shortening and growing plus-ends of MTs. Thirdly, some motor proteins depolymerize MTs. MCAK (Mitotic centromere-associated kinesin) is the major depolymerase controlling the dynamics of MT plus-end assembly at kinetochores. MCAK uses ATP to catalytically depolymerize MTs by accelerating the rate of dissociation of tubulin from MT ends (Hunter et al. 2003). It facilitates smooth chromosome movements through increase in the turnover rate of kMT attachments (Wordeman et al. 2007).

2.1.4. Mitosis and cancer therapy

Classical therapies that are used to treat cancer, such as chemotherapy and radiotherapy, are based on cytotoxic effects to cells after interference with cell division. Their effects can result from DNA damage or defects in MT-mediated processes. The proper segregation of chromosomes to daughter cells requires rapid MT dynamics, which makes mitosis extremely sensitive to MT-targeted drugs, such as vinca alkaloids and taxanes (Jordan and Wilson 2004). These drugs interfere with normal MT assembly and/or disassembly and produce prolonged mitotic arrest, which is followed by apoptosis or “mitotic slippage” due to adaptation (Rieder and Maiato 2004). After mitotic slippage cells enter the following G1-phase but because of a cytokinesis failure they are now tetraploid cells with reduced viability. The drawback of these classical approaches is that they damage also healthy tissue, especially those tissues with a high replacement rate. Furthermore, the development of resistance to MT drugs by mechanisms, such as mutations in transporters leading to drug efflux or mutations in the tubulin genes, poses a clinical problem (Fojo and Menefee 2007).

Our understanding of cancer has increased tremendously as the genes and signaling pathways that are deregulated in cancer are uncovered (Vogelstein and Kinzler 2004). With better knowledge of cancer genes and pathways, the aim is now to produce drugs

that eliminate tumor cells while sparing normal cells and tissues (Sawyers 2004). Next generation anti-cancer drugs are designed to interfere with specific molecular targets that have critical roles in tumor growth or progression. Targeting mitotic regulators provides more specificity via the opportunity to damage dividing cells while interphase cells are left unharmed. Furthermore, the consequence of the inhibition of mitotic regulators is often massive chromosome mis-segregation, which leads to apoptosis (Kops et al. 2005). The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer. It is first important to understand how different proteins and pathways function in normal cells so that their activities can be targeted appropriately in cancer cells.

2.2. The spindle checkpoint (SC)

2.2.1. Principle of the SC

Maintenance of correct chromosome number is necessary for the development and survival of an organism. As cells progress through mitosis, the SC detects errors in the attachment of MTs to chromosomes. It delays cell cycle progression from metaphase to anaphase, i.e. the separation of sister chromatids, until all chromosomes have aligned in a bipolar fashion at the metaphase plate (Fig. 4; reviewed in (Musacchio and Salmon 2007)). This is important, since daughter cells would inherit an incorrect number of chromosomes if cells would proceed into anaphase in the presence of erroneous attachments (Fig. 5).

The SC is activated in early mitosis when many kinetochores lack proper attachment to MTs and it stays active until the establishment of correct bipolar attachments. Even a single improperly attached chromosome is sufficient to block mitotic progression. It produces cell-wide inhibition of the proteolytic machinery that is required for anaphase onset. The inhibitory signal that is produced at the kinetochores of improperly attached chromosomes, is thought to diffuse into the surrounding cytoplasm and to neighboring chromosomes to inhibit anaphase onset on all chromosomes (Rieder et al. 1994; Rieder et al. 1995).

The SC detects two different aspects of kMT interaction, attachment and tension. It generates cell cycle inhibitory signals in response to lack of attachment between kinetochore and MTs (Rieder et al. 1994; Rieder et al. 1995). In addition to detecting the attachment status, additional mechanisms sense incorrect attachments that do not generate tension across the kinetochores (Fig. 5; syntelic attachment) (Li and Nicklas 1995; Nicklas et al. 1995). How these incorrect attachments are converted into bipolar attachments is discussed in in 2.5.2.2. When chromosomes become aligned at the metaphase plate in a bipolar fashion, tension is generated between the sister kinetochores by kMTs having opposing directional forces, and the SC is switched off. As the generation of tension also stabilizes kMT attachments and increases their number (Nicklas and Ward 1994; King and Nicklas 2000), it is difficult to analyze these mechanisms separately. Recently, there has been debate over the existence of the

tension pathway, and it was suggested that lack of tension simply generates a small number of unattached kinetochores that keep the SC active (Pinsky et al. 2006).

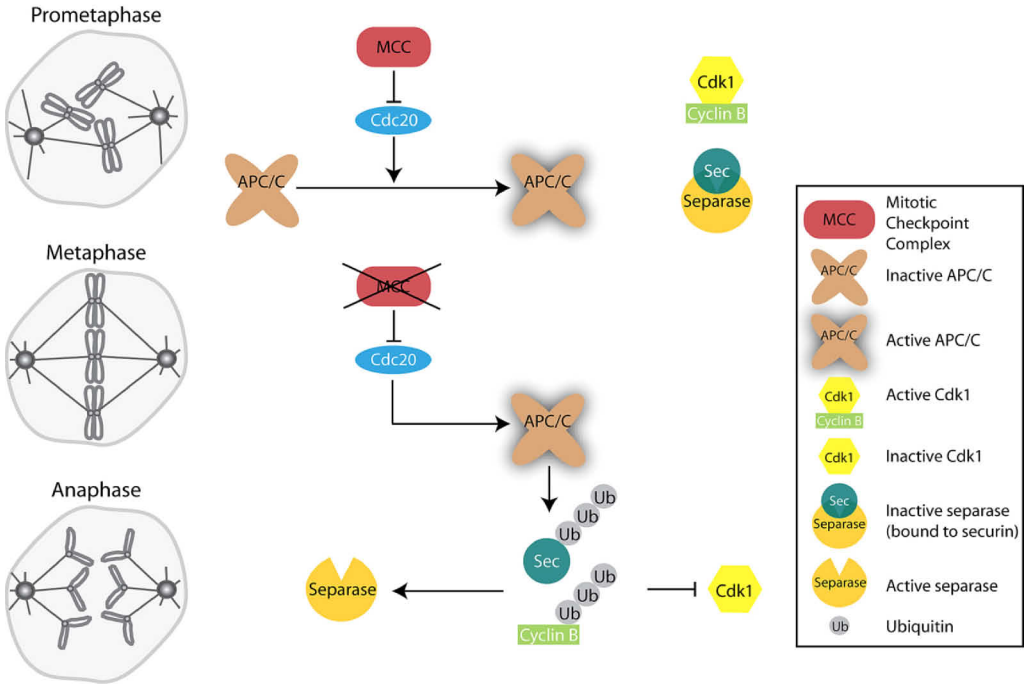

Figure 4. Molecular principle of the SC. In prometaphase, kinetochores lacking MT attachment or tension generate diffusible MCC. This prevents Cdc20-mediated activation of the APC/C. Separase is kept inactive by securin. In metaphase, kinetochores are attached and under tension and no longer generate MCC. Cdc20 activates the APC/C allowing ubiquitination of securin and cyclin B. Separase is activated and it cleaves sister chromatids to promote anaphase onset. Modified from (Musacchio and Salmon 2007).

2.2.2. Composition of the inhibitory signal

The inhibitory signals that are formed in response to lack of attachment or tension and activate the SC consist of various proteins. Two independent screens identified the genes, mutation of which bypassed the ability of budding yeast cells to arrest in mitosis in the presence of spindle disrupting agents. These genes are called Bub for budding uninhibited by benzimidazole and Mad for mitotic arrest deficient (Hoyt et al. 1991; Li and Murray 1991). Bubs and Mads form inhibitory complexes that prevent Cdc20 (cell division cycle 20)-mediated activation of the anaphase promoting complex/cyclosome (APC/C) (Fig. 4) (Kallio et al. 1998; Sudakin et al. 2001). When activated by free Cdc20, the APC/C promotes entry into anaphase by polyubiquitination of mitotic substrates, such as cyclin B and securin, which leads to their destruction by the 26S proteasome (Peters 2006).

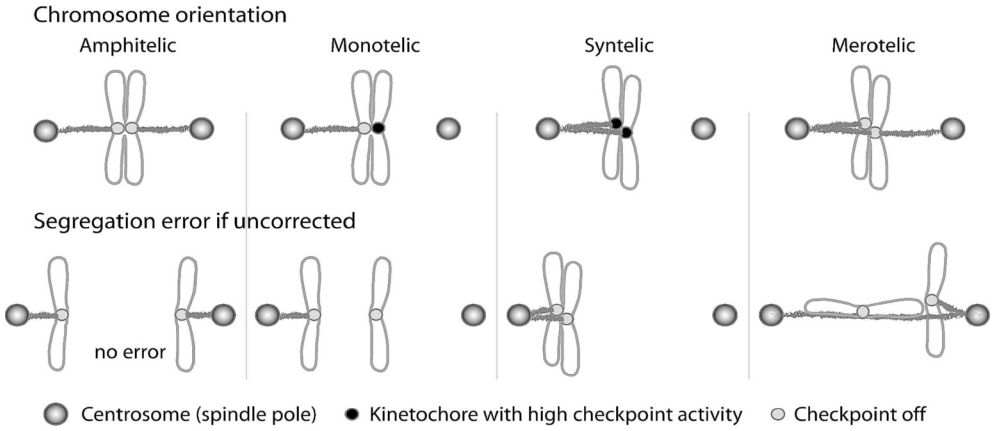

Figure 5. Description of kinetochore attachment errors, their ability to activate the checkpoint, and the consequences if they are not corrected before anaphase onset. Modified from (Maiato et al. 2004a).

The main SC complexes that keep the APC/C inactive consist of BubR1 (Bub1 related protein 1), Mad2, Bub3 and Cdc20 proteins, collectively called mitotic checkpoint complex, or MCC (Kallio et al. 1998; Sudakin et al. 2001). MCC proteins accumulate at kinetochores that are either unattached to MTs or attached in a way that does not produce tension across the sister kinetochores. BubR1 is thought to accumulate to kinetochores mainly in the absence of tension while Mad2 accumulates in the absence of attachment (Skoufias et al. 2001; Logarinho et al. 2004). According to current knowledge MCC proteins diffuse from the unattached or unaligned kinetochores throughout the cell to inhibit the APC/C.

Many other proteins are involved in the formation, release and amplification of SC inhibitory signals at kinetochores. CenpE, the kinetochore MT plus-end directed motor, has been implicated to function in the SC (Abrieu et al. 2000). It modulates BubR1 kinase activity in a MT-dependent manner, and recruits Mad1 and Mad2 to kinetochores (Chan et al. 1998; Mao et al. 2003; Mao et al. 2005). Kinases Bub1 and Mps1 (monopolar spindle 1) are essential for the checkpoint because they are required for the recruitment and retention of CenpE at kinetochores (Abrieu et al. 2001; Sharp-Baker and Chen 2001; Mao et al. 2003). Furthermore, Bub1 kinase contributes to the binding of the MCC to the APC/C synergistically with Aurora B. Bub1 also phosphorylates Cdc20 thus modifying its sensitivity towards MCC inhibition (Tang et al. 2004; Morrow et al. 2005). Finally, Aurora B contributes to SC signaling by creating unattached kinetochores that activate the SC (discussed in 2.5.2.2.) (Pinsky et al. 2006) and by recruiting CenpE, BubR1 and Mad2 to kinetochores (Ditchfield et al. 2003).

2.2.3. Switching off the SC and inhibitory signal production

The biochemical events that precede entry into anaphase are reasonably well understood (Fig. 4). When Cdc20 is relieved from inhibition upon the establishment of correct attachments and tension, it binds to the APC/C. This event together with APC/C phosphorylation by Cyclin-dependent kinase 1 (Cdk1) and possibly Plk1 leads to ubiquitin ligase activation (Golan et al. 2002; Kraft et al. 2003). The APC/C ubiquitinates securin, and it becomes degraded by the 26S proteasome. This lifts off the inhibition of separase, which is a cysteine protease responsible for the physical disjoining of sister chromatids through the hydrolysis of cohesin. The APC/C also targets cyclin B for destruction, which promotes Cdk1 inactivation and exit from mitosis. Therefore, the onset of anaphase is first temporally blocked by the rapid distribution of cell cycle inhibitory signals, and then promoted by the simultaneous cleavage of cohesin and inactivation of Cdk1.

It is, however, unclear what events are needed to switch off the SC and MCC production. No single event can be attributed to switching off the SC signaling pathway. The process involves MT capture by CenpE, which modulates SC signaling through the regulation of BubR1 kinase activity (Putkey et al. 2002; Mao et al. 2003). An antagonist of Mad2-Cdc20, CMT2/p31(comet), may also contribute to switching off the SC and activation of the APC/C (Habu et al. 2002; Xia et al. 2004; Yang et al. 2007). Dynein may participate in the silencing of the SC through removal of SC proteins from kinetochores via MT-mediated transport (Howell et al. 2001). Finally, ubiquitination of Cdc20 perturbs its interaction with the MCC and could thus drive silencing of the checkpoint (Reddy et al. 2007).

2.2.4. Dynamic association of SC proteins with kinetochores

Photo-bleaching experiments have revealed that SC proteins display fast dynamic turnover as they constantly bind to and are released from kinetochores (Kallio et al. 2002a; Howell et al. 2004; Maiato et al. 2004a). The rapid exchange of these proteins suggests that rather than stable association, kinetochore localization reflects the equilibrium between a high local concentration maintained at kinetochores, and a pool of soluble cytoplasmic protein. This exchange of protein on and off kinetochores enables rapid responses to changing conditions, and it supports models of checkpoint activation where Mad2, BubR1, Bub3 and Cdc20 dynamically exchange as part of a diffuse anaphase inhibitory signal (Howell et al. 2004). Other components such as Mad1 and Bub1 that are not part of the diffusible signal are more stably associated with kinetochores (Howell et al. 2004).

The kinetochore dynamics of Mad2 are well understood (Howell et al. 2004; Shah et al. 2004; Vink et al. 2006). A model constructed on the basis of recent studies predicts that kinetochore recruitment modifies Mad2 into an active conformation that can bind Cdc20 (Luo et al. 2000; De Antoni et al. 2005). According to the model, Mad2 exists in two conformations, open (O-Mad2) and closed (C-Mad2), which both comprise about 50% of the total kinetochore pool of Mad2. C-Mad2 represents Mad2

bound to Mad1 that is stably associated with kinetochores, whereas O-Mad2 represents free Mad2 that is highly mobile (De Antoni et al. 2005; Mapelli et al. 2007). O-Mad2 can form a closed complex with Cdc20 (C-Mad2-Cdc20), whereas C-Mad2-Mad1 functions as a kinetochore receptor for the catalytic generation of C-Mad2-Cdc20 and provides a means for SC signal amplification. Although the model is speculative, it could explain how a presumably weak SC signal that is generated by a single unattached kinetochore could be amplified.

2.2.5. SC and aneuploidy

Partial loss of SC function has been linked to the generation of aneuploidy, which is a hallmark of cancer (Kops et al. 2005). Tumors and cancer cell lines often have a chromosome number other than 46, so the cells have gained or lost whole chromosomes and are said to be aneuploidic. These changes in chromosome number are often accompanied by mutations in tumor suppressor genes, so it remains unclear what the underlying cause of oncogenic transformation is, whether it is the loss of the SC or loss of a tumor suppressor. Partial loss of SC function due to reduced levels of checkpoint proteins, such as Mad2, BubR1, Bub1 and Bub3 or motor protein CenpE, can cause gain or loss of a few chromosomes and contribute to tumorigenesis, whereas complete loss of SC is lethal (Kops et al. 2004; King 2008). Aneuploidy can also arise from other events that lead to mitotic failure and unequal partitioning of chromosomes, such as multipolar spindles, chromosome cohesion defects or improper chromosome attachments (Kops et al. 2005).

2.3. Polo-like kinase 1 (Plk1)

Plk1 is a Ser/Thr kinase that was named after Polo, a kinase whose mutation caused highly branched spindles and defective poles in *Drosophila* (Sunkel and Glover 1988; Llamazares et al. 1991). In mammals, Plk1 appears to be responsible for most functions of Plks, and has been the main focus of research, although four Plks have been identified: Plk1, Plk2 or SNK, Plk3 or Fnk/Prk, and Plk4 or Sak (Fode et al. 1994; Duncan et al. 2001). Plk2 controls entry into S-phase, whereas Plk3 plays a role in stress response pathways, and they both may have overlapping functions with Plk1 in mitosis (Lowery et al. 2005). Plk4, which has the most diverse structure of Plks, has been implicated in cytokinesis (Lowery et al. 2005). Plk1 regulates several mitotic events from entry to exit. Upon entry into mitosis, Plk1 phosphorylates factors that regulate Cdk1 activity, such as phosphatase Cdc25, CyclinB, and kinases Wee1 and Myt1. Plk1 is also involved in the DNA damage checkpoint, which delays entry into mitosis in the presence of DNA damage or other cellular stress. Plk1's G2 functions are reviewed elsewhere (van Vugt and Medema 2005), and this literature review focuses on Plk1 functions after entry into mitosis.

