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FUNCTIONAL CHARACTERIZATION OF PROTEINS REQUIRED FOR MITOTIC PROGRESSION AND THE SPINDLE ASSEMBLY CHECKPOINT

by

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To my family

Anu Kukkonen-Macchi
Functional characterization of proteins required for mitotic progression and the spindle assembly checkpoint

Department of Medical Biochemistry and Genetics, Turku Centre for Biotechnology, University of Turku and VTT Medical Biotechnology, Turku, Finland
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ABSTRACT

During mitotic cell division, the genetic material packed into chromosomes is divided equally between two daughter cells. Before the separation of the two copies of a chromosome (sister chromatids), each chromosome has to be properly connected with microtubules of the mitotic spindle apparatus and aligned to the centre of the cell. The spindle assembly checkpoint (SAC) monitors connections between microtubules and chromosomes as well as tension applied across the centromere. Microtubules connect to a chromosome via kinetochores, which are proteinaceous organelles assembled onto the centromeric region of the sister chromatids. Improper kinetochore-microtubule attachments activate the SAC and block chromosome segregation until errors are corrected and all chromosomes are connected to the mitotic spindle in a bipolar manner. The purpose of this surveillance mechanism is to prevent loss or gain of chromosomes in daughter cells that according to current understanding contributes to cancer formation. Numerous proteins participate in the regulation of mitotic progression. In this thesis, the mitotic tasks of three kinetochore proteins, Shugoshin 1 (Sgo1), INCENP, and p38 MAP kinase (p38 MAPK), were investigated. Sgo1 is a protector of centromeric cohesion. It is also described in the tension-sensing mechanism of the SAC and in the regulation of kinetochore-microtubule connections. Our results revealed a central role for Sgo1 in a novel branch of kinetochore assembly. INCENP constitutes part of the chromosomal passenger complex (CPC). The other members of the core complex are the Aurora B kinase, Survivin and Borealin. CPC is an important regulatory element of cell division having several roles at various stages of mitosis. Our results indicated that INCENP and Aurora B are highly dynamic proteins at the mitotic centromeres and suggested a new role for CPC in regulation of chromosome movements and spindle structure during late mitosis. The p38 MAPK has been implicated in G1 and G2 checkpoints during the cell cycle. However, its role in mitotic progression and control of SAC signaling has been controversial. In this thesis, we discovered a novel function for p38γ MAPK in chromosome orientation and spindle structure as well as in promotion of viability of mitotic cells.

Keywords: mitosis, kinetochore, spindle assembly checkpoint, Sgo1, CPC, p38 MAPK

Anu Kukkonen-Macchi
Solunjakautumisen säätelyyn osallistuvien proteiinien toiminnan selvittäminen

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TIIVISTELMÄ

Solunjakautumisen eli mitoosin aikana kromosomeiksi pakattu solun perintöaines jaetaan tasan kahden tytärsolun kesken. Mitoosin oikeaoppisuuden kannalta on tärkeää, että solun kaikki kromosomit ovat kiinnittyneet oikein tumasukkulan mikrotubuluksiin ja järjestäytyneet solun jakotasoon ennen kromosomien kahden kopion, sisarkromatidien, erkanemista. Mikrotubulukset kytkeytyvät kromosomeihin kinetokorien välityksellä. Kinetokorit sijaitsevat kromosomien sentromeerialueella ja koostuvat lukuisista mitoosiin ja sen säätelyyn osallistuvista proteiineista. Mitoottinen tarkastuspiste valvoo kinetokorien ja mikrotubulusten välisiä kytkentöjä, joiden puute tai virheet johtavat mitoottisen tarkastuspisteen aktivoitumiseen ja solunjakautumisen estymiseen. Mitoosi voi edetä sisarkromatidien eroamisvaiheeseen vasta, kun puutteelliset kytkennät on korjattu. Tämän mekanismin tarkoitus on estää virheellisen kromosomimäärän siirtyminen tytärsoluihin. Tässä väitöskirjatyössä selvitettiin kolmen kinetokoriproteiinin, Shugoshin 1:n (Sgo1), INCENP:n ja p38 MAP -kinaasin (p38 MAPK) tehtäviä solunjakautumisen aikana. Sgo1:n tunnettuihin tehtäviin kuuluu sisarkromatidien välisen koheesion suojaaminen sentromeerialueella. Lisäksi se saattaa osallistua mitoottisen tarkastuspisteen sekä kinetokorien ja mikrotubulusten välisten kytkentöjen säätelyyn. Tuloksemme paljastivat Sgo1-proteiinin olevan keskeinen tekijä kinetokorien järjestäytymisessä. INCENP on osa proteiinikompleksia (CPC), jonka muut jäsenet ovat Aurora B -kinaasi, Survivin ja Borealin. CPC:lla on useita keskeisiä tehtäviä mitoosin eri vaiheissa. Osoitimme INCENP:n ja Aurora B:n dynaamisen luonteen sentromeereissä sekä CPC:n osallisuuden kromosomien liikkeiden ja tumasukkulan rakenteen säätelyssä mitoosin myöhäisessä vaiheessa. p38 MAPK:n tehtävät tunnetaan suhteellisen hyvin solusyklin G1- ja G2-vaiheiden tarkastuspisteissä. Proteiinin osallisuus mitoosin ja mitoottisen tarkatuspisteen toimintaan on kuitenkin kiistanalaista ja vielä suurelta osin selvittämättä. Tässä väitöskirjatyössä kuvaamme p38γ MAPK:n ensimmäistä kertaa tärkeänä mitoosiproteiinina, joka osallistuu kromosomien orientaation ja tumasukkulan rakenteen säätelyyn sekä edistää mitoottisten solujen elinkykyä.

Avainsanat: mitoosi, kinetokori, mitoottinen tarkastuspiste, Sgo1, CPC, p38 MAPK

TABLE OF CONTENTS

ABBREVIATIONS

APC/C	Anaphase promoting complex/cyclosome
Bub	Budding uninhibited by benzimidazole
BubR1	Bub1-related kinase 1
Cdc20	Cell division cycle 20
Cdk	Cyclin-dependent kinase
CENP	Centromeric protein
CIN	Chromosomal instability
CPC	Chromosomal passenger complex
ERK	Extracellular signal regulated kinase
FRAP	Fluorescence recovery after photobleaching
Hec1	Highly expressed in cancer 1
IAP	Inhibitor of apoptosis protein
ICIS	Inner centromere Kinesin I stimulator
INCENP	Inner centromeric protein
JNK	c-Jun amino terminal kinase
Mad	Mitotic arrest deficient
MAPK	Mitogen-activated protein kinase
MCAK	Mitotic centromere associated kinesin
MCC	Mitotic checkpoint complex
MK2	Mitogen-activated protein kinase-activated protein kinase 2
MKK	Mitogen-activated protein kinase kinase
MKKK	Mitogen-activated protein kinase kinase kinase
NEB	Nuclear envelope breakdown
Plk1	Polo-like kinase 1
PP2A	Protein phosphatase 2A
Pro	Proline
recf	Recovery of fluorescence
RNAi	RNA interference
SAPK	Stress activated protein kinase
SAC	Spindle assembly checkpoint
Ser	Serine
Sgo	Shugoshin
$t_{1/2}$	Half-time of recovery
Thr	Threonine
Tyr	Tyrosine

LIST OF ORIGINAL PUBLICATIONS

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

I Pouwels J*, Kukkonen AM*, Lan W, Daum JR, Gorbsky GJ, Stukenberg T, Kallio MJ. Shugoshin 1 plays a central role in kinetochore assembly and is required for kinetochore targeting of Plk. Cell Cycle. 2007. 1;6(13):1579-85.

II Ahonen LJ, Kukkonen AM, Pouwels J, Bolton MA, Jingle CD, Stukenberg PT, Kallio MJ. Perturbation of Incenp function impedes anaphase chromatid movements and chromosomal passenger protein flux at centromeres. Chromosoma. 2009. 118(1):71-84.

III Anu Kukkonen-Macchi, Oana Sicora, Katarzyna Kaczynska, Christina Oetken Lindholm, Jeroen Pouwels, Leena Laine and Marko J. Kallio. Loss of p38gamma MAPK induces pleiotropic mitotic defects and massive cell death. Journal of Cell Science. 2010. In press.

* Equal contribution

1 INTRODUCTION

Maintenance of a correct chromosome number is essential for the survival of an organism. Somatic cell division, mitosis, produces genetically identical daughter cells while reproductive cells are formed through specialized nuclear divisions, the meiotic divisions, where the offspring cells are genetically distinct from their mother cell. During cell division, a strict control mechanism monitors the correct segregation of the chromosomes. Errors in cell division can lead to loss or gain of chromosomes (aneuploidy). In germ cells, aneuploidy is considered as the main cause of miscarriage and still birth in humans, and the origin of severe birth defects such as the trisomy 21 (Down syndrome). In mitotically dividing cells, aneuploidy is a hall mark of cancer; many cancer cells are characterized by high rates of chromosome mis-segregation, a feature referred to as chromosomal instability (CIN). Although disturbances of mitosis are connected with tumorigenesis, mitotic processes are also important targets of cancer therapies. In cancer clinics, mitotic cell proliferation can be suppressed, for example, using drugs that target the mitotic spindle apparatus, a central cell division facilitator. However, these drugs are not cancer cell selective and therefore cause adverse effects. For these reasons, part of the cell division research is aiming to identify new drugabble targets within the mitotic machinery that could assist in the development of future anti-cancer treatments.

One essential mitotic structure is a trilaminar protein complex termed the kinetochore, which assembles on the centromeric region of chromosomes. The primary function of the kinetochore is to serve as a platform for microtubule attachments during cell division. In addition to proteins required for microtubule capture and attachment, the proteins responsible for mitotic regulation and correction of erroneous kinetochore-microtubule attachments reside at the kinetochores. In normal cells, the sister chromatids can separate only when all chromosomes are attached to microtubules via the kinetochores in a bipolar manner. This process is monitored by a conserved signaling cascade called the Spindle Assembly Checkpoint (SAC). Unattached kinetochores create a diffucible “wait anaphase” signal that keeps the SAC activated and in this way prevents chromosome segregation until errors in kinetochore-microtubule attachments are corrected. The molecular target of the SAC is the activity of a mitotic ubiquitin ligase called the Anaphase Promoting Complex/Cyclosome (APC/C). Upon proper bipolar orientation of all chromosomes, the SAC is satisfied and this leads to activation of the APC/C. The APC/C then targets the anaphase inhibitor proteins for degradation, which facilitates simultaneous separation of the two copies of each chromosome and exit from the cell division phase.

Cohesion between sister chromatids is essential to avoid premature sister chromatid separation. A complex termed cohesin is responsible for cohesion and it is removed from chromosome arms already in early mitosis. In contrast, the centromeric cohesin is preserved until the onset of anaphase. When all chromosomes are properly attached to the mitotic spindle activated APC/C poly-ubiquitinates securin leading to full

activation of separase that specifically cleaves cohesin. A protein called Shugoshin (Sgo) and its orthologues in many species were discovered to have a central role in protection of centromeric cohesion in cooperation with protein phosphatase 2A (PP2A) during mitosis and meiosis. In addition, this multifunctional protein has been described in tension-sensing between sister chromatids and in the regulation of microtubule stability.

Aurora B, INCENP, Survivin and Borealin form the core of the chromosomal passenger complex (CPC). Aurora B kinase is the enzymatic heart of the CPC and it has numerous substrates during mitosis. Its localization and activity is dependent on the other members of the complex. CPC has many essential tasks in various stages of mitosis. CPC is required for recruiting SAC proteins and it is essential in the correction of erroneous microtubule-kinetochore attachments, thereby taking part in proper SAC signaling.

Four isoforms of p38 MAPK family are activated by various extracellular stresses. They take part in diverse intracellular processes. Importantly, p38 MAPKs function in the cell cycle control where they regulate both the G1/S and G2/M transitions. The role of p38 MAPKs during mitosis is less characterized. Previously, the link between p38 MAPK pathway and the SAC has been proposed. Moreover, it has been suggested that the p38 pathway regulates mitotic progression and another mitotic kinase Polo-like kinase 1 (Plk1).

In this thesis, the role of three mitotic proteins, Sgo1, INCENP and p38 MAPK, was studied. The function of Sgo1 in kinetochore assembly was clarified and the dependencies between Sgo1 and a number of important mitotic regulators were determined. The tasks of INCENP as well as its dynamics were studied at different stages of mitosis. Moreover, a novel mitotic role of a p38γ MAPK was revealed.

2 REVIEW OF THE LITERATURE

2.1 Mitosis

2.1.1 Introduction to the cell cycle

A new cell can only be born by duplicating and dividing a cell that already exists. The cell cycle is a series of events that takes place in a cell leading to its duplication. The basic function of the cell cycle is to duplicate accurately the entire genome of the cell and segregate a copy of each chromosome precisely into two genetically identical daughter cells. The eukaryotic cell cycle can be divided in four phases (Fig. 1), G1 phase, S phase, G2 phase (collectively interphase) and M phase (mitosis). There are three major checkpoints during the cell cycle (Fig. 1). Two gap phases G1 and G2 delay the cell cycle to allow cell growth. During these phases, the cell has time to monitor the environment and make sure that conditions are suitable for cell cycle progression. Some cell types continue to divide throughout the life of an organism. Other cell types remain in a so called non-dividing phase, G0, until they receive a signal from the environment to cycle again. Cells can also enter into G0 from G1 in response to lack of growth factors or nutrients for example. The first checkpoint of the cell cycle is in late G1 when cell commits to cell cycle entry and DNA duplication. In S phase the cell replicates its DNA through an organized replication machinery. The second checkpoint, the G2/M checkpoint, is at the end of the G2 phase. This checkpoint monitors that all DNA is replicated and triggers the entry into the last phase of the cell cycle, mitosis. In mitosis, duplicated DNA and other cell material divide and the cell splits into two daughter cells. The third checkpoint, termed the spindle assembly checkpoint (SAC), monitors the accuracy of chromosome segregation during mitosis.

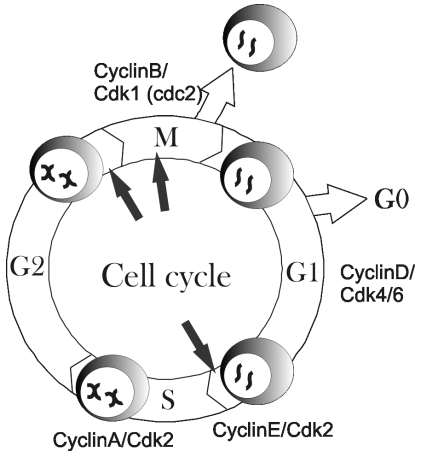

Figure 1. Phases of the vertebrate cell cycle. Three cell cycle checkpoints are marked with black arrows. The DNA content of the cell duplicates during S-phase. Cell cycle progression depends on cyclins and Cdks which are specific for each cell cycle phase.

The cell cycle control depends on cyclin-dependent protein kinases (Cdks). The activities of these kinases rise and fall during the cell cycle leading to cyclical changes in the phosphorylation of proteins that regulate the events of the cell cycle. Most

important regulators of Cdks are cyclins. Binding of Cdk to a cyclin activates Cdk and this triggers specific cell cycle events (Fig. 1).

2.1.2 Phases of mitosis

In mitosis, duplicated chromosomes divide equally between two daughter cells through systematically regulated steps. Chromosome movements during mitosis rely on a complex macromolecular structure termed the mitotic spindle where the principal components are centrosomes (also called spindle poles) and microtubules. Normal mitosis is divided into five key stages; prophase, prometaphase, metaphase, anaphase and telophase (Fig. 2). The first step of mitosis is prophase when replicated DNA condenses to form individual chromosomes and the mitotic spindle starts to assemble. Centrosomes that duplicated in S phase move apart in early mitosis/late G2. At the end of prophase nuclear envelope breakdown (NEB) marks the start of the next mitotic stage, prometaphase. In early prometaphase, chromosomes attach to the mitotic spindle through sister kinetochores, which are proteinaceous organelles assembled on the centromeric region of the sister chromatids. During prometaphase, unattached kinetochores create a signal for the SAC keeping it active until each chromosome have achieved proper bipolar connection with the spindle microtubules and moved to the cell equator. This marks the start of metaphase during which the SAC is inactivated allowing entry into the next stage, anaphase. Anaphase can be divided into early and late anaphase or anaphase A and B. At anaphase A, sister chromatids separate synchronously and move towards the opposite spindle poles. This is achieved by the shortening of kinetochore microtubules. When chromatids are fully separated, anaphase B begins. This involves elongation and sliding of polar microtubules relative to each other to drive the spindle poles to the opposite sides of the cell. At telophase, separated chromatids arrive to spindle poles and decondence. Also, the cytoplasm and other cell organelles are divided between the two daughter cells and a contractile ring of actin and myosin pinches the cell into two daughters at process termed cytokinesis to complete mitosis. Finally, the nuclear envelope reforms around the nucleus in the progeny cells.

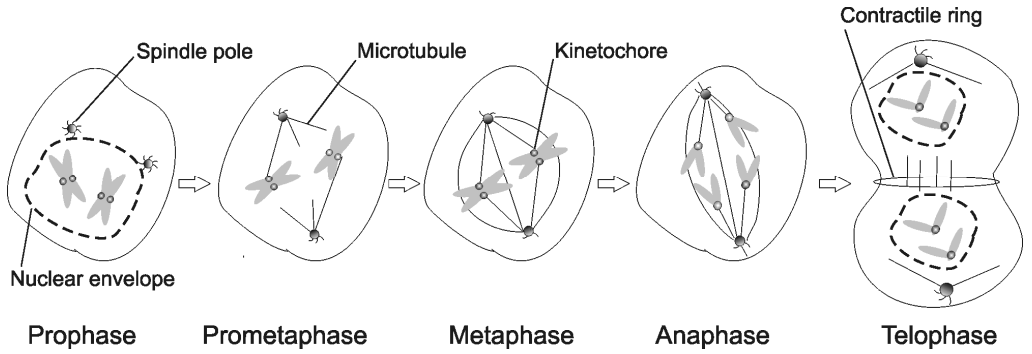

Figure 2. Mitotic phases and central structures.

2.1.3 Mitotic structures

2.1.3.1 Kinetochores and centromeres

Bipolar attachments of chromosomes to spindle microtubules are essential for accurate chromosome segregation. Kinetochores are macromolecular complexes that assemble on centromeric chromatin of the condenced chromosomes. They are composed of numerous conserved proteins and protein complexes that direct kinetochore assembly, maturation, microtubule capture and chromosome movements. The kinetochore also participates in regulation of SAC activity and therefore, is an integral component of the quality control mechanisms that detect and correct defective or nonproductive microtubule-chromosome interactions. Currently, more than 80 kinetochore protein components have been identified in humans and many of them form defined sub complexes with specific tasks (Cheeseman and Desai 2008). A trilaminar morphology of the kinetochore (Fig. 3) was revealed already more than four decades ago (Brinkley and Stubblefield 1966). Each chromosome has two sister kinetochores organized in the following manner. The inner kinetochore forms an interface with the chromatin region whereas the outer kinetochore region forms the interaction surface for the spindle microtubules. The inner and outer layers are connected by a fibrous gap termed the central kinetochore region. In the absence of microtubule attachments, a dense array of fibers called the corona can be observed extending away from outer kinetochore (Ris and Witt 1981). The outer kinetochore and fibrous corona contain the majority of known microtubule interacting proteins as well as SAC proteins (Fig. 3). The major themes in kinetochore composition and organization are conserved throughout eukaryotes (Chan et al. 2005, Cheeseman and Desai 2008).

Chromatin directs kinetochore assembly. It is important that only one centromere exists per chromosome. Many organisms lack the specific centromeric DNA sequence that determines the site of kinetochore formation. In higher eukaryotes, the centromeric DNA is normally characterized by highly repetitive tandem sequences. Human centromeric DNA is enriched in tandem repeats of a 171-base pair α-satellite DNA sequence including a motif which represents the binding site for centromeric protein CENP-B (Masumoto et al. 1989). Nevertheless, it has been contradictory whether these sequences are necessary for kinetochore assembly and maintenance (Amor and Choo 2002, Ohzeki et al. 2002, Okada et al. 2007). Nowadays, the site of kinetochore assembly is thought to be controlled by epigenetic rather than sequence based mechanisms (Allshire and Karpen 2008). The evidence for epigenetic regulation was obtained from studies of the chromosomes which contain two blocks of centromeric DNA but segregate normally because only one site is able to recruit centromere proteins and form a kinetochore. The other site is inactivated in an epigenetic manner (Agudo et al. 2000, Earnshaw and Migeon 1985, Sullivan and Schwartz 1995, Sullivan and Willard 1998, Warburton et al. 1997). However, the molecular basis of the centromere inactivation has remained unknown. In addition, kinetochores are able to form at sites lacking any normal centromeric sequences in fly and in human and propagate centromere function faithfully (Alonso et al. 2007, Choo 2001, Lo et al. 2001, Warburton 2004, Williams et al. 1998). The first human kinetochore proteins

identified were centromeric proteins CENP-A, CENP-B and CENP-C (Earnshaw and Rothfield 1985). CENP-A is a variant of histone H3 which is a core subunit of nucleosomes and it physically marks centromeres by assembling into a nucleosomal structure (Palmer et al. 1991, Sullivan et al. 1994). CENP-A nucleosomes are found at active but not at inactive centromeres and are strong candidates to carry the epigenetic centromeric mark (Black and Bassett 2008). Many studies have clearly shown that CENP-A is essential for the establishment and maintenance of centromeric function and kinetochore assembly (Blower and Karpen 2001, Heun et al. 2006, Stoler et al. 1995, Sullivan et al. 1994).

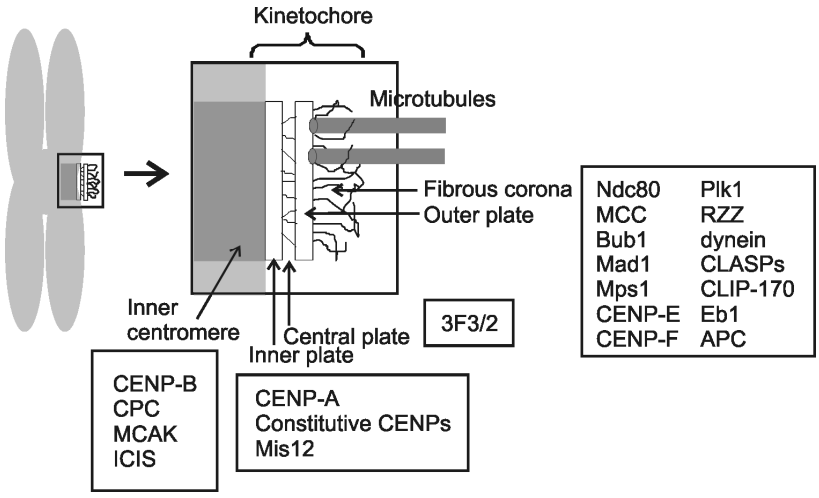

Figure 3. Kinetochore structure and major components (adapted from Maiato et al. 2004).

Kinetochore components assemble step-wise from the centromere to the outer kinetochore. Hierarchy is less linear at the outer kinetochore and fibrous corona where the assembly of components and their occupancy at these structures is transient and influenced by, for example, the microtubule attachment state. The temporal order of the kinetochore proteins also reflects the hierarchical relationship among the proteins (Cheeseman and Desai 2008). Purification of CENP-A nucleosomes revealed a network of proteins that is constitutively present at centromeres throughout the cell cycle (Foltz et al. 2006). This network includes CENP-C and 13 interacting centromeric-proteins (CENPs). Additional proteins are recruited to kinetochores in late G2 or at the specific stage of mitosis and depleted or delocalized from kinetochores in response to microtubule attachment or anaphase onset or at the end of mitosis. The other kinetochore assembly pathway in mammalian systems was found to be organized through the Mis12 complex independently from CENP-A (Goshima et al. 2003). Moreover, a protein termed KNL-1 was discovered to be a structurally important protein for kinetochore assembly (Cheeseman et al. 2004, Desai et al. 2003, Nekrasov et al. 2003). The amounts of the proteins that are mentioned above and members of Ndc80 complex do not change significantly at the kinetochores from late prophase to late anaphase and according to photobleaching assays they turn over slowly at the kinetochore (Hori et al. 2003, Joglekar et al. 2006, Mikami et al. 2005). Therefore,

these proteins together with centromeric chromatin are considered to comprise the stable kinetochore structure.

2.1.3.2 Centrosomes

Centrosomes function as microtubule organizing centres of the cell. The centrosome duplicates during interphase of the cell cycle and the duplicates separate in the beginning of mitosis / late G2 phase. The microtubules emanating from these two spindle poles form the mitotic spindle. Having only two centrosomes per cell in mitosis ensures assembly of a bipolar spindle apparatus and thereby equal partitioning of the sister chromatids and maintenance of genomic stability. Centrosome amplification elevates the potential for unequal chromosome segregation and is linked to increased chromosomal instability and tumor progression (Srsen and Merdes 2006). Centrosomes of yeast and animal cells differ fundamentally in their structure. In animal cell, the centrosome typically contains two cylindar structures, centrioles, oriented perpendicularly to each other. Each centrosome contains one old (mother) and one new (daughter) centriole born during the centrosome duplication in S phase. However, it has been demonstated that centrioles are not essential for the function of microtubule organizing centres (Basto et al. 2006, Hinchcliffe et al. 2001, Khodjakov et al. 2000). Centrioles are surrounded by the pericentriolar matrix. One component of the pericentriolar matrix, which is known to be involved in microtubule nucleation, is γ-tubulin and it appears to be present at centrosomes from species to species (Fuller et al. 1995, Gunawardane et al. 2003, Oakley and Oakley 1989). γ-tubulin forms a complex, known as the γ-tubulin ring complex based on its characteristic ring shape, with the members of a conserved protein family (Luders and Stearns 2007). Normally, microtubules organize such that the minus-end is proximal to centrosome and the more dynamic plus-end extends away. The γ-tubulin ring complex takes part in microtubule anchoring by stabilizing the minus-end of the microtubules by capping it, and thereby preventing depolymerization (Wiese and Zheng 2000). The exact mechanisms of microtubule nucleation, anchoring and release from the centrosome still remain unknown.

2.1.3.3 Microtubules

Microtubules are long hollow cylinders that are 25 nm in diameter. They consist of 12-15 parallel protofilaments that are formed by head-to-tail association of αβ-tubulin dimers. The fixed orientation of dimers gives microtubules structural polarity. Each α- and β-monomer has a binding site for GTP and GTP hydrolysis is important for microtubule dynamics. However, the GTP bound to α-monomer is stable and only the GTP bound β-monomer is exchangeable. Microtubules exhibit a specialized polymerization behavior, called dynamic instability, where growth (rescue) and rapid shrinkage (catastrophe) coexist at steady state (Mitchison and Kirschner 1984). In interphase, microtubules are typically long and undergo rare catastrophes. During mitosis, microtubules are much more dynamic. The more dynamic end of a microtubule is called the plus end where the β-subunits are exposed. The less dynamic end, where α-subunits are exposed, is called the minus end (Desai and Mitchison

1997). The plus-ends of certain type of microtubules, called interpolar microtubules, can interact with the plus-end of microtubules enamating from the other pole. This interaction leads to formation of an antiparallel array in the spindle midzone. The microtubules that attach to the kinetochores are termed kinetochore microtubules. The spindle apparatus also contains astral microtubules that radiate outward from the poles and contact the cell cortex to help in positioning the spindle in the cell (Desai and Mitchison 1997, Fig. 4).

2.1.3.4 Spindle assembly

The mitotic spindle forms as the opposite spindle poles become connected through microtubule bundles to the kinetochores. Mitotic spindle assembly is not completely understood and there are two theories trying to explain it: The one based on the centrosomes and the other on chromosomes (O'Connell and Khodjakov 2007). According to search-and-capture hypothesis microtubules nucleate from centrosomes and their plus-ends randomly explore space via their dynamic instability until the contact with a kinetochore results in capture and suppression of microtubule's dynamics. More microtubules find to the same kinetochore and attach. This process results in decrease in the number of astral microtubules and increase of kinetochore attached microtubules (Hayden et al. 1990, Kirschner and Mitchison 1986, Rieder and Alexander 1990). This mechanism, however, has been proven to be ineffective (Wollman et al. 2005) and is not applicable to the cells that lack centrosomes. The other mode of spindle assembly was suggested in meiotic frog eggs and in several acentrosomal systems where the chromatin drives the spindle formation (Heald et al. 1996, Karsenti et al. 1984, Khodjakov et al. 2000). A pathway for microtubule nucleation and stabilization in the vicinity of the chromatin that depends on the activity of a small GTPase Ran was discovered (Carazo-Salas et al. 2001, Wilde et al. 2001). Ran-GTP is present in a concentration gradient around mitotic chromosomes (Kalab et al. 2002). Interestingly, several studies with somatic cells have demonstrated that noncentrosomal microtubules can form in the vicinity of the centromere and orient through cytoplasmic nucleation followed by the attachment of plus ends to the kinetochores (Khodjakov et al. 2003, Maiato et al. 2004, Tulu et al. 2006, Witt et al. 1980). Thus, the two theories of the spindle formation are not completely exclusive. Instead, they appear to contribute simultaneously to the spindle assembly. It has been suggested that microtubules nucleating from the kinetochores are ultimately combined with those originating from the centrosomes through capture of astral microtubules by the kinetochore nucleated microtubules (Khodjakov et al. 2003, Maiato et al. 2004). The integration of different components into one common structure requires motor proteins.