2.3.1. Plk1 localization, substrate targeting and activation

Plk1 associates with spindle poles and kinetochores in early mitosis, and translocates to the spindle midzone at anaphase onset (Golsteyn et al. 1995; Arnaud et

al. 1998). These localizations likely reflect the different functions of Plk1; it associates with different substrates and regulates various events at poles, kinetochores and at the midzone (Fig. 6B).

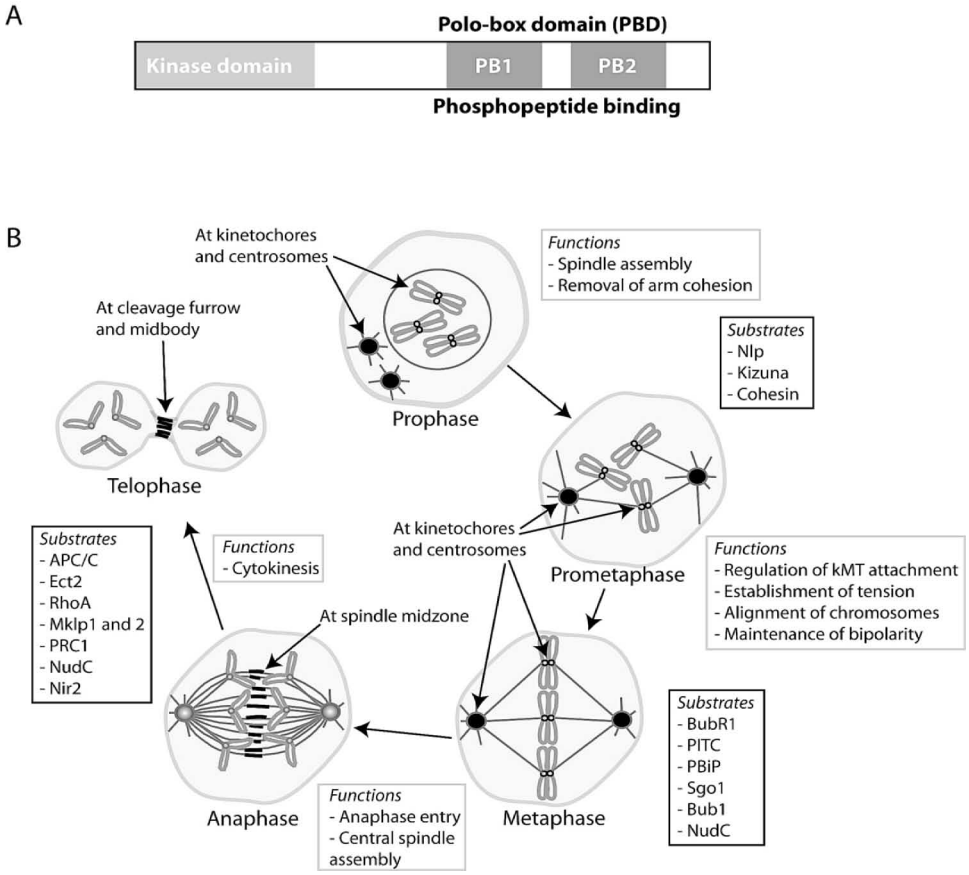

Figure. 6. The schematic structure (A) and mitotic functions (B) of Plk1.

Plk1 is composed of an N-terminal kinase domain and a non-catalytic C-terminal domain called the polo-box domain (PBD), which is composed of two polo-box motifs (Fig. 6A) (Elia et al. 2003a). The PBD constitutes a phosphopeptide-binding domain that is responsible for targeting Plk1 to its substrates in a phospho-dependent manner (Elia et al. 2003a; Elia et al. 2003b). Plk1 has been proposed to dock to particular target proteins at various subcellular sites through the PBD after they have been phosphorylated by other kinases that create "priming phosphorylations" (Elia et al. 2003a). Plk1 may also bind to target proteins by self-regulated recruitment after phosphorylation to create its own docking site (Kang et al. 2006). The kinase domain may also be involved in Plk1 recruitment to subcellular structures (Garcia-Alvarez et al. 2007).

Phosphopeptide binding to the PBD activates the N-terminal kinase domain of Plk1 possibly through structural changes that liberate it from inhibition by the PBD (Jang et al. 2002; Elia et al. 2003b). Plk1 activation requires autophosphorylation as well as phosphorylation by upstream kinases on several residues including a concerved Thr210 in the T-loop (Kelm et al. 2002). Interestingly, the catalytic activity of Plk1 seems to be adequate for some Plk1 functions, whereas other functions require kinase activity as well as correct localization (Hanisch et al. 2006).

2.3.2. Plk1 functions

2.3.2.1. Spindle assembly and removal of cohesion

Centrosomes undergo a series of events including duplication, separation and maturation to form the poles of the bipolar spindle (reviewed in (Nigg 2001)). Plk1 localizes to spindle poles to control these early mitotic events (Sunkel and Glover 1988; Llamazares et al. 1991; Lane and Nigg 1996; Liu and Erikson 2002; Sumara et al. 2004; McInnes et al. 2006). It may affect MT nucleation at spindle poles through phosphorylation of Nlp (Ninein-like protein) and recruitment of γ-tubulin, thus contributing to the formation of the mitotic spindle (Lane and Nigg 1996; Casenghi et al. 2005; McInnes et al. 2006). Plk1 is thought to control the stability of the spindle poles through phosphorylation of the centrosome protein Kizuna (Oshimori et al. 2006). It also appears to localize Aurora A, the other major centrosome controller, to the spindle poles (De Luca et al. 2006). In addition to the establishment of a bipolar spindle, Plk1 has been shown to maintain bipolarity (McInnes et al. 2006; Peters et al. 2006). The kinase is also involved in resolving chromosome arm cohesion during prophase (Sumara et al. 2002).

2.3.2.2. Chromosome alignment and anaphase onset

At kinetochores, Plk1 is important in the establishment of tension, which stabilizes kMT attachments and contributes to bipolar spindle assembly (Sumara et al. 2004; van Vugt et al. 2004). Plk1 interacts with and phosphorylates a number of kinetochore substrates, such as BuBR1, an event that is necessary for the establishment of stable kMT interactions and chromosome congression to the metaphase plate (Hanisch et al. 2006; Elowe et al. 2007; Matsumura et al. 2007). Plk1 also phosphorylates and regulates PICH, a Plk1 interacting checkpoint helicase, which was suggested to bind to centromeric DNA to monitor tension between sister kinetochores (Baumann et al. 2007). In addition to the aforementioned proteins, Plk1 recruitment to kinetochores depends on interactions with at least the following substrates: PBiP (Polo-box interacting protein) (Kang et al. 2006), Sgo1 (Shugoshin 1) (Pouwels et al. 2007), Bub1 (Qi et al. 2006) and NudC (Nuclear distribution protein C) (Nishino et al. 2006).

Plk1 and Cdk1 jointly regulate the APC/C at anaphase onset (Descombes and Nigg 1998; Shirayama et al. 1998; Donaldson et al. 2001). Plk1, like Cdk1, can phosphorylate specific APC/C subunits *in vitro* (Kotani et al. 1998; Golan et al. 2002) but it is unclear whether this is required for APC/C's ubiquitin ligase activity. Plk1

might also regulate the APC/C through the destruction of the proposed APC/C inhibitor xERP1 (*Xenopus* homolog of Early mitotic inhibitor 2) (Schmidt et al. 2005). Furthermore, Plk1 has been implicated in the timing of the metaphase to anaphase transition through complex formation with Incenp (Goto et al. 2006).

2.3.2.3. Cytokinesis and mitotic exit

At the midzone, Plk1 is involved in the regulation of cytokinesis and mitotic exit (Carmena et al. 1998; Descombes and Nigg 1998; Seong et al. 2002; Lee et al. 2005). Plk1 localization and function at the midzone appear to require interactions with many proteins. Its substrates include PRC1 (Protein regulator of cytokinesis 1) (Neef et al. 2007), Mklp1 (Lee et al. 1995; Adams et al. 1998; Liu et al. 2004), Mklp2 (Neef et al. 2003) and NudC (Zhou et al. 2003). Plk1 localization to the spindle midzone may also involve self-regulated recruitment (Neef et al. 2003; Neef et al. 2007). Furthermore, there is evidence to suggest that Plk1 associates with the midzone after Cdk1 creates a priming phosphorylation on Nir2 (Golgi-associated protein) (Litvak et al. 2004).

The precise role of Plk1 during cytokinesis is starting to unravel as small molecule inhibitors have been developed against the kinase. Earlier studies using other methods to block Plk1 function led to prometaphase arrest and hampered the detailed analysis of late mitotic functions of Plk1. Several studies using Plk1 inhibitors or chemical genetics have now shown that Plk1 triggers the initiation of cytokinesis by promoting the recruitment of RhoA GTPase exchange factor Ect2 to the central spindle in anaphase (Brennan et al. 2007; Burkard et al. 2007; Petronczki et al. 2007; Santamaria et al. 2007). This activates RhoA at the cell cortex triggering the assembly of the acto-myosin ring and ingression of the cytokinetic furrow. In addition to its role in cytokinesis, Plk1 was recently reported to control spindle elongation that is responsible for anaphase B chromosome movements (Brennan et al. 2007). Finally, the efficient degradation of Plk1 appears to be important for exit from mitosis (Lindon and Pines 2004).

2.3.3. Plk1 and cancer

Many human cancers show elevated levels of Plk1 (Eckerdt et al. 2005; Takai et al. 2005) and its expression has been linked to the invasiveness of breast carcinomas (Rizki et al. 2007) and poor patient prognosis (Takai et al. 2005). Overexpression of murine Plk1 in NIH3T3 cells caused cell transformation, manifested as foci capable of growth in soft agar, and formation of tumors in nude mice (Smith et al. 1997). Interestingly, the knockdown of Plk1 by siRNA was reported to preferably reduce the survival of human cancer cell lines, while normal cell lines were mostly unaffected (Guan et al. 2005; Liu et al. 2006). Furthermore, membrane permeable polo-box peptides that block PBD interactions were shown to inhibit cancer cell proliferation efficiently (Yuan et al. 2002). These data together with the finding that Plk1 downregulation sensitizes cancer cells to MT-targeted drugs taxanes (Spankuch et al. 2006), suggest that Plk1 is a potential anti-cancer drug target and could be inhibited specifically in cancer cells.

At present, specific Plk1 inhibitors have been developed that target the kinase domain or PBD through allosteric inhibition (Lansing et al. 2007; Steegmaier et al. 2007; Taylor and Peters 2008). Animal experiments suggest that they could be useful anti-cancer drugs, and their effectiveness is tested in ongoing phase II trials (Taylor and Peters 2008).

2.4. The 3F3/2 phosphoepitope

Phosphorylation and dephosphorylation of kinetochore proteins may link kMT attachment and tension to biochemical regulation of the SC. One such phosphorylation event is the regulation the 3F3/2 phosphoepitope, recognized by a monoclonal antibody, called 3F3/2 antibody. The antibody was originally prepared against *Xenopus* egg extracts that had been supplemented with ATPγS (Cyert et al. 1988). It recognized a large variety of thiophosphorylated proteins in the *Xenopus* extracts. Later it was found that the antibody also recognizes a small number of native phosphoproteins from mitotic cells, and that the antibody binds to proteins at kinetochores and centrosomes (Gorbsky and Ricketts 1993).

Expression of the kinetochore 3F3/2 phosphoepitope is sensitive to MT attachment and tension (Li and Nicklas 1997). The phosphoepitope is bright in early mitotic cells (Fig. 7A) but disappears by the time chromosomes reach the metaphase plate and kinetochores attain a full set of MTs whose pulling forces generate tension across the kinetochore (Fig. 7B) (Gorbsky and Ricketts 1993). Furthermore, it is differentially expressed between sister kinetochores during chromosome congression demonstrating for the first time that there is a biochemical difference between the two kinetochores of a single chromosome (Gorbsky and Ricketts 1993). The 3F3/2 phosphoepitope is expressed strongly on the leading kinetochore of a congressing chromosome, and expression is weak on the trailing kinetochore (Fig. 7C).

Elegant micromanipulation experiments showed that this kinetochore phosphorylation is controlled mainly by tension. Force generated by pulling with a micromanipulation needle caused its dephosphorylation, while relaxation of tension caused rephosphorylation (Li and Nicklas 1997). The 3F3/2 phosphoepitope is co-expressed at kinetochores that are thought to generate cell-cycle inhibitory complexes, the MCCs. Moreover, when the 3F3/2 antibody was microinjected into living cells, it caused a delay in metaphase to anaphase transition indicating that dephosphorylation of the epitope is required for anaphase onset (Campbell and Gorbsky 1995). These results raise the possibility that the 3F3/2 phosphoepitope may participate in the tension sensitive signaling pathway that controls SC activity and entry into anaphase.

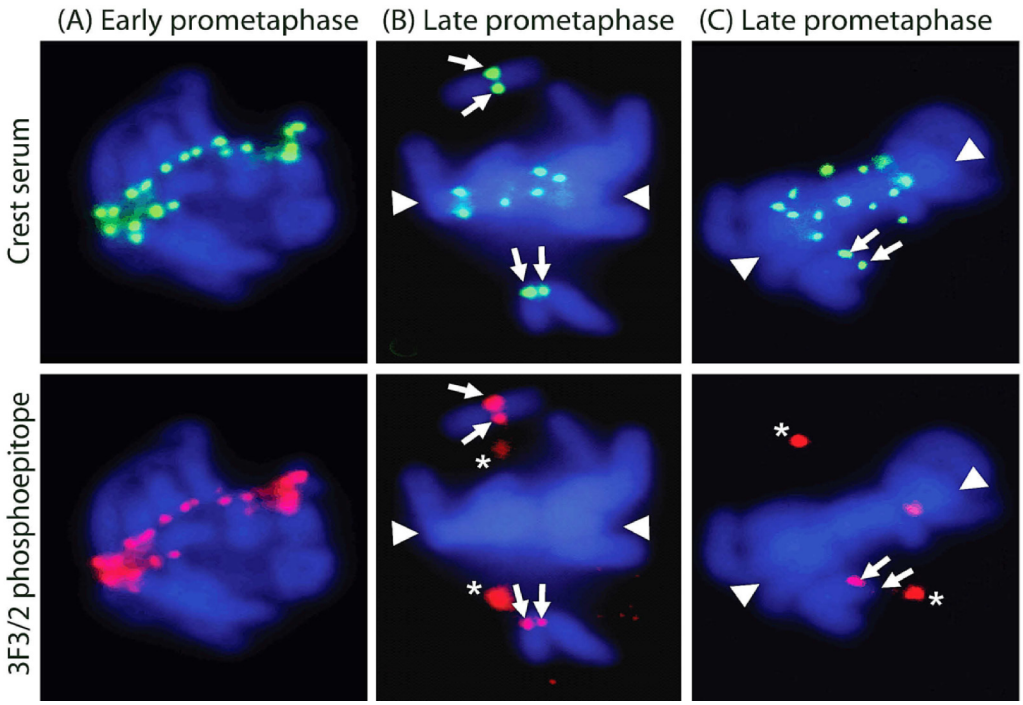

Figure 7. Differential expression of the 3F3/2 phosphoepitope during chromosome congression. All kinetochores (identified with Crest serum) express the 3F3/2 phosphoepitope in early prometaphase when there is no tension between sister kinetochores (A). In late prometaphase (B and C), the 3F3/2 phosphoepitope is expressed on kinetochores of unaligned chromosomes (arrows) that are not under tension by MT pulling forces. Expression is lost at kinetochores of metaphase aligned chromosomes that are under full tension (arrow heads show the location of the metaphase plate). The 3F3/2 phosphoepitope is expressed on the leading kinetochore of a congressing chromosome (C) marked with arrows. The 3F3/2 phosphoepitope antibody also recognizes centrosomes (marked with asterisk). PtK1 cells were fixed and labelled with Crest autoimmune serum (green) and 3F3/2 antibody (red), and DNA was stained with DAPI (blue). Images were obtained through the courtesy of Gary Gorbsky.

2.5. Aurora B and the chromosomal passenger complex (CPC)

2.5.1. CPC composition and localization

Aurora B kinase forms the CPC together with Incenp, Survivin and Borealin (Fig. 8A). The complex shows a cell division phase-dependent localization pattern (Fig. 8B). It first binds to chromosome arms in early mitosis, then concentrates to inner centromeres, and remains there until metaphase-to-anaphase transition. At this point CPC proteins move to the spindle midzone, where they colocalize with MTs, and to the cell cortex (Cooke et al. 1987). Incenp was the founding member of Inner Centromere Proteins that exhibited this localization pattern (Cooke et al. 1987). The CPC complex was identified when Aurora B and Incenp were co-precipitated from various organisms and their depletion phenotypes showed similar defects (Kim et al. 1999; Adams et al.