2.1.3.5 Motor proteins

Microtubule end-binding proteins are required to generate the core attachment site at the kinetochores, couple kinetochore movement to disassembling microtubules and affect the polymerization dynamics of the kinetochore-bound microtubules. Microtubule polarity is essential to motor protein movement along the microtubules to

transport cargo to specific cellular locations. Many different kinds of cargoes such as chromosomes, specific proteins or other microtubules are transported along the microtubules. Microtubule binding of microtubule motor proteins takes part in so called sliding-filament mechanism of the spindle assembly (Gatlin and Bloom 2010). Kinesins are microtubule-based plus-end directed motor proteins and many members of this family are involved in mitosis (Yildiz and Selvin 2005). An important kinesin in mitosis is Eg5 which drives the poleward movement of microtubules taking part in bipolar spindle establishment (Kapitein et al. 2005, Kashina et al. 1996). Blocking the function of Eg5 prevents centrosome migration and arrest cells in mitosis with monopolar spindles (Kapoor et al. 2000, Mayer et al. 1999). Some kinesins bind DNA and take part in chromosome segregation (Wang and Adler 1995) while other kinesins destabilize microtubules (Desai et al. 1999). A central microtubule destabilizing kinesin in mitosis is the centromere associated kinesin, MCAK, which is thought to mediate the poleward motion of the separated chromosomes by shrinking the length of the microtubules (Maney et al. 1998). It also contributes to correction of erroneous kinetochore-microtubule attachments by destabilizing the mal-oriented connections (Kline-Smith et al. 2004). Minus-end directed motor protein, dynein, is a multisubunit protein and has various roles both at interphase and at mitosis. During mitosis, dynein participates in spindle orientation, chromosome movement and spindle pole organization (Karki and Holzbaur 1999). Dynein interacts with another multisubunit complex, dynactin, which is involved to link dynein to the kinetochores in mitosis (Echeverri et al. 1996). In addition to motor proteins, a group of nonmotor proteins take part in the function of the microtubule plus ends such as cytoplasmic linker protein (CLIP)-170, CLIP-associating protein (Clasp)-1 and -2, end-binding protein (EB)-1 and adenomatous polyposis coli (APC). They take part in stabilization of the microtubules and link the microtubule ends to different cellular structures such as the kinetochores or the cell cortex (Jiang and Akhmanova 2010).

2.1.4 Microtubule binding at the kinetochores

Kinetochore-microtubule attachments generate forces that are required for chromosome movements during mitosis, such as biorientation of the chromosomes and chromosome segregation in anaphase. Spindle microtubules can also create rapid poleward chromosome movements during early phases of chromosome capture which are caused by transient lateral attachments of spindle microtubules with the kinetochores (Kops et al. 2010). At the end, approximately 15-20 microtubules bind end-on to a kinetochore in humans (McEwen et al. 2001). Recently, it was shown that the main microtubule attachment site is comprised by the KNL-1 protein, the Mis12 complex and the Ndc80 complex (KMN network) (Cheeseman et al. 2006, Wei et al. 2007). Furthermore, it has been shown that the four membered Ndc80 complex consisting of Ndc80 (highly expressed in cancer 1 (Hec1) in human), Nuf2, Spc24 and Spc25, is essential for kinetochore-microtubule attachments (DeLuca et al. 2002, McCleland et al. 2003). Ndc80 and Nuf2 localize to the outer regions of kinetochores (DeLuca et al. 2005) and can directly bind microtubules (Wei et al. 2007). However, the microtubule binding affinity is increased when the Ndc80 complex associates with KNL-1 and Mis12 (Cheeseman et al. 2006). In vertebrates, both the Mis12 complex

and the constitutive CENP proteins influence the Ndc80 complex localization to the kinetochores (Kline et al. 2006, Okada et al. 2006). Other proteins which might function in parallel with the KMN network include the motor protein dynein and kinesin CENP-E. Dynein accumulates on unattached kinetochores and becomes depleted when initial lateral interactions of the microtubules to the kinetochores mature into end-on attachments (King et al. 2000). It has been shown that elimination of CENP-E diminishes the number of microtubules bound to each kinetochore (McEwen et al. 2001). In addition, large coiled-coil protein CENP-F has a weak microtubule binding affinity and it associates with some dynein binding proteins (Feng et al. 2006, Vergnolle and Taylor 2007).

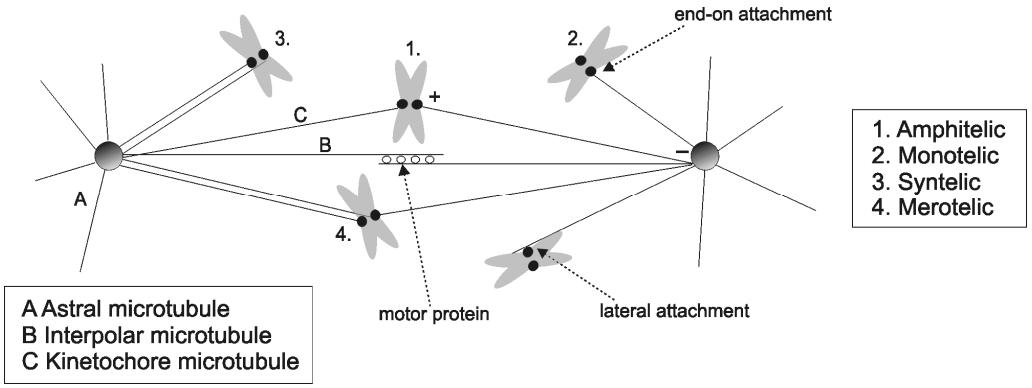

Figure 4. Microtubules and kinetochore-microtubule attachments. The mitotic spindle contains different kind of microtubules (A-C). In early stages of mitosis, the chromosomes are found in many orientations (1-4, partially adapted from Cheeseman and Desai 2008).

2.1.5 Kinetochore-microtubule mis-attachments

For accurate chromosome segregation, chromosomes must receive amphitelic (bipolar) microtubule attachments (Fig. 4). This interactions forms when sister kinetochores interact with microtubules from opposite spindle poles. However, it has been shown that erroneous microtubule-kinetochore interactions such as monotelic, syntelic or merotelic attachment types can occur in early mitosis (Fig. 4). A monotelic orientation forms when one sister kinetochore becomes attached to one pole and the other remains unattached. This leads to positioning of the chromosome close to the pole to which it is attached. Monotelic orientations are an obligatory step in chromosome congression, but usually they are transient (Rieder and Salmon 1998). The SAC can detect monotelic attachments and delay the cell in preanaphase stage to enable the formation of a correct attachment type and chromosome orientation (Musacchio and Salmon 2007, Rieder et al. 1995). A syntelic orientation is formed when sister kinetochores of a chromosome attach to the same spindle pole. The syntelic chromosome typically localizes close to this pole. However, syntelic orientations are rarely observed in normal tissue culture cells suggesting that they occur infrequently or they are short-lived (Hauf et al. 2003). Also, syntelic attachments activate the SAC (Pinsky and Biggins 2005) and become corrected. A merotelic orientation occurs when one kinetochore binds microtubules

from both spindle poles. Unlike monotelic or syntelic chromosomes, merotelically attached chromosomes can align to metaphase plate and importantly also escape the SAC control. Typically, the cells possessing merotelic orientations do not arrest in mitosis, but enter the anaphase without significant delay (Cimini et al. 2003, Cimini et al. 2004). This leads to a tug-of-war between the two attached microtubule bundles that are connected to opposite spindle poles. In anaphase, the net movement of the merotelic chromosome depends on the relative strenghts of the attached microtubule bundles. If the sizes/strenghts of the bundles are equal, the merotelic chromosome will lag behind at the spindle equator in anaphase (Cimini et al. 2004, Salmon et al. 2005). In cytokinesis, the lagging chromosomes may induce aneuploidy. Nevertheless, a correction mechanism exists before the anaphase onset for merotelic attachments to reduce the number of microtubules bound to the incorrect pole. Currently, it is thought that this pre-anaphase correction mechanism functions by promoting the turnover of the kinetochore-microtubules attached to the wrong pole at the higher rate than the correctly oriented microtubules (Cimini et al. 2006, Cimini 2007, DeLuca et al. 2006).

2.2 Spindle assembly checkpoint

2.2.1 Main principle of the SAC

Sister chromatid separation in anaphase should take place only when all chromosomes are attached to the spindle apparatus in a bipolar manner and when proper tension is applied across the sister centromeres. Since mitosis is a very complex and error-prone process, a special evolutionarily conserved control mechanism termed the spindle assembly checkpoint (SAC), has evolved to regulate the fidelity of chromosome segregation. Although the main principles of SAC are solved, many issues remain to be elucidated. Critical questions concern the mechanism of checkpoint activation in early mitosis and inactivation before anaphase. The most complex puzzle in the regulation of SAC is what becomes sensed and through which events the SAC becomes satisfied allowing mitosis to proceed. It is well established that the SAC works to monitor connections between the kinetochores and spindle microtubules. Unattached kinetochores are believed to play a major role in the SAC signal generation producing a diffusible “wait anaphase” signal (Fig. 5) that inhibits the completion of mitosis (Cleveland et al. 2003, Musacchio and Salmon 2007). The number of unattached kinetochores is progressively reducing during prometaphase but still a strong SAC signal is maintained indicating the presence of a signal amplification loop. Indeed, the onset of anaphase in vertebrate cells has been shown to be blocked even by a single unattached kinetochore (Rieder et al. 1995). The addition of spindle poisons that interfere with microtubule dynamics or impair a microtubule motor function typically blocks the anaphase onset in a SAC-dependent manner (Clute and Pines 1999, Rieder and Maiato 2004). However, the SAC can adapt to the prolonged exposure to spindle poisons and for an unknown reason fail to keep the cells at mitotic block (Rieder and Maiato 2004). It has been reported that SAC proteins are retained at the kinetochores during prolonged mitosis indicating that the cells do not escape from M phase because of the SAC signal inactivation. In fact, the mitotic slippage has been shown to occur rather via slow but continuous degradation of a key mitotic regulator, cyclin B, in the

presence of active SAC driving the cell out of mitosis (Brito and Rieder 2006). Proteins involved in the SAC have been shown to concentrate on unattached kinetochores in mitosis and become depleted from the kinetochores upon microtubule attachment and chromosome bi-orientation (Chen et al. 1996, Meraldi et al. 2004, Taylor and McKeon 1997). This is important for the progressive downregulation of SAC.

2.2.2 Molecular mechanism of the SAC

2.2.2.1 Anaphase promoting complex/ cyclosome

A regulated degradation of mitotic proteins is a key event for the ordered chromosome segregation and mitotic progression. A mitotic E3 ubiquitin ligase termed the anaphase-promoting complex/cyclosome (APC/C) has a central role in this mechanism. The APC/C ubiquitylates substrates whose destruction is mandatory for sister chromatid separation and exit from M phase. The activation of APC/C requires a protein called cell division cycle 20 (Cdc20). This protein forms an APC/C inhibitory complex with three other SAC proteins (Sudakin et al. 2001). The signal created at unattached kinetochores delays mitosis by inhibiting the dissociation of Cdc20 from the inhibitory complex and blocking the function of APC/C. When all chromosomes are properly attached to spindle microtubules, Cdc20 dissociates from the complex and activates the APC/C. The APC/C mediates the destruction of two mitotic regulators, cyclin B and securin, by the 26S proteasome (Peters 2006). Degradation of cyclin B leads to inactivation of Cdk1, a master mitotic kinase, and thereby to exit from mitosis (Murray et al. 1989). Destruction of securin results in activation of protease called separase. Securin is an inhibitor of separase, which is required to cleave cohesins, “clue proteins”, that hold the sister chromatids together. Cohesin cleavage is necessary for the sister chromatid separation in anaphase (Nasmyth 2001). At anaphase the loss of sister chromatid cohesion results in loss of interkinetochore tension. However, this does not reactivate the SAC indicating that the SAC is inhibited at anaphase. Proteolysis of cyclin B and inactivation of the Cdk1-cyclin B kinase activity possibly has an important function in the SAC inactivation (D'Angiolella et al. 2003, Potapova et al. 2006). In vertebrates, anaphase is triggered only when both securin and cyclin B are almost completely degraded (Clute and Pines 1999, Hagting et al. 2002). This means that even if the SAC reactivated in anaphase it would not have any consequence for cell cycle progression.

2.2.2.2 Mitotic checkpoint complex

The main tasks of SAC are mediated by the members of the mitotic arrest deficient (Mad), budding uninhibited by benzimidazole (Bub), and Cdc20 protein families (Hoyt et al. 1991, Hwang et al. 1998, Kim et al. 1998, Li and Murray 1991). The SAC targets the APC/C activator Cdc20, which forms a complex with Mad2, BubR1 (Mad3 in yeast) and Bub3 (Fig. 5). This complex, termed the mitotic checkpoint complex (MCC), is the possible effector of SAC and is thought to function mainly as an inhibitor of the APC/C (Sudakin et al. 2001). The MCC proteins are highly dynamic at

the kinetochores with average turnover times ranging approximately from 2 to 25 s (Howell et al. 2000, Howell et al. 2004, Kallio et al. 2002a). At least two MCC subcomplexes have been reported to form; Mad2-Cdc20 and Bub3-BubR1-Cdc20. Mad2 and BubR1 bind directly to Cdc20 on distinct sites and they have synergistic effect on APC/C inhibition (Fang 2002, Tang et al. 2001). It is still unclear how and when the MCC is created, but it appears that the binding of BubR1 to Cdc20 requires previous binding of Mad2 to Cdc20 (Chen 2002, Davenport et al. 2006). Mad2 interaction with Cdc20 inhibits the activation of the APC/C (Li et al. 1997). BubR1 associates with the APC/C in addition to Cdc20 (Chan et al. 1999). This binding might regulate the inhibition of the APC/C activity (Shannon et al. 2002, Tang et al. 2001, Wu et al. 2000). The SAC activation requires both Mad2-Cdc20 and Bub3-BubR1-Cdc20 subcomplexes (Millband and Hardwick 2002, Shannon et al. 2002). In addition, there is evidence that mitotic kinases such as Bub1, MAPK and Cdk1 phosphorylate Cdc20 to modulate its binding affinity with Mad2 and BubR1 (Chung and Chen 2003, D'Angiolella et al. 2003, Kramer et al. 2000, Tang et al. 2004b, Wu et al. 2000, Yudkovsky et al. 2000). Moreover, Mad2 appears to be phosphorylated by an unknown kinase (Wassmann et al. 2003). When all chromosomes attain bipolar orientation and move to the middle of the spindle, the production of Mad2-Cdc20 and Bub3-BubR1-Cdc20 subcomplexes ceases. Free Cdc20 is then available to activate the APC/C resulting in destruction of anaphase inhibitors such as securin. The onset of anaphase occurs and the APC/C continues to target proteins whose degradation is essential for exit from mitosis (Peters 2006). As the SAC is regulated by phosphorylation, several phosphatases likely play an important role in the SAC inactivation.

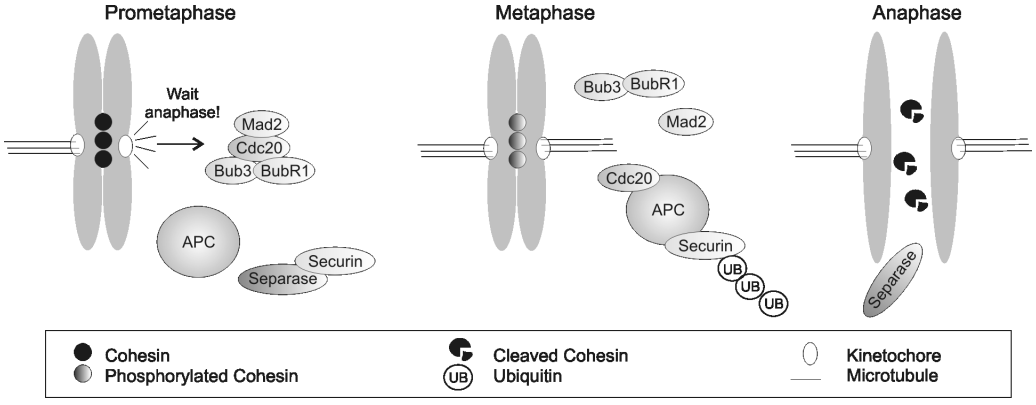

Figure 5. Main principle of the SAC (based on Musacchio and Hardwick 2002).

Mad2 binding to Cdc20 is a complex reaction. Kinetochore Mad2 was found to consist of two pools: the one being more stably bound and the other being highly dynamic (Shah et al. 2004). Nowadays, it is known that Mad2 exists in two different conformations termed open (O-Mad2) and closed (C-Mad2) which differ for the position of the C-terminal tail and have different inhibitory activities towards Cdc20 (Mapelli and Musacchio 2007). A discovery of the two conformers have provided so called template model for Mad2 regulation (De Antoni et al. 2005). Free O-Mad2 can stably bind Mad1 at unattached kinetochores. Mad1 is a protein that binds kinetochores

in a relatively stable manner during metaphase (Chen et al. 1998, Chen et al. 1999, Chung and Chen 2002, De Antoni et al. 2005). The Mad1-Mad2 complex is then able to convert inactive O-Mad2 to active C-Mad2. This form of Mad2 can bind to Cdc20 and serve as a template for activating the next free Mad2 molecule (Luo et al. 2002, Luo et al. 2004). The template model provides an explanation for the diffucible nature of the SAC signal and explains why a single unattached kinetochore can generate a robust signal to inhibit the anaphase onset (Musacchio and Salmon 2007).

2.2.2.3 Other central SAC components

In addition to the MCC, there are other proteins required for the generation, maintenance, and turning off the SAC signal. The BubR1 kinase activity is regulated by **CENP-E**, a microtubule plus-end-directed motor, when it is not attached to microtubules. It has been suggested that the prevention of BubR1 activation by CENP-E silences the SAC (Mao et al. 2003). However, the necessity of the BubR1 kinase activity in the SAC function has been challenged by another study. It was shown that defects caused by BubR1 depletion such as reduced kinetochore binding of many SAC proteins and impairment of the interaction between the MCC members could be rescued by BubR1 lacking its kinase activity (Chen 2002). Furthermore, BubR1 interacts with and phosphorylates CENP-E, which takes part in chromosome alignment (Chan et al. 1998, Chan et al. 1999, Yao et al. 2000). The effect of CENP-E depletion to the SAC has been difficult to interpret in many species, but in *Xenopus* the immunodepletion of CENP-E or antibody addition appeared to override the SAC suggesting CENP-E as a component of the SAC pathway (Abrieu et al. 2000). The **Bub1** kinase is more stable kinetochore protein than BubR1 or Bub3 (Howell et al. 2004). Bub1 levels at the kinetochore are downregulated after the kinetochore-microtubule attachment and after establishment of the inter-kinetochore tension (Taylor et al. 2001). Bub1 contributes to the centromere-kinetochore recruitment of various SAC proteins and probably to the structural stability of the centromere-kinetochore (Johnson et al. 2004, Liu et al. 2006, Sharp-Baker and Chen 2001, Vigneron et al. 2004). Bub1 also directly contributes to the formation of the complex of MCC with APC/C together with the Aurora B kinase (Morrow et al. 2005). Another kinase, **Mps1,** is a core component of the SAC (Weiss and Winey 1996). Mps1 is very dynamic at the kinetochores ($t_{1/2}$ 10 s) and it probably has more regulatory role rather than structural role in recruiting Mad1-Mad2 to the kinetochores (Howell et al. 2004). The chromosomal passenger complex (**CPC**) containing the Aurora B kinase is a candidate for being the tension-sensor of the kinetochores. It is required to arrest the cell cycle temporarily under conditions that prevent the creation of the inter-kinetochore tension (Biggins and Murray 2001). Aurora B destabilizes incorrect kinetochore-microtubule attachments generating unattached kinetochores. Thus, the CPC indirectly contributes to the regulation of SAC. However, more direct roles for the CPC in the SAC has been described, presumably in promoting the MCC assembly (Vader et al. 2008). In the chapters below, the cellular functions of CPC are described in more details.

The main event in turning off the SAC is the removal of Mad1, Mad2 and other key checkpoint components from the kinetochores after which they typically become redistributed to the spindle poles (Maiato et al. 2004). In metazoans, this requires motor protein **dynein** motility along the microtubules (Howell et al. 2001). Another mechanism of inactivation involves protein **p31^{comet}** , a Mad2 binding protein, which negatively regulates the SAC (Habu et al. 2002, Xia et al. 2004). It competes with O-Mad2 for the binding to C-Mad2 *in vitro* (Vink et al. 2006, Xia et al. 2004). Kinetochores may negatively regulate the binding of p31^{comet} and allow the O-Mad2 binding to C-Mad2. It is speculated that p31^{comet} may be reactivated at metaphase resulting in the SAC inactivation (Vink et al. 2006). Consistent with this idea, the reduction p31^{comet} delays mitotic exit after release from nocodazole-induced arrest in Hela cells (Habu et al. 2002, Xia et al. 2004).

Table 1: Summary of proteins and protein complexes assosiated to the SAC function

Protein or complex	**SAC assosiated function**
Cdk1	Cdk1 is a key mitotic kinase. Its activity requires binding with cyclin B.
cyclin B	Cyclin B is a central target for the APC/C and its degradation leads to inactivation of Cdk1. Cyclin B degradation is required for mitotic exit.
APC/C	The APC/C is a mitotic E3 ubiquitin ligase. It marks mitotic proteins for degradation facilitating mitotic progression.
securin	Securin is a central target for the APC/C. Securin binds separase. Securin degradation leads to the activation of separase.
separase	A protease separase cleaves cohesin molecules between the sister chromatids.
cohesin	Cohesins are the "clue proteins" that hold the sister chromatids together. Cohesin cleavage by separase is required for the onset of anaphase.
Cdc20	Cdc20 is an activator of the APC/C. It forms the MCC with Mad2, BubR1 and Bub3 which functions as an inhibitor of the APC/C.
Mad2	Mad2 localizes to unattached kinetochores associating with Mad1. It binds Cdc20 and inhibits the APC/C function. Mad2 is a part of the MCC.
BubR1	BubR1 is a mitotic kinase whose activity is regulated by CENP-E. It is a member of the APC/C inhibitory complex MCC.
Bub3	Bub3 is required for the kinetochore targeting of BubR1 and Bub1. It is a member of the MCC.
Bub1	Bub1 is an important mitotic kinase. It is required for the kinetochore recruitment of various mitotic proteins.
MAPK	MAPK is implicated in the SAC but the exact function is unclear.
Mad1	Mad1 recruits Mad2 to kinetochores. It is a substrate of Bub1 and Mps1.
CENP-E	CENP-E is a microtubule plus-end-directed motor. It regulates BubR1 activity.
Mps1	Mps1 is a regulatory kinase related to the SAC function. It is required for Mad1-Mad2 localization to the kinetochores.
CPC	The CPC is formed by the Aurora B kinase, INCENP, Survivin and Borealin. It is required for the correction of erroneous kinetochore-microtubule attachments and it participates in the SAC by e.g. creating unattached kinetochores.
dynein	Dynein is a microtubule minus-end-directed motor. It is required for the redistribution of the SAC components from the kinetochores to the spindle poles.
p31^{comet}	p31^{comet} binds Mad2. It is suggested to negatively regulate the SAC and take part in the SAC inactivation.

2.2.3 Attachment and tension

Both the lack of kinetochore-microtubule attachment and the absence of interkinetochore tension have been proposed to control the SAC signaling. The condition where both of them are present is believed to satisfy the SAC and turn it off enabling mitotic progression. The treatment of cells with microtubule depolymerizing

drugs (for example nocodazole) prevents the microtubule attachment to the kinetochores and satisfaction of the SAC keeping it active (Musacchio and Salmon 2007). On the other hand, when a kinetochore lacks a microtubule attachment, the kinetochore-centromere region is not under tension. Thus, the lack of attachment and the lack of tension are inseparable and many times present at the same time. For this reason, it has been difficult to distinguish the role of the attachment from that of the tension in the process of SAC satisfaction.

The hypothesis where the two inputs, attachment and tension, are involved in the SAC signaling has been suggested. According to this model, Mad2 and Mad1 become highly enriched at the unattached kinetochores and mediate signal for an unattached chromosome (Waters et al. 1998). In addition, a "low tension" signal is associated with kinetochores harbouring Bub proteins as well as a kinetochore phosphorylation that can be detected with the phospho-specific BubR1 antibodies and the 3F3/2 antibody. The 3F3/2 antibody recognizes phospho-epitope(s) at the tensionless kinetochores and one of these epitopes is reported to be phosphorylated by Plk1 (Ahonen et al. 2005, Gorbsky and Ricketts 1993, Wong and Fang 2007). Moreover, BubR1 is phosphorylated on Ser676 by Plk1 in prometaphase and this site is dephosphorylated upon establishment of interkinetochore tension (Elowe et al. 2007).

Based on other hypothesis, cells detect biorientation by monitoring the elevated tension state between sister kinetochores (McIntosh 1991). This hypothesis is supported by classical micromanipulation studies in the praying mantid spermatocytes. In these studies, the cells delayed in anaphase in the presence of a single tensionless chromosome, but when tension was artificially applied with a microneedle, the cells were able to complete cell division (Li and Nicklas 1995). Application of tension also reduced the phosphorylation state of the kinetochore associated phospho-epitope recognized by the 3F3/2 antibody (Nicklas et al. 1995, Nicklas et al. 1998). Furthermore, reducing tension with a microtubule stabilizing drug, taxol, induced accumulation of the 3F3/2 phospho-epitope to the kinetochores as well as hyperphosphorylation of BubR1 leading to mitotic delay (Elowe et al. 2007, Waters et al. 1998). Low doses of a microtubule depolymerizing drug, vinblastine, has also been shown to arrest Hela cells in mitosis with bioriented chromosomes exhibiting reduced tension (Skoufias et al. 2001). Similarly, the absence of centromere strech in budding yeast has been observed to prevent the SAC satisfaction (Stern and Murray 2001).

Eventhough, there is strong evidence that the lack of tension may stimulate the "wait anaphase" signal, it has been suggested that tension might not, after all, regulate the SAC directly. Laser ablation studies have demonstated that unattached tensionless kinetochore can mediate an inhibitory signal delaying mitotic progression (Rieder et al. 1995). Results suggested that the lack of tension was not responsible for delaying mitosis because when an unattached kinetochore in a mono-oriented chromosome was destroyed with a laser beam mitotic exit occurred. Later, a role for tension in stabilizing the kinetochore-microtubule attachments started to emerge (Kapoor et al. 2000, King and Nicklas 2000) and the checkpoint response to the lack of tension was found to be dependent on the activity of the Aurora B kinase (Biggins and Murray

2001). It is now known that Aurora B phosphorylates microtubule binding kinetochore proteins reducing their binding affinity and thereby destabilizing specific kinetochore-microtubule attachments (Cheeseman et al. 2002, Cimini et al. 2006, DeLuca et al. 2006).

Recently, it has been suggested that intrakinetochore stretching rather than interkinetochore stretching would be the more relevant parameter for the SAC satisfaction. Data leading to this suggestion was derived from experiments where the SAC was satisfied under conditions that preserve intrakinetochore stretching while interkinetochore stretching is removed, such as in low concentrations of taxol (Maresca and Salmon 2009). It was also observed that mono-oriented chromosomes underwent kinetochore stretching even if the other sister was unattached (Uchida et al. 2009). Intrakinetochore tension might also provide an explanation why the SAC becomes satisfied in cells where the last unattached kinetochore has been laser ablated (Rieder et al. 1995).

2.3 Shugoshin 1

In recent years, a new family of functionally conserved proteins called shugoshins (Sgo, Japanese for "guardian spirit") has been identified and characterized (Kitajima et al. 2004, Rabitsch et al. 2004). Sgo is neither a kinase nor a phosphatase but rather it has turned out to be a multifunctional protein required for many mitotic events. It is well established that Sgo1 and its orthologues in various species play a central role in protecting centromeric cohesion between sister chromatids both in mitosis and meiosis (Kitajima et al. 2004). Moreover, Sgo has been suggested to function in tension-sensing mechanism of the SAC and in microtubule stability (Salic et al. 2004). Recently, evidence about the function of Sgo in centrosome regulation has emerged as well (Wang et al. 2008).