2000; Kaitna et al. 2000). Later Survivin and Borealin were identified as members of the same complex (Bolton et al. 2002; Gassmann et al. 2004).

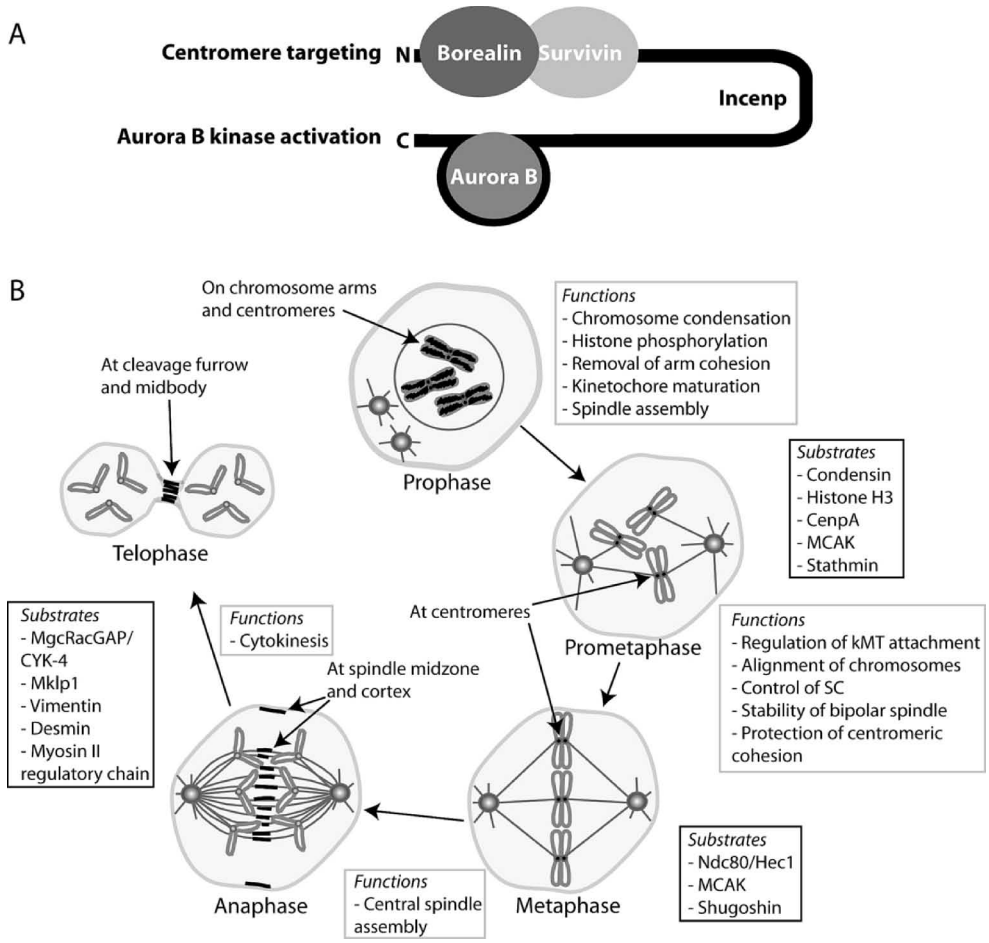

Figure 8. The schematic structure (A) and mitotic functions (B) of the CPC. Modified from (Klein et al. 2006) and (Ruchaud et al. 2007).

The Aurora kinase family is composed of Aurora A, B, and C that are Ser/Thr kinases implicated in distinct functions during mitosis. Aurora A is the centrosome-specific kinase of the family and a major controller of centrosome maturation and bipolar spindle assembly (Glover et al. 1995; Kimura et al. 1997). It promotes entry into mitosis by activating Plk1 and Cdk1 (Macurek et al. 2008; Seki et al. 2008). Aurora C is mainly expressed in the testis where it controls male meiosis together with Aurora B (Kimmins et al. 2007). Aurora C has been reported to interact with other CPC proteins (Li et al. 2004; Sasai et al. 2004; Yan et al. 2005), but its functions during somatic cell mitosis are not well known.

Aurora B kinase forms the enzymatic core of the CPC, whereas Incenp is required for Aurora B activation, and appears to function as a scaffold protein that brings the other members together. Survivin and Borealin bind near the N-terminus of Incenp, which also contains the region required for centromere and midzone targeting, whereas Aurora B binds near the C-terminus (Fig. 8A) (Ainsztein et al. 1998; Bolton et al. 2002; Klein et al. 2006). Interaction between Aurora B and Incenp, together with the phosphorylation of the C-terminus of Incenp by Aurora B in a positive feedback-loop, are necessary for full kinase activation (Bishop and Schumacher 2002; Sessa et al. 2005). Survivin and Borealin mediate CPC localization to centromeres and the midzone together with the N-terminus of Incenp (Klein et al. 2006; Vader et al. 2006).

Survivin has a dual role: in mitosis it functions as a member of the CPC, and outside of that as an inhibitor of apoptosis protein (IAP) (Li et al. 1998). Survivin is comprised of an amino-terminal BIR (Baculovirus IAP repeat) domain typical of anti-apoptotic proteins, and a C-terminal α-helical coil, and it can form homodimers (Chantalat et al. 2000; Verdecia et al. 2000). In addition to wild-type Survivin, splice variants (Survivin-2B, Survivin ΔEx3, Survivin-3B), which are truncated or alternatively spliced at the interface between exons 2 and 3 causing differences in the BIR domain or the α-helix, have been identified (Mahotka et al. 1999; Badran et al. 2004). Survivin localizes to different cellular pools (kinetochores, spindle MTs, and cytoplasm), and splice variants show distinct localization (Mahotka et al. 1999; Mahotka et al. 2002), factors that likely reflect the multiple functions of the protein (Fortugno et al. 2002). In addition to Incenp, Survivin is also phosphorylated by Aurora B, but the functional consequences of this are unclear (Bolton et al. 2002; Chen et al. 2003; Honda et al. 2003; Wheatley et al. 2004). The role of Borealin is not well understood but it may regulate Aurora B activity and facilitate the interaction between Incenp and Survivin (Vader et al. 2006; Jelluma et al. 2008).

CPC proteins are highly interdependent in their localization and function. If one of the molecules is targeted with siRNA or dominant negative mutations, other members are mislocalized and the CPC is unable to perform its mitotic functions (Kaitna et al. 2000; Adams et al. 2001b; Wheatley et al. 2001; Honda et al. 2003). According to the recently published crystal structure of the CPC, Incenp, Survivin and Borealin form a single structural unit with 1:1:1 stoichiometry (three-helix bundle) and undergo complex intermolecular interactions (Jeyaprakash et al. 2007). On the basis of this structure, it seems rational that the CPC cannot function if one of the subunits is missing from the complex. Furthermore, the authors reported that a subcomplex consisting of only Aurora B and Incenp is not adequate to perform the mitotic duties of the CPC. These findings indicating that CPC members are always together contradict a previous report proposing that different subcomplexes of the CPC, such as Aurora B-Incenp subcomplex, exist and perform distinct functions of the CPC (Gassmann et al. 2004). The existence of such subcomplexes is still under debate.

2.5.2. CPC functions

The dynamic localization pattern of the CPC molecules to chromosome arms, centromeres and the midzone reflects the functions of the complex (Fig. 8B). In early mitosis, the CPC is involved in chromosome condensation, spindle assembly, correction of kMT interactions that are inappropriate for chromosome alignment, and regulation of SC activity. In late mitosis the CPC controls the completion of cytokinesis.

2.5.2.1. Chromosome condensation and cohesion

Upon entry into mitosis, interphase chromatin that is dispersed throughout the cell nucleus becomes highly organized and compact. This condensation process provides rigidity to chromosomes and is necessary for chromosome segregation in anaphase. Aurora B contributes to chromosome condensation through recruitment and phosphorylation of condensin subunits (Giet and Glover 2001; Lipp et al. 2007). It also phosphorylates histone H3, an event that has been linked to chromosome condensation (Wei et al. 1998; Hsu et al. 2000; Adams et al. 2001b; Giet and Glover 2001).

Aurora B plays an important role in controlling cohesion between duplicated sister chromatids. As described above, cohesion is lost in two distinct phases during mitosis of vertebrate cells (Waizenegger et al. 2000). Aurora B, together with Plk1, regulates the remomal of cohesion from chromosome arms in prophase (Losada et al. 2002; Sumara et al. 2002; Gimenez-Abian et al. 2004). Aurora B also plays a role in confining cohesion to centromeres during mitosis as its depletion or chemical inhibition leads to mislocalization of Sgo1 (Shugoshin 1), the protein that protects centromeric cohesion from premature cleavage (McGuinness et al. 2005; Resnick et al. 2006; Pouwels et al. 2007).

2.5.2.2. Correction of kinetochore-microtubule attachments

Incorrect attachments are frequently formed during normal mitotic progression, and they need to be destabilized to allow the formation of correct bipolar attachments. Aurora B has a central role in this process (Tanaka et al. 2002; Lampson et al. 2004). It destabilizes incorrect attachments through regulation of MCAK localization and activity. The kinase creates inhibitory phosphorylations that regulate the ability of MCAK to promote MT disassembly (Andrews et al. 2004; Lan et al. 2004; Sampath et al. 2004). Aurora B also phosphorylates another MT destabilizing protein Stathmin/OP18 to inhibit its activity and to promote spindle assembly (Gadea and Ruderman 2006). Furthermore, Aurora B has been implicated in the regulation of Ndc80/Hec1, a central component in the establishment of stable kMT attachments at the outer kinetochore (McCleland et al. 2003; McCleland et al. 2004; DeLuca et al. 2005). Aurora B phosphorylates Ndc80/Hec1, which may weaken its interactions with MTs, therefore releasing improper attachments (Cheeseman et al. 2006; DeLuca et al. 2006; Emanuele et al. 2007).

Aurora B's role in correcting merotelic attachments deserves special attention. In merotelic attachment, one of the two sister kinetochores of a chromosome has connections to both spindle poles (Fig. 5), resulting in a situation where the kinetochore is under tension by MT pulling forces. Merotelic connections can escape SC detection and produce lagging chromatids if anaphase begins before they are corrected. Therefore merotely is a potential cause of aneuploidy (Cimini et al. 2001). Aurora B has an important role in correcting merotelic attachments, as partial inhibition of Aurora B kinase activity, which does not cause gross abnormalities in mitotic progression, produces high incidence of merotelic attachments (Cimini et al. 2006). Aurora B is thought to destabilize inappropriately attached kMTs by the recruitment and regulation of MCAK at merotelic attachment sites (Knowlton et al. 2006). How this is achieved is not straight forward, since these proteins do not co-localize at metaphase. This regulation is discussed in 2.5.3. in more detail.

2.5.2.3. SC activity

Aurora B is thought to regulate SC activity indirectly through the destabilization of incorrect attachments. This generates unattached kinetochores that are detected by the SC (Pinsky et al. 2006). Aurora B has also been proposed to function more directly in the SC. It is required to maintain SC arrest induced by drugs that promote MT disassembly (loss of attachment) or hyperstabilization (loss of tension) and therefore activate the SC (Kallio et al. 2002b; Ditchfield et al. 2003; Hauf et al. 2003). Generally Aurora B is thought to function in the tension sensitive branch of the SC since its chemical inhibition leads to more rapid escape from mitotic arrest induced by taxol (loss of tension) compared to nocodazole block (loss of attachment) (Hauf et al. 2003). Aurora B is also involved in targeting checkpoint components such as Mad2 and BubR1 to kinetochores (Ditchfield et al. 2003). Its kinase activity is needed for BubR1 retention at kinetochores as well as phosphorylation upon entry into mitosis (Ditchfield et al. 2003). Aurora B may therefore regulate SC activity by altering BubR1 activity and/or binding to kinetochores. The kinase is involved in MCC-mediated inhibition of the APC/C (Morrow et al. 2005), suggesting that it directly influences anaphase onset.

2.5.2.4. Cytokinesis

Many studies have shown that Aurora B and other CPC members regulate chromosome segregation and cytokinesis (Terada et al. 1998; Kaitna et al. 2000; Adams et al. 2001b; Giet and Glover 2001). Aurora B controls contractile ring formation, which specifies where the cytokinetic furrow develops, as well as the function of the cytokinetic furrow in multiple ways. It phosphorylates Mklp1 and MgcRacGAP/CYK-4 that are components of the centralspindlin complex, which bundles antiparallel MTs upon anaphase onset to form the spindle midzone (Mishima et al. 2002; Minoshima et al. 2003; Guse et al. 2005). Aurora B also phosphorylates a range of other proteins during cytokinesis including components of the cleavage furrow, like vimentin, myosin II regulatory light chain and desmin (Murata-Hori et al. 2000; Goto et al. 2003; Kawajiri et al. 2003).

2.5.3. Local regulation of CPC activity

At present it is not understood how tension (correct vs. incorrect attachment) regulates Aurora B's activity toward its substrates. It is important for proper mitosis that Aurora B kinase activity is modulated in response to MT attachment and tension, so that correct attachments are stabilized while incorrect attachments are destabilized. Two models described below suggest how MT attachment and tension may modify Aurora B kinase activity. The first model depicts how Aurora B activity toward its substrates may be controlled by different centromere/kinetochore localization of Aurora B, its counteracting phosphatase PP1 (protein phosphatase 1), and the substrate MCAK. The second model proposes how Aurora B activity may be regulated at various subregions of centromeres/kinetochores by priming kinases, and binding to activating or inhibitory components.

Many Aurora B substrates, such as MCAK, localize to the kinetochore, whereas Aurora B localizes to the inner centromere. High-resolution light microscopy studies suggest that their localizations completely or partly overlap at centromeres/ kinetochores that are not under tension, but they move further away as kinetochores are stretched towards opposite poles by MTs (Andrews et al. 2004; Lan et al. 2004). Because PP1 that counterbalances Aurora B phosphorylation (Murnion et al. 2001), resides in the kinetochore outer domain (Trinkle-Mulcahy et al. 2003), it possibly dephosphorylates MCAK that is pulled away from Aurora B. This regulation of MCAK by counteracting kinase and phosphatase that localize to specific regions on the centromere/kinetochore provides a model of how MT attachment and tension could regulate biochemical events at centromeres/kinetochores. Based on this model, tension distorts kinetochore structure at unstable attachment sites, such as merotelic sites, and controls whether MCAK contacts Aurora B at the centromere or PP1 at the outer kinetochore (Gorbsky 2004). However, it does not fully explain how MCAK depolymerase activity is suppressed at centromeres/kinetochores of metaphase chromosomes, since MCAK should colocalize with Aurora B in order to become inhibited. MCAK activity could also be controlled by other factors, such as ICIS (the inner centromere KinI stimulator), an activator of MCAK that has been found in complex with Incenp and Aurora B in *Xenopus* eggs (Ohi et al. 2003).

Based on the second model, the activity of Aurora B is controlled at specific subcellular locations by activating and inhibitory proteins (Rosasco-Nitcher et al. 2008). According to the recent model proposed by Rosasco-Nitcher and co-workers (Rosasco-Nitcher et al. 2008), Aurora B is first recruited to centromeres following phosphorylation by other kinases such as Plk1. At centromeres, Aurora B activity is increased by interactions with activating components, such as TD-60 (telophase disk 60) and MTs that create allosteric changes and promote autophosphorylation. Aurora B activity is suppressed by interactions with substrates that lack priming phosphorylations. The model provides an explanation to how MT attachment and tension could modify signaling at centromeres/kinetochores as it suggests that Aurora B must physically interact with MTs to become activated. The centromeres of unaligned chromosomes frequently encounter MTs that are nucleated from the spindle

poles, and centromeres of merotelically attached chromosomes contact MTs even when they are aligned at the metaphase plate. However, once chromosomes become properly aligned at the metaphase plate, centromeres are no longer in contact with MTs. Therefore at the centromeres of unaligned and merotelically attached chromosomes Aurora B activity would be high, while at kinetochores of metaphase chromosomes it would be low.

Apart from MT attachment and tension, Aurora B activity seems to be controlled in a concentration-dependent manner. Structural analysis of Aurora B in complex with the IN-box segment of Incenp suggests that full activation of Aurora B kinase occurs *in trans* so that Aurora B in one CPC phosphorylates the C-terminal TSS motif of Incenp on the neighboring CPC (Sessa et al. 2005). A recent report proposed that CPC molecules assemble on centromeric chromatin, which increases their local concentration and leads to a positive feedback loop among bound CPCs and full Aurora B activation (Kelly et al. 2007). There is an increase in local Aurora B activity, which promotes spindle assembly. In the cytoplasm the Aurora B kinase pathway is suppressed by phosphatases. In conclusion, the regulation of Aurora B and its substrates occurs at multiple levels involving activating and inhibitory proteins, high local concentration, fine-tuned subcellular localization, and MT-dependent tension.

2.5.4. Aurora B and cancer

Aurora A and B are overexpressed in a variety on tumors and cancer cell lines (Mountzios et al. 2008). There is ample proof linking Aurora A to tumorigenesis but the role of Aurora B in this process is not as well established. Overexpression of Aurora B has been reported to cause chromosome instability (Ota et al. 2002) and to promote Ras-mediated cell transformation (Kanda et al. 2005). It may therefore contribute to tumorigenesis. Overexpression of Incenp and Survivin has also been detected in cancer cells (Ambrosini et al. 1997; Adams et al. 2001a) and some of the splice variants of Survivin may play a role in cancer progression (Li 2005). Furthermore, Survivin expression in cancer cells has been linked to resistance to apoptosis, correlation with poor diagnosis, and resistance to therapy (Fukuda and Pelus 2006).

RNAi or other approaches to suppress Aurora B function lead to mitotic failure that is characterized by defects in chromosome congression and segregation, and failure of cytokinesis (Mackay et al. 1998; Adams et al. 2001b; Kallio et al. 2002b; Honda et al. 2003). Notably, targeting any member of the CPC leads to the same phenotype due to the inability of the complex to localize and function correctly (Honda et al. 2003). Failure to undergo cytokinesis leads to the generation of tetra- or polyploid cells, which have reduced viability. Tetraploid cells in general arrest in G1 and undergo apoptosis, or continue as viable 4N cells, which may later die from the generation of massive aneuploidy due to random segregation between two or more cells in the following cell cycle (Rieder and Maiato 2004). Although this could limit the replication of tumor

cells, there is some concern regarding the long-term implications of inhibiting Aurora B in normal cells.

Many inhibitors of Aurora kinases have been described in recent years and most of them have entered phase I or II clinical trials (Mountzios et al. 2008; Taylor and Peters 2008). Targeting Aurora kinases greatly inhibits tumor cell growth in cell culture and xenograft studies. The first Aurora kinase inhibitors described, ZM447439 and Hesperadin, are potent inhibitors of Aurora B, while VX-680 and ZM3 are dual Aurora A/B inhibitors, and MLN8054 inhibits Aurora A. ZM447439 and Hesperadin cause a failure to correct non-bipolar attachments and SC defects, leading to tetra- and polyploidy phenocopying the effects of Aurora B RNAi (Hauf et al. 2003; Gadea and Ruderman 2005).

New generation drug development has to a large extent focused on the discovery of compounds that inhibit the activity of kinases. Although some kinase inhibitors, such as Imatinib against chronic myeloid leukemia, CML, have proven to be successful therapies, the development of drug resistance as a result of mutations in the ATP-binding site of kinases is a potential clinical problem (Sawyers 2003; Weisberg et al. 2007). Inhibition of Aurora kinases by ZM447439 was recently reported to cause mutations in the Aurora B gene of a hypermutagenic cancer cell line making these cells resistant to several Aurora kinase inhibitors (Girdler et al. 2008). These mutations have important implications for future drug development, which should aim to target the mutated Aurora B kinase. One optional strategy would be to target Aurora B activity outside of the kinase domain. This could in principle be accomplished using small molecules or peptides that allosterically inhibit the function of the enzyme for example through blocking the interactions of different CPC subunits.

Survivin has been proposed to serve as a potential anti-cancer drug target for the development of global pathway inhibitors because the molecule integrates cellular networks that are essential for tumor cell proliferation and viability (Altieri 2008). Due to its versatile roles in mitosis and apoptosis, inhibiting Survivin might provide an opportunity to target a larger subset of cancers compared to single molecule inhibitors. So far, antisense therapies directed against Survivin have not caused toxicity in animal models and the results are encouraging (Fukuda and Pelus 2006).

3. AIMS OF THE STUDY

SC signaling is regulated by a mechano-chemical system that reacts to physical tension exerted onto sister kinetochore pairs by pulling forces of MTs. SC is turned off when kinetochores attain sufficient MT attachment and interkinetochore tension, which occur after bipolar alignment of chromosomes at the metaphase plate. However, it is not clear how changes in physical tension at kinetochores are converted into biochemical signals that control mitotic progression. The kinetochores of mitotic chromosomes that are not under tension are phosphorylated at an epitope recognized by the 3F3/2 antibody. The expression of this 3F3/2 phosphoepitope appears to overlap with SC activity in space and time; 3F3/2 is expressed at kinetochores of unaligned chromosomes that produce the "wait anaphase" signals, and its expression is shut off upon chromosome bi-orientation and generation of interkinetochore tension. The precise role of this phosphoepitope and kinases that create it are unknown. Knowledge of proteins involved in the pathway controlling 3F3/2 expression would increase our understanding on the mechano-chemical system that regulates SC signaling.

Proteins that are involved in the SC response are typically molecules that exchange, often rapidly, at kinetochores. This dynamic process is thought to play a critical role in the fast distribution of inhibitory signals that delay anaphase onset throughout mitotic cells in response to chromosome alignment errors. The CPC has been implicated to function in the SC pathway: it is required to maintain SC arrest and to target specific checkpoint proteins to kinetochores. The details of how the CPC carries out its various functions at different mitotic phases are limited. Moreover, information regarding the dynamics of CPC proteins at centromeres and how their turnover may contribute to SC signaling is limited. Understanding the dynamic biochemistry of CPC molecules is expected to reveal important data on the spatio-temporal regulation of SC signaling.

The specific aims of this thesis were:

1. To identify kinase(s) involved in the regulation of the tension sensing 3F3/2 phosphoepitope at kinetochores.

2. To identify novel mitotic functions of the CPC.

3. To analyze the dynamic properties of CPC proteins Aurora B, Incenp, and Survivin.

4. MATERIALS AND METHODS

4.1. Cell culture and reagents

HeLa, LLC-PK (porcine kidney epithelial cell line), CFPAC (human cystic fibrosis pancreatic adenocarcinoma cell line), PANC (human pancreatic carcinoma epithelial-like cell line) and PtK1 cells (rat kangaroo kidney cell line) were grown at 37°C in a humidified incubator with 5% CO_2. Cells were grown in DMEM (PtK1 cells in MEM) supplemented with 10% fetal bovine serum, 20 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mg/ml penicillin/streptomycin. Xeno S3 cells (*Xenopus* tissue culture cell line) were grown at RT in Leibovitz's L-15 medium supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin and 15% H_2O. Nocodazole, taxol (Paclitaxel; Molecular Probes), ZM447439 (a generous gift from AstraZeneca), and MG132 were used in the experiments at 0.06-10 μM, 0.6-5 μM, 2-25 μM, and 20 μM concentrations. All reagents were from Sigma unless stated otherwise. Cell culture media and supplements were from Gibco. Secondary antibodies were purchased from Jackson Immunoresearch Laboratories and Amersham Pharmacia.

4.2. Phosphorylation of detergent-lysed PtK1 cells

Kinetochore protein signaling can be analyzed and manipulated in a "lysed cell assay", in which the biochemical system of living cells is reconstituted *in vitro* in lysed cells (Campbell et al. 2000; Daum and Gorbsky 2006). This assay is particularly beneficial for investigating changes in the phosphorylation status of kinetochore-associated proteins. Kinetochore phosphoepitopes that are recognized by the 3F3/2 antibody are extremely labile and very sensitive to rapid dephosphorylation upon cell lysis. However, they can be preserved by adding the phosphatase inhibitor Microcystin-LR (Alexis Biochemicals) to the lysis buffer. Endogenous kinases that create the 3F3/2 phosphoepitope remain bound to kinetochores and active, so that when lysed and dephosphorylated cells are supplied with ATP and Microcystin-LR, the kinases recreate the 3F3/2 phosphoepitope. In order to test the activities of exogenously added kinases, the endogenous kinases can be inactivated by N-ethyl-maleimide, followed by washes and incubation in the presence of the exogenous enzyme, ATP, and Microcystin-LR.

For the lysed cell assay, PtK1 cells growing on coverslips were rinsed briefly in 50 mM Tris (pH 7.5) and 5 mM $MgSO_4$ (TM buffer) and extracted for 5 min in TM buffer containing 1% CHAPS, 1 mM DTT, and 5 μg/ml protease inhibitors (pepstatin A, leupeptin, pefabloc SC). This was followed by dephosphorylation for an additional 5 min in TM buffer containing DTT and protease inhibitors. Next, the cells on coverslips were incubated for 10 min with 5 mM N-ethyl-maleimide in TM buffer. After rinsing, cells were incubated with mitotic *Xenopus* egg extract or recombinant kinase in TM buffer containing 1 mM ATP, 1 mM DTT, protease inhibitors, and 400 nM Microcystin-LR for 20-40 min. Cells were fixed and stained for immunofluorescent analysis.

4.3. Xenopus extracts and recombinant proteins

Xenopus extracts for the phosphorylation of detergent-lysed PtK1 cells were prepared as described previously (Murray 1991; Stukenberg et al. 1997). Interphase extracts were driven into mitosis by the addition of nondegradable glutathione S-transferase (GST)-cyclin B. Extracts were immunodepleted with preimmune serum, anti-Plk1 serum, or polyclonal anti-Aurora B antibody. Plk1 and Aurora B were depleted from mitotic extract by three consecutive incubations with protein A Sepharose beads (Amersham Biosciences) coupled to antibody. The antibody-conjugated beads were subsequently washed in XB (10 mM HEPES, pH 7.7, 1 mM $MgCl_2$, 100 mM KCl, and 50 mM sucrose) containing 200 mM NaCl and 0.1% Brij 35. Preimmune, Plk1, and Aurora B -depleted extracts were analyzed by SDS-PAGE and Western blotting to determine the efficiency of depletion, and histone H1 activity was determined as previously described (Murray 1991).

Recombinant baculovirus-expressed 6His-tagged Plk1, Aurora A, and Cdk1 were purified from SF9 cells on Ni^{2+}-NTA agarose (Qiagen). Their kinase activity was determined with myelin basic protein (MBP; Invitrogen) as a substrate. For the kinase assays, recombinant protein was mixed with γ[^{32}P]ATP (Perkin Elmer Life Sciences) and MBP, and reaction was stopped by sample buffer. The reaction mixture was loaded onto an SDS-PAGE gel, which was Coomassie blue stained, dried on 3MM paper (Whatman), and analyzed for $^{32}PO_4$ incorporation with phosphoimager hardware (Molecular Dynamics) and ImageQuant 5.0 software. Purified bacterially expressed recombinant human GST-Plk1 kinase was purchased from Cyclex through the MBL International Corporation.

4.4. 3F3/2 phosphopeptide library screening

A phosphothreonine-oriented degenerate peptide library consisting of the sequence Met-Ala-X-X-X-X-pThr-X-X-X-X-Ala-Lys-Lys-Lys, in which X denotes all amino acids except Cys, Ser, and Thr, was synthesized with N-α-FMOC-protected amino acids and standard BOP/HOBt coupling chemistry. Peptide library screening was performed with protein G beads complexed to a saturating amount of the 3F3/2 antibody. 3F3/2 beads were loaded into a column and incubated with the peptide library mixture in TBS (50 mM Tris-HCl [pH 8.0] and 150 mM NaCl). Unbound peptides were removed from the column by washes with TBS containing 1% NP-40 and then with PBS (150 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mm KH_2PO_4 [pH 7.6]). Bound peptides were eluted with 30% acetic acid, lyophilized, resuspended in H_2O, and sequenced by automated Edman degradation on a Procise protein microsequencer. Selectivity values for each amino acid were determined by comparing the relative abundance (mole percentage) of each amino acid at a particular sequencing cycle in the recovered peptides to that of each amino acid in the original peptide library mixture at the same position.

4.5. Plasmids, siRNAs and transfections

Murine pShuttle-CMV-HA-EGFP-Plk1 plasmid was a kind gift from Dr. Kyung Lee (National Cancer Institute, Bethesda, USA). GFP-Survivin was created by amplification of the Survivin coding sequence from cDNA from HeLa cells and cloning of the PCR product into pEGFP-C1 (Clontech Laboratories). GFP-Survivin was transfected into HeLa or LLC-PK cells using Fugene 6 Reagent (Roche Diagnostics). GFP-Incenp plasmid was a kind gift from Erich Nigg (Max-Planck-Institute of Biochemistry, Martinsried, Germany) and was described previously (Klein et al. 2006). *Xenopus* GFP-Incenp (GFP-xIncenp) plasmid was constructed from pCS2-YFP-xIncenp by subcloning into pEGFP-C1 (BD Biosciences). pCS2-YFP-xIncenp plasmid was generated from Pet28B-xINCENP (Bolton et al. 2002) by subcloning into PCS2+YFP, which was a kind gift from David Wotton (University of Virginia, Charlottesville, USA) (Kagey et al. 2003). For construction of *Xenopus* Aurora B-YFP (xAurora B-YFP) the xAurora B (xAIRK2) coding sequence was amplified from cDNA from Xeno S3 cells and cloned into pEYFP-N1 (BD Biosciences). Aurora B-GFP was constructed by amplification of the Aurora B coding sequence from cDNA from HeLa cells and cloning of the PCR product into pEGFP-N1 (BD Biosciences). HeLa or LLC-PK cells were transfected with GFP-Plk1, GFP-Incenp or Aurora B-GFP using Effectene (Qiagen), and Xeno S3 cells were electroporated with GFP-xIncenp or xAurora B-YFP with the ECM 830 electroporator (BTX). Plk1 was silenced with siRNA targeting the sequence: 5′-GATCACCCTCCTTAAATAT-3′ (Plk1 Duplex IV, Dharmacon) (Spankuch-Schmitt et al. 2002), and CenpF using target sequence 5'-ACAACCTCCTTAAGAGTCA-3' (Dharmacon). SiRNA was transfected into HeLa cells with Oligofectamine (Invitrogen).

4.6. Mitotic-exit assay

Mitotic exit assay was used to determine if Plk1 siRNA-treated cells arrest in mitosis due to SC activation. This arrest is overcome by treatment with the Aurora B kinase inhibitor ZM447439, unless proteasome is inhibited by MG132. HeLa cells were arrested at mitosis by Plk1 siRNA or nocodazole, and collected by wash-off. These cells were subsequently incubated in 24-well tissue-culture plates with either media alone, in taxol, or in nocodazole in the presence or absence of the proteasome inhibitor MG132. ZM447439 was added to some of the wells and all cultures were incubated 4 h to allow cells to exit mitosis and to adhere to the plate surface. The wells were rinsed, and the adherent cells were fixed in 2% formaldehyde containing 1% Triton X-100 in PHEM (60 mM PIPES, 25 mM HEPES, pH 6.9, 10 mM EGTA, 4 mM $MgSO_4$) containing a 1:10 000 dilution of SyberGold (Molecular Probes). The relative number of interphase cells (the measurement of mitotic exit) was determined with a plate reader (Tecan GENios) that measured the fluorescence of SyberGold bound to nucleic acid in the fixed adherent cells.

The assay was also used to analyze whether Incenp^{-ab} causes SC inactivation and mitotic exit. Xeno S3 cells were treated with nocodazole, taxol or MG132, and subsequently injected with Incenp^{-ab}. Cells were fixed 2 h later in 2% formaldehyde

containing 1% Triton X-100 in PHEM, and stained with secondary antibody to detect injected cells and with DAPI to categorize cells into mitotic or interphase cells based on nuclear morphlogy.

4.7. Chromosome isolation

HeLa cells were transfected with Plk1 siRNA as described above. 18 h after transfection, mitotic cells were removed and discarded from the culture by shake-off and pipetting. Cells arrested in mitosis by Plk1 siRNA, or by nocodazole in the nontransfected control, were then permitted to accumulate for 3-4 h. These freshly arrested mitotic cells were harvested, and their chromosomes were isolated as described below. Some Plk1 siRNA-arrested cells were exposed to nocodazole for the final 45 min. Harvested mitotic cells were washed in 10 mM HEPES (pH 7.4), 40 mM KCl, 5 mM EGTA, 4 mM MgSO4, and 400 nM Microcystin-LR by centrifugation. Mitotic cells were lysed in PHEM containing 0.5% Triton X-100, 1 mM DTT, 400 nM Microcystin-LR, and protease inhibitors. The extracts were centrifuged through lysis buffer containing 10% glycerol over poly-L-lysine-treated coverslips at 4°C in order to collect chromosomes for immunofluorescence labeling. The chromosome-coated coverslips were then fixed and processed for immunofluorescence analysis.