2.3.1 Sgo family and structure

In higher eukaryotes and fission yeast, the Sgo family consists of Sgo1 and Sgo2. In addition, several alternative splice variants of Sgo1 have been found in mouse and human cells (McGuinness et al. 2005). However, only two of them, with molecular weights about 70 kDa (Sgo1) and 42 kDa (sSgo1), have been extensively characterized to date (Wang et al. 2006). In budding yeast, only a single Sgo1 exists which possibly handles alone many functions which are carried out by Sgo2 and Sgo1 splice variant in higher eukaryotes. Mei-S332 from *Drosophila* functionally and structurally resembles Sgo and was identified and characterized long before Sgo1 from other species was found (Kerrebrock et al. 1995, Tang et al. 1998). The vertebrate Sgo proteins and their yeast or invertebrate counterparts exhibit only marginal homology. Nevertheless, all Sgo family members are conserved in certain amino acids that are known to be critical for *Drosophila* Mei-S332 and fission yeast Sgo1 function (Kitajima et al. 2004, Rabitsch et al. 2004, Tang et al. 1998). Human Sgo1 contains two putative destruction motifs that are generally present in the APC/C substrates, D-box and KEN-box (Glotzer et al. 1991, Pfleger and Kirschner 2000). Available evidence indicates that

Sgo1 is degraded through the APC/C pathway (Indjeian et al. 2005, Kitajima et al. 2004, Salic et al. 2004, Tang et al. 2004a). Mei-S332 levels, however, remain similar throughout meiosis in *Drosophila* and only dislocation is suggested at the onset of anaphase (Moore and Orr-Weaver 1998). Despite of two recognition sites for separase, human Sgo1 does not appear to be a target of separase (Salic et al. 2004, Tang et al. 2004a). In addition, there are two putative nuclear localization signals in human Sgo1 and many consensus sites for phosphorylation by mitotic kinases such as Cdk, Aurora kinases, PKA and Plk1. Importantly, in human Sgo1 there is an overlap between D-box and phosphorylation motifs of Cdks, Aurora kinases and PKA suggesting that they might be involved in the regulation of Sgo1 stability (Wang and Dai 2005, Fig. 6).

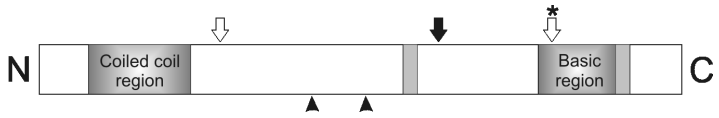

Figure 6. Schematic representation of human Sgo1 structure (partially adapted from Wang and Dai 2005). The putative motifs for nuclear localization signals (light grey boxes), D-boxes (white arrows), KEN-box (black arrow) and separase recognition sites (black arrowheads) are marked. D-box motif near the C-terminus overlaps with Cdk, Aurora kinase and PKA phosphorylation motifs (marked with *).

2.3.2 Chromosome cohesion

Sister chromatids are held together by a multi-subunit protein complex, cohesin (Haering and Nasmyth 2003). Sister chromatid cohesion is established in S phase of the cell cycle and it is maintained during G2 and early stages of cell division. In vertebrates, cohesion along chromosomal arms is destroyed during prophase whereas centromeric cohesion is retained until the onset of anaphase (Waizenegger et al. 2000). In yeast, cohesion retains both at the centromeres and at the chromosome arms until the anaphase onset.

Premature dissolution of centromeric cohesin causes errors in chromosome segregation and may lead to aneuploidy and genomic instability. Haploid germ cells are produced through meiotic cell divisions. Meiosis consists of two cell divisions, without intervening DNA replication, leading to the formation of four haploid gametes. During meiosis I, homologous chromosomes pair and go through an evolutionary important process called recombination. During recombination chiasmata are formed connecting the sister chromatid from one homologue with the sister chromatid of the other homologue. This process requires the removal of cohesion from chromosome arms (Watanabe 2004). Centromeric cohesion is maintained until the onset of anaphase II when it is cleaved by separase as in mitosis. The loss of centromeric cohesin causing premature sister chromatid separation during meiosis I may lead to abnormal chromosome number in forming gametes. This is suggested to be a contributing factor for spontaneous abortions and infertility (Nasmyth 2001).

The mitotic cohesin complex consists of four subunits, a heterodimer of Smc1 and Smc3, and two regulatory subunits Scc1/Rad21 and Scc3/SA in human (Haering and Nasmyth 2003) and it is suggested to form a ring-shaped structure (Gruber et al. 2003). In vertebrates, cohesion is removed from chromosome arms under control of Plk1 and probably Aurora B at prophase independently from the separase activity (Hauf et al. 2005, Sumara et al. 2002, Fig. 7). At the onset of anaphase, active separase cleaves the cohesin subunit Scc1 (Waizenegger et al. 2000). Also in yeast, the removal of centromeric cohesin requires the separase activity. The process is regulated by phosphorylation of Scc1 subunit by Cdc5, the yeast polo-like kinase (Alexandru et al. 2001). In recent years, several molecules have been under investigation to explain the molecular mechanism how centromeric cohesion is protected from dissociation from DNA in early stages of mitosis. Heterochromatin has been suggested to play a role in the maintenance of centromeric cohesion since it is important for the recruitment of cohesin at the centromeres (Bernard et al. 2001, Kitajima et al. 2003). Also, the Sgo family has been shown to function as a protector of centromeric cohesion (Fig. 7).

2.3.3 Function of Sgo in cell division

Subcellular localization of Sgo supports the idea of its essential roles during mitosis. Some studies have shown Sgo1 to localize at the inner centromere region from early mitosis until the initiation of anaphase (Kitajima et al. 2004, McGuinness et al. 2005, Tang et al. 2004a). On the other hand, it has also been detected at the inner and outer kinetochore (McGuinness et al. 2005, Salic et al. 2004). The discrepancy could be explained by the used antibodies, which may recognize different isoforms. For Sgo1 splice variant sSgo1 a totally different mitotic localization pattern has been reported. sSgo1 has been detected at the mitotic spindles and centrosomes during prophase, metaphase and anaphase (Wang et al. 2006). In addition, a Sgo family member Sgo2, has been observed at the inner centromere of unattached kinetochores redistributing towards the outer kinetochore regions after the establishment of tension between the sister kinetochores (Gomez et al. 2007). In meiosis of lower eukaryotes, genetic studies and the localization of Sgo1 homologs suggests a role for it in regulation of cohesion (Katis et al. 2004, Kerrebrock et al. 1995, Kitajima et al. 2004, Marston et al. 2004, Moore and Orr-Weaver 1998, Rabitsch et al. 2004).

2.3.3.1 Sgo in protection of centromeric cohesion

Sgo1 was first characterized in yeast genetic studies in regulation of sister chromatid cohesion (Indjeian et al. 2005, Katis et al. 2004, Kitajima et al. 2004, Marston et al. 2004, Rabitsch et al. 2004). In human cells, the depletion of Sgo1 has been described to induce mitotic arrest coupled with sister chromatid mis-segregation using RNAi and expression of dominant negative mutants (Kitajima et al. 2005, McGuinness et al. 2005, Salic et al. 2004, Tang et al. 2004a). The silencing of Sgo1 in Hela cells have also been shown to diminish the ectopically expressed myc-Scc1 at the centromeres during prometaphase (Kitajima et al. 2005, McGuinness et al. 2005). These results suggest that Sgo1 is required for the centromeric localization of the cohesin subunit, Scc1, or protection of the centromeric cohesin from dissociation. In meiotic yeast cell,

Sgo1 protects chromatids from mis-segregation through regulating the localization of Rec8 (Scc1 in mitosis) and possibly protecting it from separase cleavage during anaphase I (Kitajima et al. 2004, Rabitsch et al. 2004). Timelapse confocal microscopy studies revealed that the cells lacking Sgo1 experience difficulties in chromosome congression and lagging chromosomes can be observed. Furthermore, at the moment when anaphase starts in normal cells, metaphase plate seems to collapse leading to abnormal chromosome segregation in the absence of Sgo1 (McGuinness et al. 2005). These data suggest that human Sgo1 has a central role in protecting the centromeric cohesion in early mitotic phases.

It has been proposed that the task of Sgo1 in the protection of centromeric cohesion is to prevent cohesin phosphorylation (McGuinness et al. 2005). Protein phosphatase 2A (PP2A) associates with Sgo1 in human and yeast cells (Kitajima et al. 2006, Riedel et al. 2006, Tang et al. 2006). PP2A is a heterotrimeric Ser/Thr phosphatase (Janssens and Goris 2001). Immunoprecipitates of Sgo1 were found to contain a catalytic subunit PP2A-C, a scaffold subunit PP2A-A and several isoforms of the specific variant PP2A-B to which Sgo1 was shown to bind (Tang et al. 2006). Similarly as Sgo1 depletion, PP2A silencing by RNAi in human cells and deletion mutants of yeast caused cohesion defects (Kitajima et al. 2006, Riedel et al. 2006, Tang et al. 2006). It was suggested that PP2A counteracts phosphorylation of cohesin by Plk1 thus preventing its dissociation from the chromatin. For example, PP2A dephosphorylated cohesin fragment *in vitro* that had been previously shown to be phosphorylated by Plk1 (Kitajima et al. 2006). *In vivo*, the artificial recruitment of PP2A to the chromosome arms prevented Rec8 phosphorylation and release in yeast meiosis (Riedel et al. 2006). In this system, Sgo1 is required for the PP2A localization while the retention of Rec8 is independent from Sgo1. This suggests that the role of Sgo1 is to bring PP2A to the correct location where it neutralizes cohesin phosphorylation. However, in human cells the localization of Sgo1 is dependent on PP2A and not vice versa (Kitajima et al. 2006, Tang et al. 2006). In human, Sgo2 is associated with PP2A and required for proper PP2A localization (Kitajima et al. 2006, Tanno et al. 2010). Furthermore, Sgo2 functions in the protection of centromeric cohesion. Unexpectedly, in human Sgo1 was still detected at the centromeres in the absence of PP2A in Sgo2 depleted cells. This was explained by the hypothesis that also cytoplasmic PP2A may contribute to the localization of Sgo1 (Kitajima et al. 2006). This theory was more or less revoked by the results showing that Bub1 depletion preventing the targeting of PP2A abolished the localization of Sgo1. In this case, cytoplasmic PP2A pool was present but did not rescue the Sgo1 localization (Tang et al. 2006). It was suggested that PP2A is required to counteract phosphorylation of Sgo1 by Plk1 which would otherwise lead to Sgo1 removal from the chromosomes and premature removal of centromeric cohesin. The mechanism orchestrated by Sgo and PP2A collaboration seems to be conserved from yeast to human. How cells inactivate the shield of cohesin at the right moment still remains unclear.

Bub1 is required to restrict the localization of Sgo1 and PP2A to the kinetochore region both in yeast and human (Kitajima et al. 2004, Riedel et al. 2006). It has been shown that in Bub1-deficient Hela cells Sgo1 is dimished from the kinetochores and a weak

Sgo1 staining can be observed at the chromosome arms (Kitajima et al. 2005, Tang et al. 2004a). This is consistent with the observation that ectopically expressed Scc1 localized along chromosome arms was lost from the centromeres upon Bub1 depletion (Kitajima et al. 2005). The reported consequences of Sgo1 mis-localization, however, are controversial. On one hand, it has been shown that Bub1-deficient cells show the identical phenotype to Sgo1 depletion with the premature loss of sister chromatid cohesion suggesting that the depletion of either protein causes unprotection of the chromosomal cohesin (Tang et al. 2004a). On the other hand, Bub1 depletion was observed to create loosened centromeric cohesion and increased pairing between sister chromatid arms. Thus, it was proposed that the presence of Sgo1 at the chromosome arms prevented the dissociation of cohesin resulting in the tighter arm cohesion in the absence of Bub1 (Kitajima et al. 2005). In some studies, but not all, inactivation of the Aurora B kinase decreased Bub1 levels at the kinetochores (Ditchfield et al. 2003, Hauf et al. 2003, Meraldi and Sorger 2005). Generally, it has been shown that in human cells in the absence of CPC, Sgo1 is delocalized from the kinetochores and observed along the chromosome arms (Kitajima et al. 2005, Riedel et al. 2006). Moreover, the CPC proteins INCENP and Aurora B are directly regulating MEI-S332 in *Drosophila* meiosis (Resnick et al. 2006). INCENP can bind both MEI-S332 and Aurora B possibly bringing them together. Furthermore, Aurora B phosphorylation is essential for the stable centromere localization of MEI-S332 in *Drosophila* (Resnick et al. 2006). Recently, Sgo2 was also shown to be phosphorylated by Aurora B and this phosphorylation was required for PP2A recruitment to the kinetochores in human cells (Tanno et al. 2010).

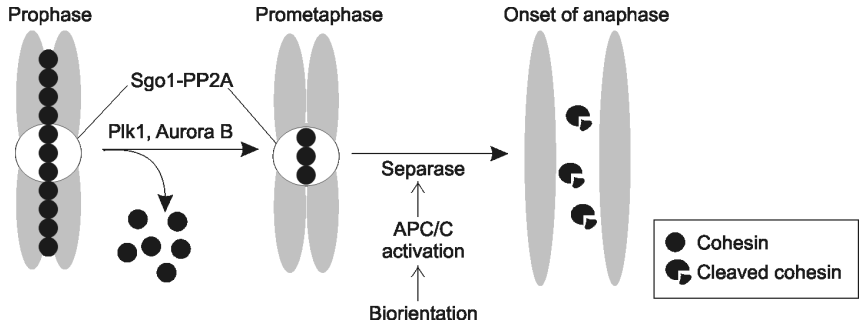

Figure 7. Sgo1 function in protection of centromeric cohesion during mitosis (partially adapted from McGuinness et al. 2005).

Neither Sgo nor PP2A is able to protect the centromeric cohesion alone and thus only when they are together there is a proper protection present. Importantly, it was observed that the depletion of Plk1 rescued the premature sister chromatid separation in PP2A-deficient cells but not in Sgo1-deficient cells (Tang et al. 2006). This implies that Sgo1 do more than just protect cohesin from Plk1 phosphorylation. Since neither Plk1 nor Aurora B is required for cohesin dissociation from the chromosome arms in the absence of Sgo1, it has raised a question whether there are additional protein kinases taking part in the "prophase dissociation" pathway (McGuinness et al. 2005). Alternatively, this pathway includes some events, other than cohesin phosphorylation,

that Sgo1 prevents. Although, the PP2A-independent function of Sgo1 gets support from studies in meiotic yeast cells (Brar et al. 2006, Kitajima et al. 2006) this aspect of Sgo1 function remains to be determined.