4.8. Antibody injection

The Incenp^{-ab} that was used for micro-injections was characterized previously (Bolton et al. 2002). For injections and live cell analysis Xeno S3 cells were grown on 35 mm chambers with glass bottom (MatTek, Corp.), and for fixed cell analysis cells were grown on coverslips. Antibody microinjections were performed at a needle concentration of ~5 mg/ml (microinjection buffer: 0.1 M KCl, 1.7 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with the Narishige micromanipulator and manual micro syringe pump system using needles pulled with Narishige PN-30 (World Precision Instruments, Inc.). In fixed cell analysis injected cells were identified by including FITC-dextran (Molecular Probes) into the antibody solution or by incubating coverslips in FITC-conjugated secondary antibody.

4.9. Image capture and analysis

Images (excluding FRAP; see 4.10) were captured with a Zeiss Axioplan II microscope equipped with 63X (N.A. 1.4) and 100X (N.A. 1.4) objectives, Hamamatsu Orca 2 camera (Hamamatsu Photonics), and Metamorph imaging software (Universal Imaging Corp). Confocal images for Incenp and Aurora B FRAP were captured using Zeiss LSM510 META confocal microscope with 63X (N.A. 1.4) objective using LSM5 3.2 software with Physiology option (Carl Zeiss Corporation). Images for Survivin FRAP were captured with Zeiss Axiovert 200M microscope equipped with 63x (N.A. 1.4) and 100x objectives (N.A. 1.4), Hamamatsu Orca ER CCD camera, and Metamorph software.

Immunofluorescent kinetochore protein analysis was performed by collecting Z-section image stacks from all fluorophores. DAPI staining was used to identify prometaphase cells or chromosomes, and Crest labeling was used to identify kinetochore regions. Regions were drawn around crest signals and transferred to corresponding Z-section image stacks of other fluorophores, and integrated fluorescent intensities minus the background were calculated.

4.10. Fluorescence recovery after photo-bleaching (FRAP)

HeLa and LLC-PK cells transfected with Survivin-GFP, GFP-Incenp or Aurora B-GFP plasmid or Xeno S3 cells electroporated with GFP-xIncenp or xAurora B-YFP plasmid were used for FRAP experiments. Experiments with HeLa and LLC-PK cells were performed at 37°C and 5% CO_2 in a humidified chamber, and experiments with Xeno S3 cells at RT without CO_2 controller. In FRAP, a small region of interest (ROI), typically one or a few centromeres, was targeted with intense light at 488 nm wavelength to photo-bleach GFP or YFP. The region was then followed by taking images at certain intervals using low level light to measure how fast, and to what extent recovery in this area occurs.

For Incenp and Aurora B FRAP, three images were captured with the confocal microscope prior to photo-bleaching, after which a small ROI was bleached with laser irradiation. Fluorescence recovery in this area was followed by scanning the sample with 488 nm low intensity laser irradiation at regular intervals. The duration of filming was typically 5 to 10 min and scans were taken every 5 to 20 s. Acquired data was corrected for background and fitted to f(t)=A(1-exp(kt)) using FRAPCalc® (Rolf Sara, Turku Centre for Biotechnology) to calculate half-time of recovery ($t_{1/2}$) and total recovery of fluorescence (recf). Graphs representing FRAP curves were plotted with Prism 3.0 software (GraphPad Software, Inc.).

Survivin-GFP photo-bleaching experiments were performed using a Micropoint Laser System (Phototonic Instruments) and the Axiovert microscope. The laser was aligned to the target region using phase-contrast optics and a set of pre-bleach images was captured. A short exposure of laser irradiation (5-10 pulses each 4 nanos) was used to reduce the fluorescence intensity of the target. Time-lapse sequences were captured using 30 to 500 millis exposure times. Recovery curves were calculated and drawn similarly to Incenp and Aurora B FRAP.

4.11. Cell fixation and immunofluorescence labeling

Cells growing on coverslips were fixed and extracted for 15 min in 60 mM Pipes, 25 mM HEPES, pH 6.9, 10 mM EGTA, 4 mM $MgSO_4$ (PHEM) containing 2% paraformaldehyde, 0.2% glutaraldehyde (optional) and 0.5-1% Triton X-100 or CHAPS. The coverslips were rinsed in 10 mM MOPS, pH 7.4, 150 mM NaCl, 0.05% Tween-20 (MBST) and blocked for one h in 20% boiled normal goat serum (BNGS) in MBST. Primary antibodies were diluted into MBST containing 5% BNGS and

coverslips were incubated 1 h at RT. Cells were stained with antibodies against Plk1, Cdc20, CenpE, Mad2, Spc24, Bub1, BubR1, AIM1, Hec1, survivin, Cdc20, Aurora B, pMCAK, tubulin, Crest, or 3F3/2. Fluorescent secondary antibodies were diluted into MBST/ 5% BNGS, and incubations were carried out 1 h at RT. DNA was counter stained with DAPI before mounting in Vectashield (Vector Laboratories).

4.12. Cell extraction, immunoprecipitation and Western blotting

Mitotic HeLa or LLC-PK cells were harvested by shake-off and centrifugation followed by a wash in PBS (pH 7.2), flash freezing in liquid nitrogen, and storage at −70°C. For cell extracts, cell pellets were thawed in 20 mm Tris (pH 7.7), 100 mM KCl, 50 mM sucrose, 0.1 mM CaCl2, 1 mM MgCl2, and 0.5% Triton X-100 (APC buffer) containing protease inhibitor cocktail (Amresco) and 400 nM Microcystin-LR, vortexed and centrifuged. For some experiments, 0.5% SDS (from 10% stock) was added to break down protein complexes, extract was incubated 2 min at RT and vortexed, and diluted 1:10 into APC buffer for immunoprecipitation (IP). Proteins of the cleared extract were used for IP with the 3F3/2 antibody and separated by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were subsequently blocked in 5% milk/ TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween), incubated with primary antibodies, and HRP-conjugated secondary antibodies (diluted in TBST), and detected using chemiluminescent Western blotting kit from Amersham Pharmacia. For IP, the 3F3/2 antibody was conjugated to protein A beads, the beads were washed 3 times in APC buffer and used for IP at 4°C for 2 h or overnight. Beads were subsequently washed 3 times in APC buffer containing protease inhibitor coctail and Microcystin-LR, buffer was removed, and SDS-PAGE loading buffer was added.

5. RESULTS

5.1. Plk1 regulates 3F3/2 phosphoepitope expression during mitosis (I)

We set out to determine which kinase functions in the tension sensing pathway through the regulation of 3F3/2 phosphoepitope expression. Using the lysed cell assay and mitotic extracts that were depleted of Plk1 or Aurora B, two key mitotic kinases, we studied if these kinases are responsible for generating the 3F3/2 phosphoepitope. To this end, PtK1 cells attached to coverslips were permeabilized, dephosphorylated, and treated with N-ethylmaleimide to inactivate endogenous kinetochore bound kinase(s) that create the 3F3/2 phosphoepitope. These cells were then rephosphorylated using mitotic extracts (preimmune control extract or extracts depleted of specific mitotic proteins), or purified recombinant kinases in the presence of Microcystin-LR. Generation of the 3F3/2 phosphoepitope was studied using immunofluorescence labeling with the 3F3/2 antibody.

We demonstrated that the 3F3/2 phosphoepitope was generated on PtK1 kinetochores by mitotic extract from *Xenopus* eggs (I, Fig. 1C), showing that the extract contains kinase activity needed for this phosphorylation. Detection of 3F3/2 labeling in lysed cells after incubation with Plk1 or Aurora B-depleted mitotic extracts showed that the phosphoepitope could be generated in the absence of Aurora B but not when Plk1 was depleted (I, Fig. 1C). The supplementation of Plk1-depleted extract with recombinant Plk1 rescued this activity (I, Fig. 1C). Furthermore, recombinant Plk1 by itself could create the 3F3/2 phosphoepitope (I, Fig. 1E), demonstrating that Plk1 was the sole protein in the extract that was responsible for generating the phosphoepitope. In contrast to this, purified Aurora A (similar kinase activity to Aurora B, which we did not succeed to synthesize) or Cdk1 that were used as controls, could not generate the phosphoepitope (I, Fig. 1E). Our results implied that Plk1 is involved in the SC response through generation of the tension sensing phosphoepitope recognized by the 3F3/2 antibody. This finding also served as a proof-of-concept of the lysed cell assay in the study of mitotic signaling.

We next performed a peptide library screen in order to determine the consensus phosphorylation motif recognized by the 3F3/2 monoclonal antibody. To accomplish this, bead-immobilized 3F3/2 antibody was incubated with a phosphothreonine oriented peptide library and the bound phosphopeptides were sequenced to analyze the relative amount of amino acids in each position of the peptide. The 3F3/2 binding motif showed strong selection for Phe and Tyr in the pThr-4 position, Leu and Met in the pThr-3 position, Glu and Asp in the pThr-2 position, and Asn in the pThr+1 position (I, Fig. 2A). On the basis of this data, the optimal 3F3/2 binding motif is (F/Y)-(L/M)-(D/E)-(I/L/M)-(pThr/pSer)-N (I, Fig. 2B). The 3F3/2 motif and the epitope created by Plk1 based on mapped phosphorylation sites from known *in vivo* substrates containing (hydrophobic aa such as L/M)-(D/E)-X-(S/T)-(aliphatic)-(D/E) showed partial but significant overlap (I, Fig. 2 B and C), which suggested that Plk1 could create a phosphoepitope similar to the 3F3/2 on a number of its substrates.

When staining various cell types with an antibody against Plk1, we noticed that the kinase accumulated at kinetochores to a variable extent. More Plk1 was present at kinetochores of unaligned chromosomes, and after treatment with nocodazole or taxol to hyperactivate the SC (I, Fig. 3). This result was confirmed by expressing a GFP-fusion of the enzyme (I, Fig. 3). Using GFP-Plk1 we could also exclude the possible epitope masking by MTs in the immunofluorescence assays. The localization pattern suggested that Plk1 accumulated at kinetochores of unaligned chromosomes that emit SC signals during normal mitotic progression and in cells treated with MT drugs. Interestingly, Plk1 accumulated at kinetochores in the absence of tension (taxol- treated cells; I, Fig. 3), which is similar to the behavior of the 3F3/2 phosphoepitope. Plk1 was found to colocalize with the 3F3/2 phosphoepitope indicating that these proteins could interact at kinetochores (I, Fig. 4).

To get further insighs into Plk1 biology, we used Plk1 siRNA to knockdown protein expression. We analyzed SC activity, 3F3/2 phosphoepitope expression, and the kinetochore association of various mitotic proteins in Plk1 silenced cells. As described earlier (Spankuch-Schmitt et al. 2002; Sumara et al. 2004), Plk1 knockdown resulted in monopolar spindles (I, Fig. 5B) and induction of apoptosis (data not shown). Plk1 silenced cells exhibited mitotic arrest that could be overcome using Aurora B inhibitor ZM447439 (I, Fig. 5C). Since Aurora B maintains SC activity, this observation indicated that Plk1 silenced cells were arrested due to an active SC. Plk1 siRNA treatment resulted in the diminished expression of the 3F3/2 phosphoepitope *in vivo*, and restricted the ability of kinetochores to create the phosphoepitope *in vitro* on isolated chromosomes (I, Fig. 6), consistent with our results from the lysed cell experiment. Furthermore, after Plk1 siRNA treatment, the kinetochore association of proteins Mad2, Cdc20, CenpE, and Ndc80/Hec1 was reduced, whereas Bub1 and BuBR1 remained at normal levels (I, Fig. 7A). The finding that some of the analyzed proteins remained at kinetochores at normal levels indicated that kinetochore structure was intact in Plk1 depleted cells. Despite of the lowered level of Mad2 at kinetochores, the checkpoint was apparently functional. This could indicate that BubR1 binding was sufficient to maintain SC activity, or the reduced but not totally abolished Mad2 level was sufficient to maintain SC-mediated arrest. It should be noted that the diminished expression of the 3F3/2 phosphoepitope did not abrogate the checkpoint. In summary, Plk1 was needed for the expression of the 3F3/2 phosphoepitope and for the normal kinetochore-association of the SC proteins Mad2 and Cdc20, the motor protein CenpE, and the outer kinetochore component Ndc80/Hec1, proposing that Plk1 contributes to SC signaling and the establishment of kMT attachments.

5.2. Plk1 targets an unknown kinetochore protein (unpublished results)

Within this project we also searched for the kinetochore protein that contains the 3F3/2 phosphoepitope (unpublished results). Previous work conducted in the Gorbsky laboratory had identified two proteins that contain the 3F3/2 phosphoepitope, the APC/C (Daum et al. 2000) and topoisomerase IIα (TopoIIα) (Daum and Gorbsky 1998). On the basis of its co-localization with the 3F3/2 phosphoepitope at the middle

to outer kinetochore, the APC/C could be the kinetochore protein that harbors the mitotic 3F3/2 phosphoepitope. TopoIIα is a DNA decatenating protein that untangles DNA strands (Watt and Hickson 1994) and localizes to the inner centromere (Null et al. 2002). It is therefore at the right place to function as a tension sensor and has characteristics that fit this proposed role. TopoIIα has also been suggested to play a role in the timely separation of sister chromatids at anaphase onset (Porter and Farr 2004). However, even though the 3F3/2 antibody was reported to bind to TopoIIα (Daum and Gorbsky 1998), its localization at the inner centromere does not correspond to the localization of the 3F3/2 phosphoepitope at kinetochores making it an unlikely target. Moreover, casein kinase II was shown to create the 3F3/2 epitope on TopoIIα (Daum and Gorbsky 1998). The third candidate protein was Plk1 itself, since it has autophosphorylation capacity (Kelm et al. 2002), it co-localized with the 3F3/2 phosphoepitope (I, Fig. 4), and was pulled down in 3F3/2 immunoprecipitation (IP) (Fig. 9A).

To assess whether the APC/C is the 3F3/2 phosphoepitope containing kinetochore protein targeted by Plk1, we silenced Plk1 by siRNA, collected mitotic cells, and prepared extracts that were used for IP by bead-immobilized 3F3/2 antibody. Sodium dodecyl sulfate (SDS) was added to some extracts to break down protein complexes and to insure that proteins bound to the 3F3/2 antibody directly, and were not co-precipitated with some other protein. We then analyzed the abundance of Cdc27, an APC/C subunit that contains a 3F3/2 phosphoepitope, in the IPs using Western blotting, and compared this to the control sample with normal Plk1 level. The results indicated that the 3F3/2 phosphosite on Cdc27 was not affected by Plk1 knockdown, but the protein was less phosphorylated as indicated by a mobility shift (Fig. 9B), suggesting that Plk1 phosphorylates Cdc27 on some other residue(s). Identical experiments carried out with TopoIIα ruled out the protein as a candidate (data not shown). To assess whether Plk1 itself contains the 3F3/2 phosphoepitope, recombinant Plk1 was incubated with ATP, and subjected to 3F3/2 IP. Plk1 did not bind to the antibody, suggesting that the kinase did not create the phosphoepitope on itself, at least not under these experimental conditions (personal communication with J.R. Daum). Furthermore, addition of SDS to the cell extract abolished Plk1 binding to 3F3/2 IP, indicating that Plk1 co-precipitated with another protein instead of direct binding (Fig. 9B). It should be noted that the 3F3/2 antibody does not perform well in Western blotting; therefore reciprocal IP and blotting assays could not be performed.

In addition to these proteins, we attempted to characterize new 3F3/2 phosphoepitope proteins in 3F3/2 IPs by Western blotting and mass spectrometry, and using siRNA against various kinetochore proteins followed by immunofluorescent labeling with the 3F3/2 antibody. We also analyzed the phosphorylation status of some kinetochore proteins after silencing Plk1 with siRNA. The results indicated that CenpF is phosphorylated by Plk1 as its electrophoretic mobility shifted as a result of Plk1 knockdown (Fig. 9C). Therefore, Plk1 directly or indirectly phosphorylates CenpF, possibly at a 3F3/2 phosphoepitope-like sequence. Interestingly, it was reported by co-workers that Plk1 siRNA diminishes CenpF kinetochore binding by about 80%

(Pouwels et al. 2007). In silver stained gels of 3F3/2 IPs, a ~350 kDa protein corresponding to the size of CenpF was less phosphorylated after Plk1 siRNA treatment compared to control (Fig. 9D). However, we could not verify by mass spectrometric methods that the corresponding band is CenpF (data not shown). Furthermore, siRNA against CenpF did not remove the 3F3/2 epitope from kinetochores (Fig. 9E) arguing against the possibility that CenpF would contain the 3F3/2 phosphoepitope. Other proteins found in 3F3/2 IPs included Incenp, Aurora B, and BubR1, but they bound to the antibody regardless of whether they were phosphorylated or not (data not shown). In addition to Cdc27, Plk1 exhibited phospho-dependent binding to the 3F3/2 antibody (Fig. 9A). As described above, this binding was abolished by SDS treatment (Fig. 9B), indicating that Plk1 did not bind directly to the 3F3/2 antibody.

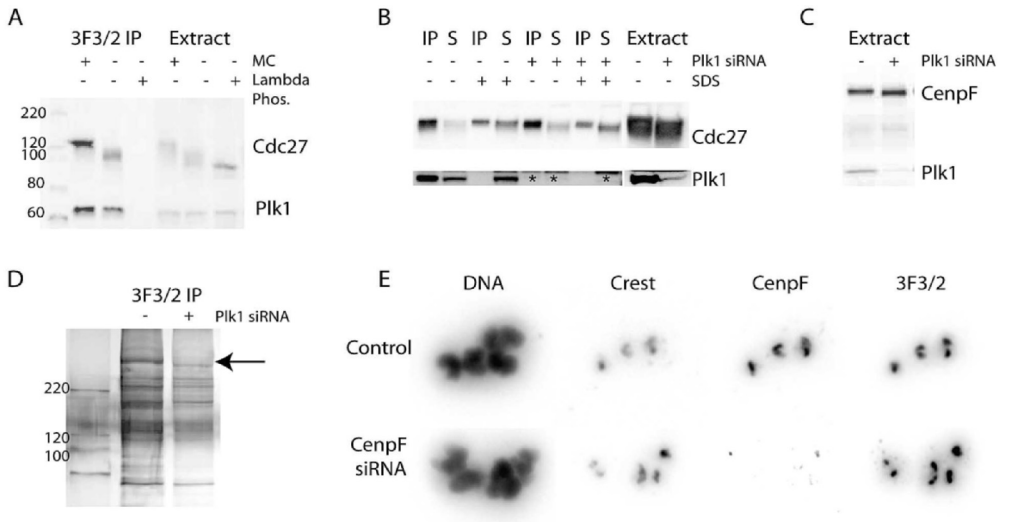

Figure 9. Analysis of potential 3F3/2 phosphoepitope proteins. (A) Cdc27 and Plk1 bound to 3F3/2 immunoprecipitation (IP) in the presence of the phosphatase inhibitor Microcystin-LR (MC), and binding was abolished by lambda phosphatase treatment. (B) The amount of Cdc27 in 3F3/2 IP was not affected by Plk1 siRNA. Cdc27 underwent a mobility shift indicating that Plk1 phosphorylates the protein, but not at the 3F3/2 phosphoepitope. Plk1 binding to 3F3/2 IP was abolished by SDS treatment and all Plk1 was present in the supernatant (S; extract after IP). Note that Cdc27 retained binding after SDS treatment indicating direct binding to 3F3/2 antibody. Asterisk (*) indicates residual amounts of Plk1. (C) CenpF underwent a mobility shift in Plk1 siRNA-treated extract indicating that it is phosphorylated by Plk1. (D) Silver stained gel of 3F3/2 IPs demonstrating a mobility shift in a high molecular weight (~350 kDa) protein after Plk1 siRNA treatment (marked with arrow). (E) Images of fluorescently labeled chromosomes from control and CenpF-silenced cells showing the presence of the 3F3/2 phosphoepitope on chromosomes with low CenpF level. Panels A-C represent Western blots. All cells were incubated with nocodazole to accumulate mitotic cells before they were harvested and used for extracts or chromosome isolation.

Recently Wong and Fang (Wong and Fang 2007) reported that in *Xenopus* extract BubR1 is targeted by Plk1 at the 3F3/2 phosphoepitope (see discussion). Results from

the Gorbsky laboratory showed that siRNA against BubR1 did not reduce the kinetochore 3F3/2 phosphoepitope in human cells (personal communication with G.J. Gorbsky), suggesting that the findings of Wong and Fang are species-specific.

5.3. Survivin localizes to distinct mitotic structures (II)

Based on previous reports (Li et al. 1998; Skoufias et al. 2000; Uren et al. 2000; Wheatley et al. 2001; Fortugno et al. 2002), the localization of Survivin was controversial. To investigate the exact localization of Survivin in HeLa and LLC-PK cells, we used anti-Survivin antibody and transfection of GFP-Survivin plasmid, and analyzed the distribution of endogenous and exogenous Survivin in different phases of the cell cycle. Survivin accumulated near centromeres in early G2 before Aurora B (II, Fig. 1A and B). In prophase, Survivin localized to the cytosol and nucleus, and accumulated at spindle MTs and centromeres with some label at chromosome arms (II, Fig. 1A). More detailed analyses of mitotic LLC-PK cells showed that centromere accumulations of Survivin underwent notable stretching upon bipolar MT attachment (II, Fig. 1C). Survivin accumulations were bipartite, with a gap between the two centromeres when they were under tension (II, Fig. 1C). Survivin translocated to the spindle midzone and colocalized with MTs after anaphase onset (II, Fig. 2). Our results support the notion that many pools of Survivin exist in cells, and that chromosome-associated Survivin undergoes dynamic changes in its distribution during mitosis.

5.4. CPC proteins bind to centromeres in a dynamic manner (II, III)

To shed light on CPC regulation at kinetochores we determined if Aurora B, Incenp and Survivin are dynamic components of inner centromeres. We studied by Fluorescence recovery after photo-bleaching (FRAP) the mobility of GFP-Survivin, GFP-Incenp, and Aurora B-GFP or YFP in various cell types at different mitotic organelles and in different drug treatment conditions. Cells were transiently transfected with plasmids encoding individual fusion proteins, and used for photo-bleaching assays in living cells after their localization to inner centromeres was confirmed.

These studies showed that all three fusion proteins exhibited dynamic association with inner centromeres. The turnover of Survivin appeared to be fast with a half-time of recovery ($t_{1/2}$) typically 4 to 8 s at centromeres in early mitosis (prophase to metaphase) in both HeLa and LLC-PK cells (Table 1). Incenp and Aurora B exhibited mobility at centromeres at a moderate rate, turning over with a $t_{1/2}$ of ~40-110 s depending on the cell type (Table 1). Turnover of Survivin was rapid at early mitosis, whereas recovery was either slow or completely absent before mitosis (II, Table 1), or at the end of mitosis (Table 1, telophase). Similarly to Survivin, Incenp (Table 1) and Aurora B (data not shown) were immobile in telophase. Interestingly, we observed rapid recovery of GFP-Incenp to MTs of the spindle midzone in early anaphase cells ($t_{1/2}$ = 18 s; Table 1). This recovery slowed down at about the time of cleavage furrow contraction (data not shown). These observations are based on LLC-PK cells expressing GFP-Incenp at high levels, and the findings could not be verified using

other cell lines that expressed the fusion protein weakly. Inability to do so could be due to technical limitations. Anaphase measurements with Aurora B showed similar results (Table 1). Survivin did not exhibit mobility after anaphase onset (Table 1), but this could be due to the fact that measurements were carried out by bleaching GFP-Survivin at the cleavage furrow. Also Incenp and Aurora B exhibited limited recovery at this late mitotic stage.

Table 1. Dynamics of Survivin, Incenp and Aurora B at different mitotic phases in the indicated cell types and experimental conditions. This table is based on original publications II and III and unpublished results.

Mitotic phase/ location	Treatment	n	$t_{1/2}$ (s)*	Recf (%)*	Ref.
GFP-SURVIVIN					
LLC-PK					
prophase/ inner centromere	-	7	7±4	92±9	II
prometaphase/ inner centromere	-	5	5±1	79±8	II
metaphase/ inner centromere	-	10	5±3	93±8	II
prometaphase/ inner centromere	nocodazole	10	74±18	77±11	II
metaphase/ inner centromere	taxol	10	10±8	78±9	II
prometaphase/ inner centromere	MG132	5	4±2	83±8	II
prometaphase/ inner centromere	MG132+ZM447439	8	67±27	18±13	II
anaphase/ central spindle	-	3	87±23	8±4	II
telophase/ midbody	-	5	204±108	11±3	II
HeLa					
prometaphase/ inner centromere	-	10	7±4	79±8	II
prometaphase/ inner centromere	nocodazole	10	31±10	71±10	II
telophase/ midbody	-	5	144±42	9±5	II
GFP-INCENP					
XenoS3					
metaphase/ inner centromere	-	8	53±19	83±8	III
prometaphase/ inner centromere	nocodazole	6	69±21	86±10	III
metaphase/ inner centromere	MG132	8	38±9	80±13	III
metaphase/ inner centromere	MG132+inj.	4	n.d.	9±2	III
LLC-PK					
prometaphase/ inner centromere	-	11	115±32	76±14	III
metaphase/ inner centromere	-	6	100±37	85±16	III
anaphase/ midzone microtubule	-	5	18±9	49±15	unp.
telophase/ midbody	-	4	n.d.	2±2	unp.
prometaphase/ inner centromere	nocodazole	9	108±31	72±9	III
prometaphase/ inner centromere	taxol	5	107±34	72±9	III
metaphase/ inner centromere	MG132	7	83±26	77±10	III
metaphase/ inner centromere	MG132+ZM447439	8	92±22	44±12	unp.
HeLa					
metaphase/ inner centromere	-	5	79±27	54±17	unp.
AURORA B-GFP (OR YFP)					
XenoS3					
metaphase/ inner centromere	-	3	43±7	88±18	III
metaphase/ inner centromere	MG132	5	31±5	69±11	III
metaphase/ inner centromere	MG132+inj.	4	n.d.	10±4	III
LLC-PK					
metaphase/ inner centromere	-	4	60±12	62±20	unp.
anaphase/ midzone microtubule	-	3	27±9	79±19	unp.
HeLa					
metaphase/ inner centromere	-	4	62±16	44±9	unp.

* Mean ± s.d.; $t_{1/2}$ (s) = Protein turnover half-time in seconds; Recf = Mobile protein fraction; Ref. = Number of original publication; Inj. = Injected with Incenp^{-ab}; n.d. = not determined due to low recf value; unp. = unpublished data

We next determined whether the recovery rates of Survivin and Incenp were altered by MT attachment or interkinetochore tension. These events control the spreading of inhibitory SC complexes and progression through mitosis, and could therefore induce changes in CPC turnover. HeLa, LLC-PK and XenoS3 cells expressing the fusion proteins were treated with nocodazole to induce kinetochores that are free of MTs, or taxol to remove tension but maintain MT attachment. These studies indicated that Survivin turnover was dependent on MT attachment as nocodazole slowed down $t_{1/2}$ to 31 s in HeLa and to 74 s in LLC-PK cells (Table 1). Addition of taxol to the culture medium slowed down recovery $t_{1/2}$ to 10 s in LLC-PK cells (Table 1). We did not observe significant changes in the turnover of Incenp upon treatment with nocodazole or taxol prior to photo-bleaching experiments (Table 1). In summary, Survivin, Incenp, and Aurora B were all dynamic components of inner centromeres, and they became stably associated with the midbody. MT attachment affected the recovery rate by which Survivin was recruited to centromeres, whereas Incenp was recruited at a constant rate independent of MT attachment or tension.

5.5. Blocking CPC reduces its turnover and causes mitotic exit (II, III)

We next asked whether Aurora B kinase activity is needed for the turnover of CPC molecules at centromeres. LLC-PK cells expressing GFP-Survivin or Xeno S3 cells expressing GFP-Incenp were maintained in mitosis by MG132 treatment, which arrests the cell cycle at metaphase due to proteasome inhibition. Otherwise, cells would exit mitosis upon treatment with the Aurora B kinase inhibitor ZM447439 (Kallio, McCleland et al. 2002). MG132 treatment, which was used as a control in this experiment, had no effect on the turnover of Survivin, and slightly increased the turnover of Incenp (Table 1). When cells were first treated with MG132, and subsequently with ZM447439, the turnover of Survivin and Incenp was significantly altered with total recovery (Recf) limited to 18 and 44% (Table 1). Therefore the kinase activity of Aurora B modulated the dynamic association of Survivin and Incenp with inner centromeres, and was needed for their full recovery.

In another approach, we targeted the CPC using anti-Incenp antibody ($Incenp^{-ab}$) injection, and measured the turnover of Incenp and Aurora B in XenoS3 cells. Recf for either fusion protein was only about 10% compared to 70-80% in control cells treated with MG132 (Table 1). Besides this effect on turnover, injection of $Incenp^{-ab}$ caused escape from mitotic arrest induced by nocodazole or taxol, but not from MG132-induced arrest indicating that SC was bypassed (III, Fig. 1D). Previously it was shown that ZM447439 causes premature exit from mitosis through SC inactivation (Kallio et al. 2002b). To determine the mechanism of action of $Incenp^{-ab}$ we analyzed whether it inhibited the kinase activity of Aurora B *in vitro* and *in vivo* toward known substrates. Based on the analysis of incorporation of γ-^{32}P-ATP on histone H3 and phosphorylation of T95 on MCAK, $Incenp^{-ab}$ reduced Aurora B kinase activity by ~40% (III, Fig. 1 E-H). Therefore, antibody injection likely affected Aurora B kinase activity at the intracellular concentrations we used. $Incenp^{-ab}$ may also interfere with other aspects of CPC functions such as interactions with substrates at centromeres. To

summarize, CPC turnover at centromeres was inhibited by Incenp^{-ab} in a similar manner to chemical inhibition of Aurora B. Interestingly, both methods caused premature SC inactivation. This finding proposes that the centromeric turnover of CPC molecules is essential for the maintenance of SC activity.

5.6. CPC regulates chromosome movements in anaphase (III)

Functional dissection of the CPC's functions at specific time points during mitosis has been hampered by the lack of appropriate tools. Cells, in which any CPC member is targeted with siRNA knockdown or expression of dominant negative mutants, enter mitosis with defects in CPC localization and function. We set out to determine the effects of temporally controlled CPC inhibition by using microinjection of Incenp^{-ab} at various points during mitotic progression. Cells injected with either buffer/non-specific IgG or Incenp^{-ab} were visualized live with fluorescence or phase-contrast microscopy, or after fixation and staining with anti-tubulin antibody to label the spindle apparatus. Injection of Incenp^{-ab} antibody in prophase did not influence NEB, but severely disrupted chromosome congression in the following prometaphase (III, Fig. 1 A and B). Time-lapse movies (III, supplementary material) indicated that initial MT attachments could be formed as chromosomes underwent fast movements toward spindle poles, but the alignment of many chromosomes to the metaphase plate was blocked. This could be due to inability of Aurora B to resolve erroneous kMT attachments that typically form at the beginning of chromosome capture soon after NEB. Based on the analysis of fixed cells, the spindles of injected cells were malformed, MTs were not properly polymerized, and there were no clear MT attachments to kinetochores (III, Fig. 2 A and B). The observed defect in kMT formation could be due to the inability of kinetochores to form stable end-on attachments, or errors in MT polymerization at plus-ends that are embedded in the kinetochores. Therefore, Incenp^{-ab} did not only prevent the correction of improper attachments, but it also interfered with the establishment of robust kMTs. This could contribute to the observed defects in chromosome congression and SC signaling.

When Incenp^{-ab} was injected into metaphase cells, chromosomes remained in a metaphase-configuration, but underwent reduced oscillation and the spindle shrunk, which was evident from the measurement of pole-to-pole distances (III, Fig. 3 C-F). Therefore, at metaphase the antibody seemed to interfere with normal MT dynamics (polymerization at plus-ends and/or depolymerization at minus-ends) while MT attachment to kinetochores was not lost. Our results suggest that the CPC contributes to the regulation of normal MT dynamics that is necessary for spindle formation and function.

When Incenp^{-ab} was injected into early anaphase cells, i.e. after cohesion had been removed, sister chromatid segregation was severely disrupted (III, Fig. 4 A and B). Mostly the phenotype was due to defects in spindle elongation at anaphase B when MTs elongating and sliding relative to each other drive the separation of the spindle poles (III, Fig. 5 A-D). In some cells also anaphase A, during which the poleward

movement of sister chromatids by depolymerisation of kMTs occurs, was affected (III, Fig. 4A). The latter was not as robust phenotype possibly because the full effect of Incenp^{-ab} was not immediate, and at the time of injection, anaphase A movements were already in motion. Defective chromosome movements were followed by cytokinesis errors; the cleavage furrow cut through the poorly separated chromatin mass (III, Fig. 5A) or furrow formation failed resulting in the formation of polyploid progeny cells (III, Fig. 4 A-C). In injected anaphase cells that did not undergo cytokinesis, the midzone MTs were abnormally bundled, and were reduced in number compared to control cells (III, Fig. 5 E and F). Furthermore, astral MTs were elongated compared to control cells (III, Fig. 5 E and G). In summary, blocking CPC in early anaphase resulted in defects in chromatid movements, spindle elongation, and cytokinesis.