2.3.3.2 Sgo in tension sensing, SAC and microtubule dynamics

Sgo1 was identified from a genetic screen for mutants that do not respond to the absence of tension during mitosis in budding yeast (Indjeian et al. 2005). The results indicated that Sgo1 is able to sense and/or mediate tension, promote bi-orientation of the microtubules and delay cell cycle progression into anaphase in the absence of tension. It was suggested that Sgo1 possibly takes part in the SAC signaling by monitoring tension and promoting the detachment of the microtubules that are not attached. When tension is created after the completion of bioriented attachments between microtubules and kinetochores, Sgo1 is inactivated enabling the initiation of anaphase (Indjeian et al. 2005). This resembles the results from Ipl1p (yeast Aurora B) mutants in budding yeast. These mutants are defective specifically in mitotic arrest caused by the lack of tension at the kinetochores but arrest normally at preanaphase if the microtubules are disassembled (Biggins and Murray 2001). Another data suggested that Sgo1 might do more than sense tension. In *Xenopus* egg extracts, the frog Sgo (xSgo) was identified in an assay for proteins that bind and stabilize microtubules (Salic et al. 2004). This suggests a role for Sgo in regulation of microtubule dynamics. Also the observation that human Sgo localize to the inner and outer kinetochore regions, where the microtubules penetrate, provides further support for the idea. In addition, RNAi studies suggested that human Sgo promotes the stability of kinetochore-attached microtubules during mitosis (Salic et al. 2004). Sgo2 is required for sensing tension in fission yeast meiosis and mitosis through targeting Aurora B to the kinetochores (Kawashima et al. 2007, Vanoosthuyse et al. 2007). Furthermore, Sgo2 phosphorylation by Aurora B was shown to recruit microtubule destabilizing kinesin, MCAK, to the kinetocores (Tanno et al. 2010). In budding yeast, Sgo1 is required for sister kinetochore bi-orientation in meiosis (Kiburz et al. 2008).

2.3.3.3 Sgo at the centrosomes

Recently, the Sgo family proteins have been proposed to have functions at the centrosomes (Wang et al. 2008). The integrity of the centriole cohesion is known to be dependent on separase function (Tsou and Stearns 2006). The loss of premature centriole cohesion (centrosome splitting) is a potential source for chromosomal instability and aneuploidy. Sgo1 splice variant sSgo1 has been shown not only to localize at the centrosomes but also to be involved in the maintenance of cohesion between centrioles (Wang et al. 2008). This function resembles the role of Sgo1 in the maintenance of centromeric cohesion. In addition, the spindle pole localized Plk1 takes part in proper localization of sSgo1, highlighting the same regulators both at the centromeres and centrosomes (Wang et al. 2008). When putative Plk1 phosphorylation sites were mutated in sSgo1, sSgo1 was significantly reduced at the centrosomes and the centriole splitting was observed (Wang et al. 2006, Wang et al. 2008). This opens a

possibility that other kinases such as Bub1 and Aurora B may also play a role in the regulation of sSgo1.

2.4 Chromosomal passenger complex

The diverse and essential functions of the chromosomal passenger complex (CPC) during mitosis and meiosis have been extensively studied. In early mitosis, the CPC is suggested to participate in the regulation of mitotic chromosome structure and formation of the mitotic spindle, chromosome segregation, SAC signaling and regulation of centromeric cohesion. In addition, it has been shown to have functions at anaphase and cytokinesis in late mitosis. The term "chromosomal passengers" was initially used to describe proteins with a characteristic localization during mitosis. These proteins associate along the chromosome arms during prophase and accumulate at the inner centromeres at prometaphase and metaphase. At the onset of anaphase, they leave chromosomes and remain in the midzone of central spindle finally concentrating in the midbody at cytokinesis. This mobile localization reflects the multiple functions of CPC during mitosis (Fig. 9). Also, a multifunctional mitotic kinase, Plk1, has similar localization pattern during mitosis. In most organisms, the CPC consists of four core members the Aurora B kinase, Inner centromeric protein (INCENP), Survivin and Borealin (Fig. 8). Aurora B is the enzymatic core of the complex and three other non-enzymatic members control the targeting, activity and stability of the Aurora B kinase (Lens et al. 2006). There is growing evidence that the CPC plays a role also in meiotic division. However, the localization and the tasks of the complex differ between mitosis and meiosis (Ruchaud et al. 2007).

2.4.1 Members of the CPC

INCENP (Sli15 in budding yeast) was the first complex member discovered in a monoclonal antibody screen as a novel component of the mitotic chromosome scaffold (Cooke et al. 1987). Structurally, INCENP is a basic protein including coiled-coil region but otherwise lacking any recognizable motifs (Mackay et al. 1993). Homologues for INCENP can be found from all eukaryotes. The Aurora B kinase binds to a conserved strech near the C-terminus of INCENP known as IN box and phosphorylates INCENP at two serine residues within the region (Adams et al. 2000, Bishop and Schumacher 2002, Honda et al. 2003). The N-terminus of INCENP has motifs sufficient for the centromeric targeting and transfer to the spindle midzone (Ainsztein et al. 1998). Survivin and Borealin bind to the N-terminus of INCENP suggesting that they may be the primary targeting subunits of the complex (Gassmann et al. 2004b, Honda et al. 2003, Klein et al. 2006, Sessa et al. 2005). INCENP gene has turned out to be essential in many species (Adams et al. 2001b, Cutts et al. 1999, Honda et al. 2003, Kaitna et al. 2000, Kim et al. 1999). Perturbation of INCENP leads to mislocalization of both Aurora B and Survivin from the kinetochores and defects in chromosome congression and failure of cytokinesis (Ainsztein et al. 1998, Mackay et al. 1998).

Aurora B, a Ser/Thr kinase, is conserved among species. The first Aurora kinase (Aurora A) was discovered from *Drosophila* where it localized to the spindle poles (Glover et al. 1995). Aurora B (Ipl1p in budding yeast) was isolated in a screen for kinases causing growth arrest when overexpressed (Terada et al. 1998). Aurora B binds and phosphorylates INCENP in a two-step process which is required for the full activity of the kinase (Bishop and Schumacher 2002, Honda et al. 2003, Yasui et al. 2004). Yeasts have only one Aurora kinase. *Drosophila* has two and mammals three Aurora kinases known as Aurora A, B and C (Adams et al. 2001a, Nigg 2001). They have different function or tissue specificity. Aurora A has functions in the spindle assembly and cell cycle progression. Aurora C is highly expressed in testis and in many cancer cell lines where it assosiates with the spindle poles (Bernard et al. 1998, Hu et al. 2000, Kimura et al. 1998, Tseng et al. 1998). It can bind the members of the CPC and rescue Aurora B loss of function in various cell types (Sasai et al. 2004, Yan et al. 2005). The numerous substrates of Aurora B include many important mitotic regulators. For example, Aurora B is suggested to be required for the maintenance of CENP-A phosphorylation during mitosis and this phosphorylation may be essential for the centromeric localization of Aurora B during mitosis (Kunitoku et al. 2003). Moreover, Aurora B is involved in the correction mechanism of erroneous kinetochore-microtubule attachments via inducing a destabilization of improper connections by phophorylating MCAK (Andrews et al. 2004, Lan et al. 2004).

Survivin is a conserved and essential gene (Uren et al. 2000). The protein has important functions both in mitosis and programmed cell death, apoptosis. Moreover, Survivin is highly expressed in the number of tumours (Ambrosini et al. 1997). Survivin belongs to the family of inhibitor of apoptosis proteins (IAPs). In its structure, it carries a baculoviral IAP repeat (BIR) Zn^{2+} finger motif. X-ray crystallography studies show that Survivin forms a butterfly-shaped dimer where the Zn^{2+} finger motif is required for the dimerization (Chantalat et al. 2000, Muchmore et al. 2000, Verdecia et al. 2000). The localization of Survivin is dependent on INCENP (Wheatley et al. 2001). Survivin is phosphorylated by Aurora B and it can bind all the other members of the CPC. It has been shown that mutating the Aurora B phosphorylation site containing residue Thr117 perturbs the binding of Survivin to INCENP leading to its mislocalization in mitosis (Carvalho et al. 2003, Wheatley et al. 2004). Some studies suggest that the binding of Survivin to Aurora B is required for the Aurora B activity (Bolton et al. 2002, Chen et al. 2003). In another study, Survivin was not detected to influence the activity of Aurora B (Honda et al. 2003). The centromere targeting of CPC has been unclear. Recently, it was shown that Survivin directly binds Haspin phosphorylated histone H3 mediating the recruitment of CPC to the kinetochores (Wang et al. 2010, Kelly et al. 2010). Depletion of Survivin by RNAi or function neutralizing antibodies in human cells caused defects in chromosome alignment, SAC activity and cytokinesis (Carvalho et al. 2003, Kallio et al. 2001, Lens et al. 2003).

Borealin (also known as Dasra-B) was discovered in screens for new components of the mitotic chromosome scaffold and for novel *Xenopus* chromosome-binding proteins. Borealin is conserved among vertebrates but homologs have not been identified in yeast. Many vertebrates, but not human, have a second distantly related protein called

Dasra-A which may have a similar function. Borealin binds to the CPC associating with Survivin and INCENP and is phosphorylated by Aurora B. Borealin is required for the function of CPC. The depletion of Borealin by RNAi causes a dramatic increase in kinetochore attachment errors and failure of cytokinesis (Gassmann et al. 2004b, Sampath et al. 2004).

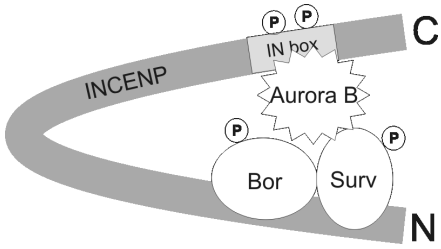

Figure 8. Schematic representation of the CPC. Core members of the complex INCENP, Aurora B, Survivin (Surv) and Borealin (Bor) are illustrated. Aurora B kinase phosphorylates the other members of the complex.

In addition to the core members of the CPC, other proteins interact transiently with the complex to perform essential functions. **TD-60** (telophase disk 60 kDa) was originally identified as a guanine nucleotide exchange factor (GEF) (Mollinari et al. 2003). It has a typical chromosomal passenger behavior. TD-60 mislocalizes if any of the CPC members is perturbed. Moreover, when TD-60 is partially depleted the CPC is still able to localize correctly, but is not fully active (Andreassen et al. 1991, Martineau-Thuillier et al. 1998).

2.4.2 Function of the CPC during mitosis

2.4.2.1 CPC in chromosome structure and spindle formation

The function of CPC in regulation of chromosome structure is not well understood. One of the many substrates of Aurora B is histone H3 which phosphorylation on Ser10 is an indication of Aurora B activity and it correlates with chromosome condensation (Crosio et al. 2002, Giet and Glover 2001, Hsu et al. 2000, Murnion et al. 2001). However, the role of this phosphorylation in the mitotic chromosome structure is not clear. A complex called condensin is required for the chromosome architecture throughout mitosis (Gassmann et al. 2004a). Some studies have shown that the CPC may be required for the loading of condensin to mitotic chromosomes (Giet and Glover 2001, Hagstrom et al. 2002, Kaitna et al. 2002). However, the other studies indicate that condensin is loaded normally despite of Aurora B depletion (Losada et al. 2002, MacCallum et al. 2002). Due to this discrepancy, the role of CPC in chromosome condensation and structure remains to be clarified.

The CPC is implicated in the two pathways required for the spindle assembly, centrosome-driven and chromatin-driven pathways. In *Drosophila*, the bipolar spindle formation is disrupted if the CPC function is perturbed (Adams et al. 2001b). In human, bipolar spindles form but appear to disassemble during metaphase in the absence of Borealin causing the formation of ectopic poles leading to defects in chromosome segregation and cytokinesis (Gassmann et al. 2004b). The targets for the CPC in the centrosome-driven spindle assembly are not known. Moreover, it has been

shown that in *Xenopus* egg extracts a perturbation of the CPC blocks the chromatin-driven spindle assembly (Gadea and Ruderman 2005, Sampath et al. 2004). This pathway is thought to be regulated by the CPC and Ran-GTP together. However, in recent years the cooperation between the CPC and a protein called Stathmin have been under investigation. Stathmin is a microtubule-stablizing protein implicated in the spindle assembly (Andersen et al. 1997). Aurora B phosphorylation negatively regulates Stathmin activity (Gadea and Ruderman 2006, Kelly et al. 2007). Aurora B phosphorylation on MCAK is also required for supporting the bipolar spindle formation in *Xenopus* egg extracts (Ohi et al. 2004, Sampath et al. 2004, Tulu et al. 2006). In budding yeast, Ipl1p is required for the spindle disassembly after the completion of anaphase (Buvelot et al. 2003).

2.4.2.2 CPC in kinetochore-microtubule attachments and chromosome segregation

It is unlikely that all chromosomes initially form bioriented attachments. Instead, kinetochores may initially bind microtubules in any configuration. Therefore, kinetochore-microtubule connections must be regulated. In principle, a mechanism where only the correct attachments are stabilized and the incorrect attachments destabilized would be sufficient to ensure the biorientation of each chromosome through a trial-and-error process. On the other hand, it has been suggested that the stability of the attachments might depend on tension across the centromere. The CPC function is essential for the biorientation of mitotic chromosomes at metaphase in eukaryotes reflecting the necessity of the CPC in the trilaminar kinetochore assembly and in the regulation of kinetochore-microtubule attachments (Liu and Lampson 2009). It was first suggested in budding yeast that Ipl1p is required for the accurate chromosome segregation and regulation of microtubule-kinetochore attachments (Biggins et al. 1999, Chan and Botstein 1993, Cheeseman et al. 2002, Francisco et al. 1994). Later on, the CPC was described in tension-sensing mechanism in yeast cells (Biggins and Murray 2001, Tanaka et al. 2002). The CPC is discovered to be important in higher eukaryotes in the detection and correction of improper kinetochore-microtubule attachments as well as in the detection of lack of tension between sister kinetochores. Evidence confirming this was obtained from experiments with the inhibition of Aurora B using small molecule inhibitors and function neutralizing antibodies (Ditchfield et al. 2003, Hauf et al. 2003, Kallio et al. 2002b). The inhibition of the kinase stabilized the incorrect attachments, for example the number of syntelic attachments was increased (Hauf et al. 2003). After wash-out of the inhibitor, the improper microtubule-kinetochore interactions were corrected through destabilization of the incorrect attachments while the bipolar connections remained stable (Lampson et al. 2004). The function of the CPC in detecting and correcting improper kinetochore-microtubule attachments seems to be mainly carried out by Aurora B phosphorylation of the key kinetochore and centromere molecules. However, INCENP and Survivin may also have a role in this task. INCENP is known to associate with microtubules (Wheatley et al. 2001) but the significance of this finding has been unclear. In a study in yeast, INCENP-Survivin (Sli15-Bir1) complex has been implicated in the microtubule binding and in tension detection (Sandall et al. 2006). A Ser/Thr phosphatase, protein phosphatase 1 (PP1) has been suggested to oppose the role of the

Aurora B kinase during mitosis (Francisco et al. 1994, Emanuele et al. 2008, Liu et al. 2010). PP1 isoforms have been detected at the outer kinetochores in vertebrates (Trinkle-Mulcahy et al. 2003) and this phosphatase has been shown to counteract Aurora B phosphorylation stabilizing the kinetochore-microtubule attachments (Liu et al. 2010, Sassoon et al. 1999).

The Ndc80 complex is essential for the microtubule binding to the kinetochore, chromosome congression, and SAC activity (McCleland et al. 2003, McCleland et al. 2004, Welburn and Cheeseman 2008). In vertebrates, Hec1 has been recently identified as a key kinetochore target for Aurora B. The tasks of Hec1 at the kinetochore include the regulation of kinetochore-microtubule attachments and chromosome segregation (DeLuca et al. 2003). It has been shown that Aurora B phosphorylates the N-terminus of Hec1 *in vitro*, which results in negative regulation of microtubule binding of the KMN network (Cheeseman et al. 2006). This domain is also suggested to be required for the microtubule turnover at the kinetochores (DeLuca et al. 2006). It has been shown that there are also other targets of Aurora B within KMN network (Welburn et al. 2010). These observations suggest that phosphorylation of the proteins of KMN network by Aurora B may be one mechanism how kinetochores release the improper microtubule attachments. One regulator of kinetochore-microtubule attachments was suggested to be a protein called the inner centromere KinI stimulator (ICIS) that assosiates with MCAK stimulating its microtubule depolymerizing activity (Ohi et al. 2004). ICIS has also been isolated in a complex with Aurora B and INCENP. It has been proposed to be an inner centromere factor promoting the disassembly of laterally attached microtubules and thereby possibly reducing merotelic attachments. Later, it was shown that Aurora B phosphorylates MCAK itself at the centromeres inhibiting its microtubule depolymerizing activity (Andrews et al. 2004, Lan et al. 2004). The depletion of MCAK is shown to result in chromosome congression and segregation defects due to inappropriate kinetochore-microtubule attachments (Kline-Smith et al. 2004). However, the effects were not as severe as in inhibiting the CPC proteins, suggesting that there are additional targets of Aurora B taking part on this function in vertebrates (Kline-Smith et al. 2004, Maney et al. 1998). Merotelic attachments are one main reason leading to aneuploidy in cultured cells (Cimini et al. 2001). Moreover, multipolar spindles and lagging chromosomes, which may involve merotelic attachments, have been identified in cancer cells (Cimini 2008). The CPC is enriched at unattached kinetochores as well as merotelically attached kinetochores (Knowlton et al. 2006, Vagnarelli and Earnshaw 2004). It has been suggested that Aurora B can promote the accumulation of MCAK and regulate its activity at improper microtubule attachment sites (Andrews et al. 2004, Lan et al. 2004). In addition, a balance between Aurora B activity and a counteracting phosphatase has been suggested to regulate MCAK in merotelic attachments (Murnion et al. 2001, Sugiyama et al. 2002).

Although the exact mechanism remains to be solved, it is obvious that the CPC with its catalytic subunit, Aurora B is an essential regulator of kinetochore-microtubule attachments and a facilitator of the release of improperly connected microtubules. How the regulation of kinetochore-microtubule attachments is selective in a way that only the incorrect connections become destabilized remains unknown. Sensitivity to tension

which forms between the properly attached sister kinetochores is proposed to explain this by two models (Tanaka et al. 2002). In one model, tension could cause a conformational change in the kinase or in a regulatory protein resulting in the inhibition of the kinase activity. In the other model, the kinase activity is constant, but the forces pulling the sister kinetochores to opposite directions change the distance between the kinase and the substrate when the kinetochores are under tension. The increased distance between the kinase and the substrate would decrease the phosphorylation of the substrate without directly inhibiting the kinase. Experimental data is supporting the second model (Fuller et al. 2008, Liu et al. 2009). The results indicate that the tension-sensing mechanism fails when Aurora B is positioned closer to its kinetochore substrates leading to constitutive phosphorylation and destabilization of microtubule attachments.

2.4.2.3 CPC in the SAC signaling

Knowing that unattached kinetochores maintain the activity of SAC, the CPC is indirectly involved in the SAC function by regulating the kinetochore-microtubule connections and generating unattached kinetochores in response to incorrect attachments. However, more direct roles for the CPC in the regulation of SAC have been suggested. Disruption of the CPC function both in yeast and mammals impairs the SAC activity (Biggins and Murray 2001). INCENP and Survivin are required for the SAC function in presence of a microtubule hyperstabilizing agent taxol, a treatment that decreases the tension at the kinetochores (Carvalho et al. 2003, Lens et al. 2003, Vader et al. 2006). Moreover, Aurora B has been described in the checkpoint response in normal cells as well in presence of taxol using selective small molecule inhibitors ZM447439 and Hesperadin and microinjection of inhibitory antibodies (Ditchfield et al. 2003, Gadea and Ruderman 2005, Hauf et al. 2003, Kallio et al. 2002b). A microtubule destabilizing drug, nocodazole, induced mitotic arrest was only mildly affected by Aurora B inhibition or by the knock-down of the CPC members (Hauf et al. 2003). Therefore, apparently the CPC is not absolutely required to signal the presence of unattached kinetochores but the kinetochores that are not under tension such as the incorrectly attached kinetochores.

It is not yet clear what the downstream targets of the CPC are in exerting the direct control over the SAC. There is evidence to consider the SAC proteins BubR1 and Bub1 as targets of the CPC. INCENP and Survivin are required for the kinetochore recruitment of BubR1 (Carvalho et al. 2003, Lens et al. 2003, Vader et al. 2006). Aurora B activity has been suggested to be required for the localization of both BubR1 and Bub1 (Ditchfield et al. 2003, Vigneron et al. 2004). In fact, Aurora B promotes the association of BubR1 with the APC/C maintaining the SAC in cooperation with the Bub1 kinase (Morrow et al. 2005). However, Bub1 and BubR1 have additional duties to the checkpoint control at the kinetochores. Both of them are required for the kinetochore-microtubule attachments (Ditchfield et al. 2003, Lampson and Kapoor 2005, Meraldi and Sorger 2005) and Bub1 also in sister chromatic cohesion (Kitajima et al. 2005, Tang et al. 2004a). Therefore, Aurora B dependent localization of these proteins could be involved in the other functions as well. In budding yeast, the

phosphorylation of BubR1 (Mad3 in yeast) is proved to be involved in the SAC function (King et al. 2007). In human, the direct phosphorylation of BubR1 by Aurora B has not been able to show possibly because in metazoans BubR1 function is controlled by a more complex way (Elowe et al. 2007). Still, the phosphorylation status of BubR1 is dependent on Aurora B activity (Ditchfield et al. 2003). Recently, it was shown that BubR1 is a substrate for Plk1 (Elowe et al. 2007). The phosphorylation is thought to occur at the kinetochores. Since Aurora B regulates the kinetochore localization of BubR1 it may indirectly promote BubR1 phosphorylation by Plk1. Therefore, the reduction of BubR1 phosphorylation in Aurora B deficient cells could just reflect BubR1 mislocalization. The kinetochore recruitment of BubR1 depends also on KNL-1 (Kiyomitsu et al. 2007). Aurora B phosphorylates Hec1, but it is not known whether this is involved in the SAC signaling (Cheeseman et al. 2006, DeLuca et al. 2006). Ndc80 complex recruits the SAC proteins Mps1, Mad1 and Mad2 to the kinetochores and is probably required for the SAC activity (Martin-Lluesma et al. 2002, McCleland et al. 2003).

The CPC containing INCENP that lacks its coiled-coil domain is still able to support proper chromosome alignment but fails to maintain the SAC signal (Vader et al. 2007). This observation indicates that the coiled-coil domain of INCENP is important for the CPC function in regulation of the SAC. Moreover, INCENP may cooperate with Cdk1-cyclin B in the regulation of anaphase onset. Cdk1 phosphorylation on INCENP creates a binding site for the Plk1 kinase. The interaction is required for Plk1 recruitment to the kinetochores. The interaction is essential also for regulating the timing of the metaphase-to-anaphase transition (Goto et al. 2006). However, the detailed mechanism for this is unknown, but it is likely to involve the regulation of the SAC.

2.4.2.4 CPC in anaphase and cytokinesis

After the inactivation of SAC and separation of sister chromatids the CPC transfers from the centromeres to the spindle midzone in early anaphase and later to the equatorial cortex. The dynamics of the complex decreases as it assosiates with the central spindle (Beardmore et al. 2004, Murata-Hori et al. 2002). The transfer of INCENP to the spindle midzone requires motor proteins. In budding yeast, this transfer is triggered by separase activation of the Cdc14 phosphatase (Pereira and Schiebel 2003). The role of this phosphatase in vertebrates is less well defined. Nevertheless, the mitotic kinesin-like protein-2 (MKLP2) can bind both Aurora B and Cdc14 and is required for the CPC and Cdc14 localization to the midzone (Gruneberg et al. 2004). The binding of MKLP2 to Plk1 is also required for Plk1 targeting to the central spindle (Neef et al. 2003). However, it is not known if this requires the motor protein activity of MKLP2 or the phosphatase activity of Cdc14 which could release INCENP from Plk1. Similar interaction between MKLP2, CPC, and Plk1 has been detected in *Drosophila* (Cesario et al. 2006).

The members of CPC are essential for regulation of cytokinesis. INCENP has been shown to accumulate at the equatorial cortex before there is any sign of furrow

formation even before myosin II accumulation (Eckley et al. 1997). However, the normal localization of the CPC to the cleavage furrow is not required for the initiation of furrowing but is essential for the completion of cytokinesis (Honda et al. 2003, Schumacher et al. 1998, Terada et al. 1998). It has been shown in many species that Aurora B is required for the spindle midzone localization of another kinesin-like protein, MKLP1 (Giet and Glover 2001, Severson et al. 2000). Aurora B phosphorylates MKLP1 in *C. elegans* and human (Guse et al. 2005). Loss of MKLP1 and MKLP2 results in spindle midzone and cytokinesis defects (Gruneberg et al. 2004, Severson et al. 2000). MKLP1 forms a complex with Rac GTPase activating protein-1 (MgcRacGAP) (Mishima et al. 2002). Aurora B is shown to phosphorylate MgcRacGAP, which is important for the spindle assembly and completion of cytokinesis (Jantsch-Plunger et al. 2000, Minoshima et al. 2003). Moreover, Aurora B phosphorylation on vimentin appears to be required for the modulation of vimentin filament network and cleavage furrow formation (Goto et al. 2003, Yasui et al. 2004). The CPC interaction with a protein EVI5 (ecotropic viral integration site-5) has an unknown function in cytokinesis (Faitar et al. 2006). Finally, Aurora B phosphorylation on CENP-A has been observed to be important in the timely regulation of the completion of cytokinesis (Zeitlin et al. 2001). However, the details of this pathway remain unknown.

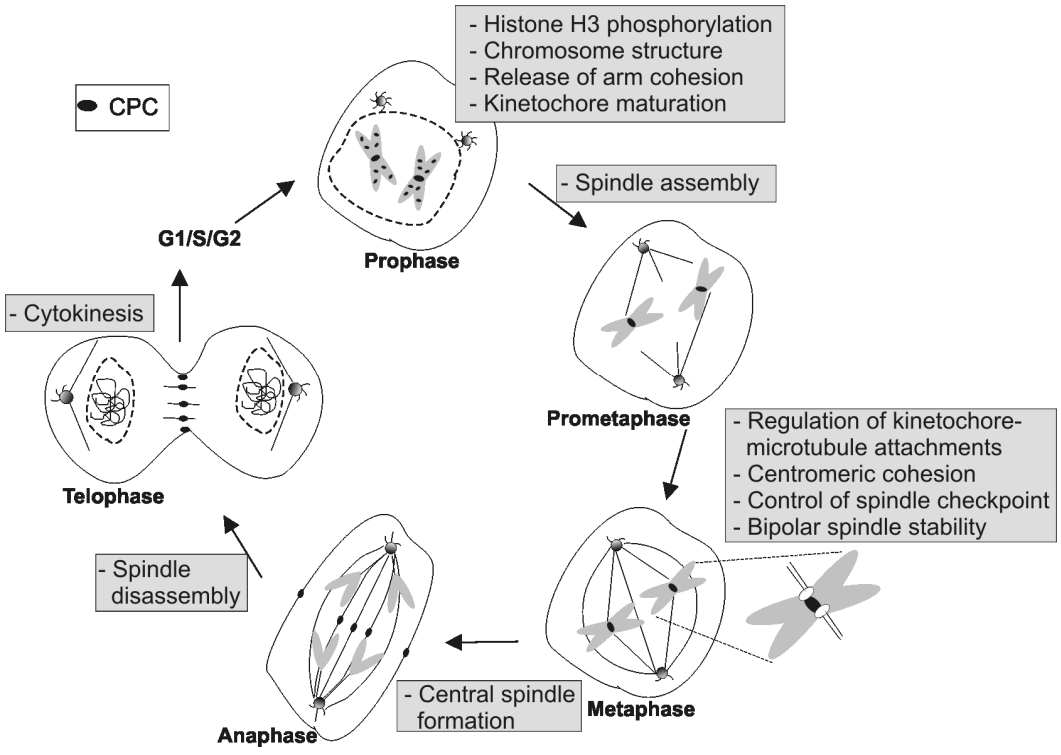

Figure 9. Localization and tasks of the CPC during mitosis (adapted from Ruchaud et al. 2007).