6. DISCUSSION

6.1. Role of Plk1 and the 3F3/2 phosphoepitope in SC signaling

The 3F3/2 phosphoepitope is a mysterious post-translational modification on a kinetochore-bound protein that has remained unidentified for over a decade (Gorbsky and Ricketts 1993). It is present on the kinetochores of early mitotic cells, and lost upon the establishment of proper kMT attachments. Classic micromanipulation experiments demonstrated that the kinetochore 3F3/2 phosphoepitope responds to changes in interkinetochore tension within seconds: it becomes dephosphorylated when the kinetochore is under tension, and phosphorylated when tension is lost (Nicklas et al. 1995; Li and Nicklas 1997). It is differentially expressed on the leading and trailing sister kinetochores, which likely have a different MT attachment and tension status, suggesting that its expression is controlled locally in subregions of a sister kinetochore pair depending on their binding to MTs.

When this thesis work began, it was postulated that the 3F3/2 phosphoepitope is somehow linked to the SC as it is expressed at kinetochores that generate inhibitory signals to control SC activity. Furthermore, injection of the 3F3/2 antibody into mitotic cells delayed the onset of anaphase, indicating that it must be dephosphorylated to allow anaphase onset (Campbell and Gorbsky 1995). We set out to discover more details of how the 3F3/2 phosphoepitope is involved in the mechano-chemical system that controls SC activity.

We sought to identify the kinase that regulates the expression of the tension sensing 3F3/2 phosphoepitope at kinetochores. We showed by several methods that Plk1 is responsible for generating the 3F3/2 phosphoepitope (I). Furthermore, our results demonstrated that not only did Plk1 generate the 3F3/2 phosphoepitope on kinetochores of unaligned chromosomes (I, Fig. 1), but it also accumulated to these unaligned kinetochores while metaphase kinetochores showed low levels of the kinase (I, Fig. 3). In addition to this, loss of MTs or tension from metaphase cells led to the recruitment of higher amounts of Plk1 to the kinetochores (I, Fig. 3C) as well as rapid rephoshorylation of the 3F3/2 phosphoepitope (I, Fig. 4B) (Campbell and Gorbsky 1995). The finding that Plk1 levels were particularly high on unattached kinetochores implies that Plk1 may have important roles either in promotion of kMT attachments or in regulation of the SC.

Several reports have recently linked Plk1 to the SC. It interacts with a number of substrates that have roles in SC signaling. Plk1 phosphorylates BubR1 and Bub1, and the DNA helicase PICH, that are all necessary for proper SC function (Qi et al. 2006; Baumann et al. 2007; Elowe et al. 2007). However, Plk1 function *per se* does not appear to be essential for SC signaling, because inactivation of Plk1 by RNAi or pharmacologic inhibition causes a prolonged arrest in prometaphase due to activation of the SC (Sumara et al. 2004; van Vugt et al. 2004; McInnes et al. 2006; Lenart et al. 2007). In our experiments, Plk1 depletion resulted in the reduced kinetochore association of the checkpoint proteins Mad2 and Cdc20 (I, Fig. 7A and 8). Despite of

this, Plk1 siRNA-treated cells exited mitosis when SC was experimentally inactivated (I, Fig. 5C), confirming that the prometaphase arrest was due to an active SC.

The significance of the 3F3/2 phosphorylation event for kinetochore function and tension sensing remains to be fully explored. While 3F3/2 expression pattern follows changes in SC activity, its molecular participation in the SC has not been directly established. Our results showed that when 3F3/2 expression was severely reduced, the checkpoint could be activated (I, Fig. 5C) and checkpoint proteins accumulated to kinetochores (I, Fig. 7A). Therefore, the results suggested that 3F3/2 expression was not necessary to establish and maintain the checkpoint. However, Plk1 siRNA treatment did not abolish Plk1 and the 3F3/2 phosphoepitope completely, so it is possible that the residual amounts of Plk1 and the 3F3/2 phosphoepitope at kinetochores were adequate to perform their functions.

Regulation of the 3F3/2 phosphoepitope by Plk1 could also reflect the status of kMT attachments. Based on recent findings, it is becoming clear that Plk1 promotes the formation of stable kMT attachments. Electron microscopic studies demonstrate that when Plk1 is inhibited, end-on attachments between MTs and kinetochores cannot be formed, whereas the formation of lateral associations appears normal (Lenart et al. 2007). Plk1 promotes the establishment of stable kMT attachments by phosphorylating at least two substrates, BubR1 and NudC (Nishino et al. 2006; Elowe et al. 2007; Matsumura et al. 2007). It is likely that Plk1 functions through cooperative phosphorylation of several target proteins at the kinetochore. These findings suggest that Plk1 and the 3F3/2 phosphoepitope may function to promote the formation of stable kMT attachments, which are necessary to satisfy the SC.

To identify Plk1's target protein at kinetochores, we analyzed kinetochore proteins with known 3F3/2 phosphosites as well as searched for unidentified 3F3/2 phosphoepitope containing proteins. Plk1 has been implicated to control APC/C activation through phosphorylation (Descombes and Nigg 1998; Kotani et al. 1998; Shirayama et al. 1998; Golan et al. 2002). Because the 3F3/2 antibody binds to the APC/C (Daum et al. 2000), we analyzed whether Cdc27, a subunit of the APC/C that contains the 3F3/2 phosphoepitope, was affected by Plk1 knockdown. Based on our analysis from Plk1 siRNA-treated cell extracts, the 3F3/2 phosphoepitope on Cdc27 was not affected by the absence of Plk1 (Fig. 9B). However, the results indicated that Plk1 phosphorylates Cdc27 on some other residue(s), as it was not fully phosphorylated (shift in electrophoretic mobility) in the Plk1 siRNA extract (Fig. 9B). As our analysis detected the total pool of Cdc27 present in mitotic cells, it remains possible that another APC/C subunit carrying the 3F3/2 phosphoepitope or a minor kinetochore pool of Cdc27 is targeted by Plk1. Similar experiments carried out to analyze TopoIIα and CenpF ruled them out as candidate 3F3/2 phosphoepitope containing Plk1 target proteins.

Recently, Wong and Fang reported that Plx1 (*Xenopus* Plk1 homolog) creates the 3F3/2 epitope on BuBR1 in *Xenopus* extract after priming phosphorylation by Cdk1,

and that residue T650 is targeted by both kinases (Wong and Fang 2007). It was proposed by the authors that BubR1 is the kinetochore-located 3F3/2 phosphoepitope containing protein that senses tension. However, there are some indications that argue against BubR1 being the ubiquitous tension sensing protein in vertebrate cells. SiRNA-mediated silencing of BubR1 did not abolish or even reduce 3F3/2 phosphoepitope expression at kinetochores of vertebrate cells (personal communication with G.J. Gorbsky). Elowe and co-workers studied another phosphorylation on BubR1 (S676), which is created by Plk1 (Elowe et al. 2007). While S676 phosphorylation was regulated in an identical fashion to the 3F3/2 phosphoepitope, the authors reported that it was not the one identified by the 3F3/2 antibody. Therefore, although it is evident that Plk1 controls BubR1 through phosphorylation, based on these data, BubR1 does not contain the 3F3/2 phosphoepitope in vertebrate cells.

There is evidence to suggest that the 3F3/2 phosphoepitope is expressed on several proteins and might be created by multiple kinases. We showed that Plk1 creates the 3F3/2 phosphoepitope corresponding to an identical tension-dependent pattern seen *in vivo* (I). It was shown previously that casein kinase II creates the 3F3/2 phosphoepitope on TopoIIα (Daum and Gorbsky 1998). In addition, the MAP kinase pathway was implicated in the regulation of 3F3/2 expression (Shapiro et al. 1998). It is evident from the multiple proteins that are pulled down by the 3F3/2 antibody (Fig. 9D), that there are several proteins carrying the 3F3/2 phosphoepitope in mitotic cells. Furthermore, basal expression of the 3F3/2 phosphoepitope at kinetochores of metaphase chromosomes in mantids and grasshoppers (Li and Nicklas 1997) suggests that at least a proportion of the 3F3/2 phosphoepitope is not controlled by tension. Based on these data, the bulk 3F3/2 phosphoepitope at kinetochores reacts to changes in tension, but other proteins containing the 3F3/2 phosphoepitope may exist that are not relevant to kinetochore tension and SC signaling.

What properties should the tension sensing 3F3/2 phosphoepitope protein(s) have? Firstly, it should localize to the same layer of the kinetochore where the 3F3/2 phosphoepitope resides. Electron microscopic studies revealed that the phosphoepitope localizes mainly to the electron-lucent middle layer of the kinetochore (Campbell and Gorbsky 1995). This kinetochore zone is composed of an array of structures that appear to connect the more electron dense inner and outer plates. The localization of many kinetochore proteins partially overlaps the localization of the 3F3/2 phosphoepitope but few proteins appear to localize solely to the middle layer. Interestingly, the localization of PICH and Sgo1 closely resembles that of the 3F3/2 phosphoepitope: they localize between outer kinetochore proteins but outside of inner centromere proteins (Indjeian et al. 2005; Baumann et al. 2007; Pouwels et al. 2007). Furthermore, they have been suggested to function as tension sensors. In fact, the depletion of Sgo1 reduces the kinetochore 3F3/2 phosphoepitope (Pouwels et al. 2007), but since it also leads to Plk1 mislocalization, the reduction is likely due to mislocalized Plk1. Secondly, the candidate protein may react to changes in tension and fall off the kinetochore as cells progress towards metaphase similarly to Bub1, Bub3 and BubR1 (Skoufias et al. 2001; Logarinho et al. 2004). The protein may also

associate with kinetochores throughout mitosis, as *in vitro* studies suggest that the protein is present at kinetochores also in metaphase and anaphase (Campbell et al. 2000). However, the 3F3/2 phophoepitope-bearing protein could be a diffent molecule in metaphase and anaphase cells. Thirdly, the conformation of the 3F3/2 phosphoepitope containing protein may change upon MT attachment and tension, or it may have high turnover, which allows rapid concentration changes in response to alterations in MT attachments and interkinetochore tension.

How is 3F3/2 expression regulated? It could occur at three levels: at the level of the substrate, the upstream kinase (Plk1), or an antagonistic phosphatase. Intriguingly, the kinetochore has a "memory" since the differential 3F3/2 expression pattern seen *in vivo* can be reproduced on the cell cytoskeleton *in vitro*. Both the endogenous kinetochore-bound kinase, as well as an exogenous kinase, produce the characteristic phosphorylation pattern seen *in vivo* when phosphorylation is carried out for short periods of time (~5 min) (Campbell et al. 2000). Therefore, although Plk1 accumulated to kinetochores of unaligned chromosomes, its concentration cannot solely dictate to what extent the kinetochore becomes phosphorylated, otherwise all kinetochores would become equally phosphorylated in the presence of an exogenous kinase. The counteracting phosphatase is clearly in close proximity as it rapidly dephosphorylates the epitope upon cell lysis. It is possible that the phosphatase localizes to a dinstinct subregion of the kinetochore and counteracts Plk1 activity in a tension-mediated manner similarly to the model that explains how MCAK is regulated by Aurora B and its counteracting phosphatase. The accessibility of the substrate to regulatory proteins, kinases and phosphatases, likely contributes to the differential expression pattern. Accessibility may be dependent on the level of substrate accumulation at kinetochores as well as conformational changes in the molecule. *In vitro* phosphorylation assays carried out for extended periods of time with endogenous or exogenous kinase led to 3F3/2 phosphoepitope expression also at kinetochores of metaphase and anaphase cells (Campbell et al. 2000). These results suggest that the tension sensing 3F3/2 phosphoepitope protein is present at kinetochores in all mitotic stages unless the protein is of different identity. Therefore, the differential expression pattern is most likely achieved through modifications in the susceptibility of the substrate to become phosphorylated. This could be achieved through conformational changes in the substrate upon MT attachment and tension. Figure 10 summarizes the findings of this part of the thesis and proposes a model for the regulation of the 3F3/2 phosphoepitope.

6.2. Role of CPC turnover in Aurora B kinase function

Unlike previously reported (Delacour-Larose et al. 2004; Delacour-Larose et al. 2007), our findings suggested that not only Survivin, but also Aurora B and Incenp associated with the inner centromeres in a dynamic manner, circulating continuously between the centromere-bound and a free cytoplasmic fraction (Table 1). When Incenp was blocked with antibody injection or Aurora B kinase activity was inhibited with small molecules, the dynamic turnover of CPC components at inner centromeres was limited (Table 1). However, the CPC molecules were able to bind to centromeres in the

absence of Aurora B kinase activity and upon injection with Incenp^{-ab} (data not shown), suggesting that Aurora B kinase activity was not needed for their initial association with inner centromeres. Because photo-bleaching analyses showed that Survivin, Incenp and Aurora B cannot undergo dynamic turnover in the presence of Aurora B kinase inhibitor or Incenp^{-ab} (Table 1), their release from the centromere must be blocked. In agreement with our data, dephosphorylation of Survivin at T117 (Aurora B phosphorylation site) converted mobile Survivin to stably associated with the centromere (Delacour-Larose et al. 2007). Together the data imply that Aurora B kinase activity regulates the turnover of CPC molecules at centromeres. Also other post-translational modifications, such as ubiquitination, have been proposed to control the dynamic association of Survivin with centromeres (Vong et al. 2005).

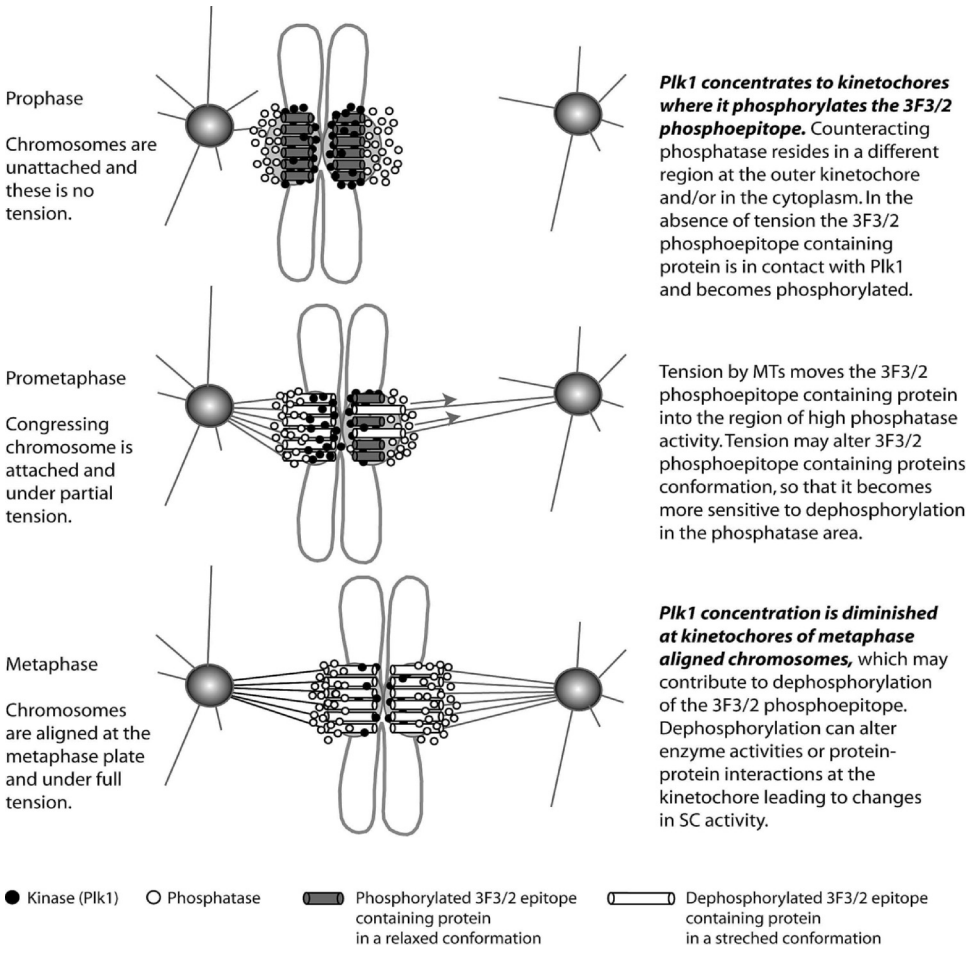

Figure 10. Summary of the central findings of the original publication I (***in bold Italic***) and a model for the regulation of the 3F3/2 phosphoepitope by MT-induced tension. The model is based on I, (Gorbsky 2004) and (Campbell and Gorbsky 1995).

Based on the FRAP data, Survivin underwent fast exchange at inner centromeres while Incenp and Aurora B exhibited moderate turnover (Table 1). Furthermore,

Survivin turnover $t_{1/2}$ was found to be dependent on kMT attachment, whereas Incenp turnover $t_{1/2}$ was independent of MTs (Table 1). These differences point to the possibility that at least a subpopulation of Survivin turns over independently of the Aurora B-Incenp pair, and that this could be mediated by bound MTs. How this could be accomplished remains unclear due to the strict interdependencies between the subunits. If there would be two pools of Survivin at the centromere, one bound to the CPC and the other free, one would assume that the recovery curve for Survivin would be biphasic. Our results implied that all Survivin recovers rapidly. It remains possible that higher expression levels of GFP-Survivin compared to GFP-Incenp and Aurora B-YFP, which we observed, caused the faster recovery $t_{1/2}$. In principle this should not be possible if Survivin associates with Aurora B and Incenp, which undergo moderate turnover. We cannot rule out the possibility that GFP-Survivin might dimerize with endogenous Survivin, which could affect the turnover. It remains a possibility that the different microscope setups we used (see 4.9. and 4.10.) affected these results, but also other studies have reported very fast recovery of Survivin (Delacour-Larose et al. 2004). The main conclusion of these findings is that all studied CPC molecules undergo constant exchange between the inner centromere and the cytoplasm.

We can not fully explain why in our experiments Aurora B and Incenp were mobile in contrast to the results obtained by Delacour-Larose and co-workers (Delacour-Larose et al. 2004; Delacour-Larose et al. 2007). Their experiments demonstrated that both transiently and stably expressed Aurora B-GFP fusion proteins were immobile, whereas GFP-Survivin was highly mobile. Both groups performed analysis with transiently transfected HeLa cells (Table 1) (Delacour-Larose et al. 2004) and with the same construct (Aurora B-GFP), yet the results were strikingly different. Delacour-Larose and co-workers used also stably transfected NIH-3T3 cells and came to the same conclusion as with transiently transfected HeLa cells. Their results with transiently expressed GFP-Incenp (immobile) (Delacour-Larose et al. 2007) were also contradictory to our studies, which showed high mobility at centromeres in different cell types including HeLa, LLC-PK and Xeno S3 (Table 1). Based on these data, transient expression in our experiments is not a likely explanation to the discrepancy in the results. In agreement with our data, Murata-hori and Wang have published that Aurora B exhibits dynamic turnover at centromeres (Murata-Hori and Wang 2002).

Antibody injection against Incenp prevented the turnover of CPC components at centromeres (III, Fig. 3), and it also resulted in the premature inactivation of the SC and a forced mitotic exit without cytokinesis (III, Supplemental Fig. S1). Similarly, the Aurora B kinase inhibitor ZM447439 reduced Survivin and Incenp turnover at centromeres (Table 1), and induced SC override and precocious mitotic exit (Kallio et al. 2002b). Furthermore, Incenp^{-ab} partially inhibited Aurora B kinase activity (III, Fig. 1 E-H). Therefore, we speculate that CPC turnover, which depended on active Aurora B kinase, plays a critical role in the maintenance of an active SC, although this is based on indirect observations. Aurora B inhibition by Hesperadin was shown to lead to loss of BubR1 and Bub1 from kinetochores (Hauf et al. 2003). Therefore, Aurora B

function is required for efficient kinetochore recruitment of BubR1 and Bub1, which may be necessary for prolonged SC signaling.

A picture is starting to emerge, in which high local concentrations of active Aurora B kinase mediate specific mitotic functions of the CPC, such as spindle assembly and cytokinesis (Kelly et al. 2007; Fuller et al. 2008). Aurora B kinase becomes activated by autoactivation *in trans* when it is in close proximity with other CPC molecules. Full Aurora B activation appears to occur when Aurora B on one CPC phosphorylates the N-terminal TSS motif of Incenp on another CPC (Sessa et al. 2005). Such close proximity may occur at inner centromeres or at the spindle midzone. Our results regarding the mobility of Aurora B and other CPC molecules at inner centromeres fit the proposed model of activation. Our studies showed that CPC molecules associate with inner centromeres transiently, which indicates that they are constantly released into the cytoplasm and new molecules can bind. We propose that this activates Aurora B kinase locally and provides a mechanism for Aurora B autoactivation at inner centromeres (Fig. 11A). Based on this model, Aurora B/CPC binds to the inner centromeres to become activated by another Aurora B/CPC, after which the complex is released from the inner centromeres into the cytoplasm. This model explains how Aurora B could perform its mitotic functions at regions outside of the centromere, such as at the kinetochore. Injection of Incenp^{-ab}, which blocked CPC turnover at centromeres, may function by preventing the CPC activation and release step, and/or block CPC interactions with substrates causing SC failure (Fig. 11B).

6.3. Spatial and temporal regulation of anaphase by the CPC

To overcome the biological limitation of using genetic perturbation of the CPC, which results in defective CPC function upon mitotic entry, we used a unique technique to study the mitotic tasks of the complex at specific time points during mitotic progression. Previously, the CPC was implicated in the regulation of numerous mitotic events, such as chromosome condensation, metaphase congression, MT dynamics (Rosa et al. 2006), spindle stability (Gassmann et al. 2004), resolution of incorrect kMT attachments, SC activity, chromosome segregation and cytokinesis (see Review of the literature). Our results showed for the first time that the CPC was needed in anaphase for the poleward movements of chromatids and for spindle elongation (III, Fig. 4 and 5). These functions were in the previous studies masked by earlier mitotic defects.

We made some interesting observations when Incenp^{-ab} injected live cells expressing GFP-tubulin, or fixed cells labeled with anti-tubulin antibody, were imaged. Injection of the antibody in early anaphase cells led to abnormal bundling of MTs in the spindle midzone (III, Fig. 5). This suggested that MT dynamics/associations were perturbed. We hypothesize that these defects were due to inhibition of motor proteins that are responsible for anti-parallel sliding of MTs in the spindle midzone (Sharp et al. 2000; Brust-Mascher et al. 2004; Kwon et al. 2004). They could also be caused by defects in the function of the centralspindlin complex, which has MT bundling activity

and is phosphorylated by Aurora B (Mishima et al. 2002; Minoshima et al. 2003; Guse et al. 2005). Interestingly, a similar phenotype was reported after chemical inhibition of Plk1 (Brennan et al. 2007) and the kinase interacts with Incenp (Goto et al. 2006) suggesting that there may be an interplay between Plk1 and the CPC in anaphase. It remains possible that Incenp^{-ab} injection also influenced anaphase movements through effects on astral MTs, since they were abnormally long in the injected cells (III, Fig. 5). Our results together with those of Miyauchi and co-workers (Miyauchi et al. 2007) suggest that Aurora B kinase activity is also required for proper regulation of astral MT dynamics.

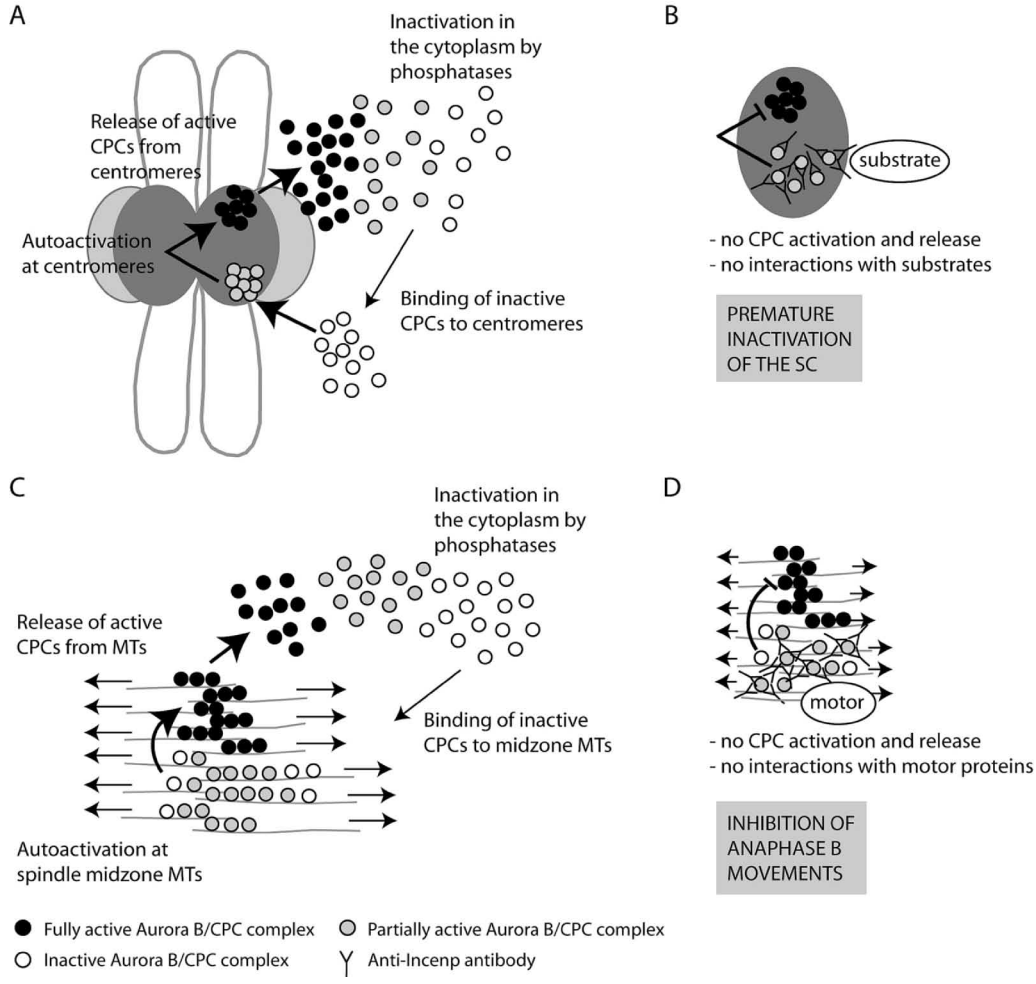

Figure 11. Model of the Aurora B phosphorylation gradient near centromeres (A) and the spindle midzone (C) followed by proposed mechanisms of the Incenp^{-ab} (B and D). The model is based on II, III, (Sessa et al. 2005), (Kelly et al. 2007) and (Fuller et al. 2008).

Very recently, it was proposed that Aurora B kinase forms a phosphorylation gradient in anaphase cells, which provides spatial information about cleavage furrow positioning (Fuller et al. 2008). The authors used different substrates of Aurora B to

show that the closer the substrate is to the localization of Aurora B, the more it gets phosphorylated. The authors provided further evidence that Aurora B becomes fully activated at the spindle midzone. Similarly to the proposed mechanism of Aurora B activation at centromeres, Aurora B would then be released from the midzone into the cytoplasm to perform its anaphase and telophase functions. In line with these results, we detected a high rate of recovery of GFP-Incenp and Aurora B-GFP at midzone MTs when the fusion proteins were expressed in LLC-PK cells and photo-bleached (Table 1). This mobility was highest during early anaphase and was diminished when the cleavage furrow contracted (data not shown). In telophase cells, all CPC molecules were immobile (Table 1; data not shown). Although the findings regarding the anaphase recovery were restricted to only one cell type that expressed GFP-Incenp and Aurora B-GFP at high levels, the data suggests that Incenp turnover at midzone MTs may play a role in anaphase chromatid movements.

Our results support the proposed mechanism for Aurora B activation in anaphase cells (Fuller et al. 2008). We assume that Aurora B and Incenp, and possibly other subunits, are in a complex at this cell cycle stage, which is very likely taking into concideration the interdependency of the subunits (Jeyaprakash et al. 2007). To form the phosphorylation gradient, the CPC needs to dissociate from the midzone MTs. Our results indicated that GFP-Incenp and Aurora B-GFP recovered to anaphase MTs with a high rate (Table 1), which indirectly suggests that they also dissociate from MTs with the same rate. One would expect that there is a limited number of binding sites for Incenp/Aurora B on these MTs, so that in order for new molecules to associate, others must dissociate. Recovery was limited after the cleavage furrow contracted (data not shown), and was absent at the midbody (Table 1, data not shown) suggesting that at this point the CPC becomes immobile. Interestingly, injection of Incenp^{-ab} into anaphase cells blocked normal anaphase progression when injection was performed within 5 min after anaphase onset, but if introduced at later time points cytokinesis appeared normal (III, Fig. 4). This approximately coincided with the loss of GFP-Incenp/Aurora B-GFP mobility at midzone MTs pointing to the possibility that Incenp^{-ab} prevented Incenp/Aurora B turnover and thereby caused the observed cellular phenotype. Therefore, we propose that CPC turnover at the midzone MTs produces the Aurora B phosphorylation gradient, which is important for mitotic progression until the cleavage furrow contracts. This seems to be the commitment point for the execution of cytokinesis and the point after which CPC turnover is no longer necessary for normal mitotic exit. The results of this part of the thesis are summarized in Fig. 11 C and D.

6.4. Targeting Plk1 and Aurora B in cancer therapies

Future cancer therapies target specific molecules that have crucial roles in processes such as cell division and cell death. Targeting these molecules provides a means to eliminate cancer cells that are highly proliferative, whereas normal cells are minimally damaged. In combination with cancer cell specific delivery, such therapies could be powerful tools in the treatment of this disease.

Both Plk1 and Aurora B are necessary for mitotic progression and their overexpression has been linked to cancer cells with high proliferative index (Eckerdt et al. 2005; Takai et al. 2005; Mountzios et al. 2008). Furthermore, these kinases, at least Plk1, may be more crucial for the survival of cancer cells compared to normal cells (Guan et al. 2005; Liu et al. 2006). Therefore, they are possible targets for future anti-cancer therapies, and studies evaluating Plk1 and Aurora kinase inhibitors are ongoing. There is increasing interest in siRNA-based methods to target cell division and the apoptotic pathway (Takeshita and Ochiya 2006; Pirollo and Chang 2008). SiRNA against Plk1 or Aurora B, or other members of the CPC complex, leads to cell death or reduced viability demonstrating that these molecules could be targeted by siRNA to reduce cell proliferation. Furthermore, knockdown of Plk1 may reduce the survival of cancer cells compared to normal cell populations (Guan et al. 2005; Liu et al. 2006). This effect could be mediated through the transcriptional activator and tumor suppressor p53: Plk1 physically interacts with p53 (Ando et al. 2004), and its depletion preferentially reduces the survival of p53-defective cells (Guan et al. 2005; Liu et al. 2006). This is an important finding that should be considered when new strategies are developed to target cancer cells that often have mutated p53.

Studies with Incenp^{-ab} demonstrated that the CPC can be blocked using other approaches than kinase inhibitors. The antibody binds to the C-terminus of Incenp and is expected to interfere with Aurora B-Incenp interaction, which is needed for full kinase activation. The results suggest that small molecules that are targeted against the Aurora B-Incenp interface could be beneficial in inhibiting Aurora B kinase activity allosterically. This provides an alternative method to block CPC function outside of the Aurora B kinase ATP-binding pocket, which is likely to be prone to mutations (Girdler et al. 2008). Future studies are needed to evaluate the potential of this approach.

7. CONCLUSIONS

Mitotic regulation of the 3F3/2 phosphoepitope was, and still is, of great interest to the cell division field. While the 3F3/2 phosphoepitope was discovered already over a decade ago, and was shown to respond to kinetochore tension, its precise role in SC signaling has remained unclear. We were able to identify Plk1 as a regulator of this phosphoepitope and implicated the kinase for the first time in the SC response. Although the identity of the kinetochore target of Plk1 carrying the 3F3/2 phosphoepitope is still under debate, our findings revealed a key player in the mitotic mechano-chemical pathway that responds to physical tension applied at kinetochores. Future experiments are needed to reveal the precise role of Plk1 and the 3F3/2 phosphoepitope in SC signaling.

The CPC has been a focus of intense reseach since the discovery of Incenp, the first inner centromere protein that showed the characteristic movement pattern from chromosome arms to centromeres and to the midbody during mitosis. Since then, Incenp has been shown to interact with other proteins that together form the CPC. Many CPC roles in mitosis have been uncovered, and recently crystal structures have shed light on the molecular structure of the complex. The spatio-temporal control of CPC activity in mitotic cells is now gaining more attention. CPC regulation may involve interactions with activating and inhibiting co-factors, and it may be controlled through physical forces that are imparted by MTs that stretch centromeres and kinetochores to control Aurora B's access to its substrates. Our studies on CPC mobility implied that all CPC molecules undergo constant turnover at inner centromeres of early mitotic cells. Therefore, we propose that another level of control on CPC activity is enforced by its rapid dynamics. This dynamics may contribute to CPC activation at sites of high local concentration.

In summary, our studies identified new functions of two key mitotic kinases, Plk1 and Aurora B. The studies provide valuable information of how mitotic progression is orchestrated by these kinases. Information of the different functions of important cell-cycle regulators can be utilized in the design of strategies for cancer therapies in the future. Our studies using Incenp^{-ab} targeted against the C-terminus of Incenp, which is important for Aurora B activation, demonstrated that Aurora B function can be blocked by other means than inhibition of kinase activity by small molecules. This provides insighs into the design of drugs that would induce cell killing via the inhibition of normal CPC function.

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Leena Ahonen

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