Table 2: Summary of Aurora B substrates

Substrate	Aurora B phosphorylation related function
Histone H3	Histone H3 phosphorylation is an indication of Aurora B activity and it may be required for the mitotic chromosome structure.
CENP-A	CENP-A phosphorylation may be essential for the centromeric localization of Aurora B during mitosis and in the regulation of the completion of cytokinesis.
condensin	Phosphorylation of condensin may be required for the condensin loading on mitotic chromosomes.
Sgo1	Phosphorylation of Sgo1 is essential for the stable kinetochore localization of the protector of centromeric cohesion, Sgo1, at least in *Drosophila*.
Sgo2	Phosphorylation of Sgo2 is required for PP2A and MCAK kinetochore recruitment.
Hec1	Phosphorylation of Hec1 negatively regulates the microtubule binding of the KMN network. The phosphorylation may take part in the release of improperly attached microtubules from the kinetochores.
MCAK	Phosphorylation of MCAK inhibits its microtubule depolymerizing activity.
Stathmin	Phosphorylation of microtubule-stabilizing protein Stathmin negatively regulates Stathmin activity.
MgcRacGAP	Phosphorylation of MgcRacGAP is required for the spindle assembly and completion of cytokinesis.
MKLP1	Aurora B is required for the spindle midzone localization of MKLP1.
vimentin	Phosphorylation of vimentin is required for the modulation of vimentin filament network and cleavage furrow formation.
EVI5	Phosphorylation of EVI5 has an unknown function in cytokinesis.

2.5 p38 mitogen-activated protein kinases

2.5.1 Introduction to MAPK pathways

Mitogen-activated protein kinases (MAPKs) are evolutionally conserved Ser/Thr kinases which link the extracellular signals to the intracellular signaling network that regulates numerous fundamental cellular processes including embryogenesis, differentiation, apoptosis and proliferation. The MAPK cascades consist of three sets of kinases, MAPKs, MAPK kinases (MKKs) and MAPKK kinases (MKKKs). Many MAPKs activate specific effector kinases, MAPK-activated protein kinases (MAPKAPKs), and are inactivated by MAPK phosphatases. MAPKs can be divided into three main groups, extracellular signal regulated kinases (ERK) 1/2, c-Jun amino terminal kinases (JNK) 1/2/3 and p38 α/β/γ/δ MAPKs. In addition, ERK3/4, ERK5 and ERK7/8 have been established. However, the function and regulation of these three pathways is not completely understood. Different MAPKs activate in response to different stimuli and are regulated by specific upstream kinases. In general, ERKs are activated by mitogenic and proliferative stimuli, whereas JNKs and p38 MAPKs are known to respond to environmental stress such as UV irradiation, heat, osmotic shock and inflammatory cytokines (Johnson and Lapadat 2002, Raman et al. 2007a).

2.5.2 p38 MAPK pathways

The p38 MAPKs are involved in inflammation, cell growth, cell differentiation, cell cycle, cell death and, on the other hand, also in cell survival (Fig. 10). The p38 MAPKs are mainly activated by a dual phosphorylation on Thr180 and Tyr182 by an upstream MKK called MKK6. Other MKKs, such as MKK3 and in some cases MKK4, have also been suggested to activate the p38 MAPKs. The activation of the p38 MAPKs mediates a signal transduction into the nucleus to turn on the responsive genes. The

p38 MAPKs also transduce signals to other cellular components to execute different cellular responses (Zarubin and Han 2005).

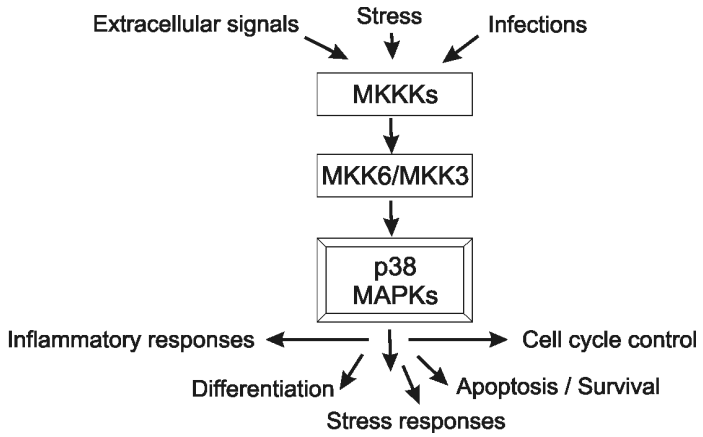

Figure 10. The p38 MAPK cascade and some intracellular effects (partially adapted from Mayor et al. 2007).

Four p38 MAPK isoforms have been identified. p38α was first isolated as a 38-kDa protein and molecular cloning revealed that it belonged to the MAPK family (Han et al. 1994). Three homologues, p38β, p38γ (or ERK6, SAPK3) and p38δ (or SAPK4), were cloned in mammals (Jiang et al. 1996, Jiang et al. 1997, Lechner et al. 1996, Li et al. 1996). These four p38 MAPK isoforms display approximately 60 % sequence identity in amino acid level. The isoforms are encoded by different genes and they differ in their tissue expression pattern and biological function. Human p38α and p38β are ubiquitously expressed whereas p38γ is shown to be predominantly expressed in skeletal muscle and p38δ is enriched in lung, kidney, testis, pancreas, and small intestine. However, the p38 MAPK isoforms are co-expressed and co-activated in many cells. Therefore, the exact roles of each isoform have not been entirely identified. The activation and function of the p38α isoform is the best characterized in the variety of signaling events. All isoforms phosphorylate the Ser-Pro or Thr-Pro MAPK consensus motifs, but some substrate specificity has been reported (Thornton and Rincon 2009). The isoforms also differ in their sensitivity to the commonly used p38 MAPK chemical inhibitors, such as SB203580. This inhibitor specifically inhibits p38α and p38β, but not p38γ or p38δ (Bain et al. 2007, Cuenda et al. 1995). In addition, the disruption of p38α gene results in embryonic death in knockout mice because of placental defects whereas loss of p38β, p38γ or p38δ does not perturb the normal development (Adams et al. 2000, Allen et al. 2000, Beardmore et al. 2005, Mudgett et al. 2000, Sabio et al. 2005). This indicates that there is some redundancy in the function of the family members and since all isoforms are expressed in murine tissues (Beardmore et al. 2005), it is possible that some isoforms can compensate for the lack of the others. On the other hand, the loss of p38α cannot be compensated by the other family members. Although indications for the compensatory activity of the isoforms have been obtained the mechanisms are yet to be revealed (Beardmore et al. 2005, Sabio et al. 2005).

2.5.3 p38 MAPK in cell cycle regulation

The p38 MAPKs are shown to have a role in regulation of cell cycle progression and in the cell cycle checkpoints. In response to stress stimuli that causes DNA damage, cells undergo cell cycle checkpoints to allow time for the DNA repair before moving to the next phase of the cell cycle. The p38 MAPKs have functions in two major checkpoints in the regulation of the cell cycle, the G1/S prior to and during the DNA synthesis and the G2/M prior to cell division (Thornton and Rincon 2009).

Several mechanisms how the p38 MAPK can activate the G1/S cell cycle checkpoint, for example in response to osmotic stress, reactive oxygen species and cellular senescence, have been described. The p38 MAPK mediated tumor suppressor, p53, activation in the G1/S results in accumulation of a protein p21 (Kishi et al. 2001). In this checkpoint, p21 is established to inactivate Cdk2, a member of the cyclin/Cdk complex, which activation is important for cell cycle progression. It has also been reported that the p38 MAPK can directly phosphorylate and stabilize p21 (Kim et al. 2002). The formation of the cyclin D/Cdk4/6 complex is essential for the transition to S phase. The p38 MAPK activation has been shown to reduce cyclin D1 levels by two ways; by negatively regulating cyclin D1 transcription level (Lavoie et al. 1996), and directly phosphorylating cyclin D1 leading to its degradation (Casanovas et al. 2000). A cyclin-dependent protein kinase phosphatase, Cdc25A, regulates also the G1/S transition. The p38 MAPK can phosphorylate Cdc25A promoting its degradation and hence contributing to the establishment of the G1/S checkpoint (Goloudina et al. 2003).

The p38 MAPK is activated in response to double strand DNA break, which can be caused by for example UV light, γ-irradiation or chemotherapeutic drugs, causing the establishment of the G2/M checkpoint. Different p38 MAPK family members can have distinct functions in the induction of the G2/M checkpoint depending on the experimental system. The p38 MAPK activation may be dependent on certain protein kinases that serve as DNA damage sensors called ATM and ATR (Reinhardt et al. 2007). However, these kinases do not directly activate the p38 MAPK. Alternative mechanism to activate the p38 MAPK for example in response to UV and topoisomerase II inhibitors has been suggested (Mikhailov et al. 2004, Reinhardt et al. 2007). The p38 MAPK can contribute to the induction of the G2/M in two ways. Firstly, the p38 MAPK activates p53 leading to the transcription of p53 target genes (Hermeking et al. 1997, Huang et al. 1999, She et al. 2000, Zhan et al. 1999). These proteins can inactivate the Cdk1/cyclin B complex, which is essential for the transition from G2 to M, either directly or indirectly and enforce the G2/M checkpoint. Secondly, the p38 MAPK can induce the G2/M checkpoint by phosphorylating and inhibiting a phosphatase Cdc25B (Bulavin et al. 2001), which is an activator of the Cdk1/cyclin B complex and drives cell cycle progression. This inactivation of Cdc25B is suggested to occur through the p38 MAPK substrate MAPKAPK-2 (MK2) (Manke et al. 2005). The role of the active p38 MAPK in the regulation of the G2/M checkpoint and mitotic entry through the modulation of Cdc25B in absence of stress has also been recently revealed (Cha et al. 2007).

2.5.4 p38 MAPK in mitosis

The role of p38 MAPK in mitotic transit is well established but how the p38 MAPK takes part on mitotic progression and SAC signaling remains controversial. Moreover, the number of reports describing the function of p38 MAPK in mitosis is very limited. The p38 MAPK was first suggested to play a role in mitosis and SAC more than 10 years ago when Takenaka et al. showed that p38 MAPK, but not ERK or JNK, was activated in mammalian culture cells when the cells were arrested in mitosis by disrupting the mitotic spindle with nocodazole. It was also observed that the addition of the recombinant p38 MAPK to *Xenopus* cell-free extracts caused an arrest of the extracts in mitosis. Moreover, an injection of activated p38 MAPK in cleaving *Xenopus* embryos induced mitotic arrest. Inhibition of p38 MAPK with a chemical inhibitor in mammalian culture cells appeared to suppress the activation of the SAC by nocodazole. Based on these observations, the conclusion that the p38 MAPK might be a component of the SAC in somatic cells was drawn (Takenaka et al. 1998). In another study, the activity of p38 MAPK was evaluated using a kinase assay in mammalian cells mitotically arrested by microtubule interfering drugs. The results suggested that p38 MAPK was specifically activated in response to mitotic arrest by the spindle disruption stimulating cell death (Deacon et al. 2003). Furthermore, a chemical inhibition of p38 MAPK during normal mitosis appeared to have no effect on mitotic entry or exit (Deacon et al. 2003). The role of p38 MAPK in the SAC is supported by a recent study showing that the p38 MAPK signaling is required for mitotic arrest caused by Cdc20 proteolysis in response to a spindle poison cadmium (Yen and Yang 2010). Moreover, p38α has been shown to negatively regulate cardiomyocyte proliferation *in vivo* (Engel et al. 2005).

On the other hand, more direct role for p38 MAPK in mitotic progression has been suggested. The activity of the p38 MAPK has been shown to increase during non-stressed conditions in normal mitosis in developing rat retina (Campos et al. 2002). Furthermore, the active p38 MAPK has been found to distribute throughout the cytoplasm (Fan et al. 2005) and also concentrate on the spindle poles in mitotic Hela cells (Cha et al. 2007, Lee et al. 2010, Tang et al. 2008). The active p38 MAPK was observed at the centrosome(s) throughout the cell cycle but the accumulation appeared to be enhanced in a microtubule-dependent manner when the cells entered mitosis (Lee et al. 2010). It was also reported that the duration of mitosis was not changed due to a chemical inhibition of the p38 MAPK in nocodazole co-treated cells compared to controls arguing against the necessity of p38 MAPK in the SAC (Lee et al. 2010).

The mitotic role of p38 MAPK has also been tried to clarify by knocking down the p38 MAPK pathway members. Fan et al. discovered that the RNAi mediated knockdown of p38α inhibited cell proliferation and caused mitotic arrest while a chemical inhibition of the p38 MAPK activity did not cause the same effect. Ectopic expression of a kinase negative p38α was able to rescue the lethal phenotype caused by p38α depletion. Moreover, also the overexpression of wild type or kinase negative p38α caused inhibition of cell proliferation as the RNAi depletion of p38α. These results led to a conclusion that p38α isoform has a role in mitotic progression independent of its kinase

activity (Fan et al. 2005). Consistently, another study suggested that the p38 MAPK pathway was required for mitotic progression based on the depletion of various p38 MAPK pathway members (Tang et al. 2008). This study, however, showed that a chemical inhibition of the p38 MAPK activity caused mitotic defects such as lagging chromosomes and mitotic block suggesting that also the kinase activity was required for mitotic progression. This conclusion is supported by a recent report which showed that a chemical inhibition of the p38 MAPK activity mildly and transiently prolonged mitosis between the nuclear envelope breakdown (NEB) and anaphase onset suggesting a role for p38 MAPK in timely satisfaction of the SAC probably due to slightly elongated spindles. However, any other mitotic defects were not observed (Lee et al. 2010).

Interestingly, a link between the well known mitotic kinase Plk1 and the p38 MAPK pathway in mitosis has recently been discovered (Tang et al. 2008). It was observed that the phosphorylated p38 MAPK and its activated substrate MK2 colocalized with Plk1 at the mitotic spindle poles during prophase and metaphase. Furthermore, MK2 was shown to phosphorylate Plk1 *in vitro* and *in vivo* on Ser326. This phosphorylation was observed to occur throughout mitosis concentrating on mitotic structures using phospho-Plk1 Ser326 antibody. The physical association of Plk1 and MK2 was confirmed with the immunoprecipitation approach. Importantly, this phosphorylation was shown to be critical for the mitotic function of the Plk1 kinase (Tang et al. 2008).

So far, the literature describing the role of the p38 MAPK in mitosis has been concentrating almost exclusively to the role of p38α isoform while the specific function of the other isoforms has not been reported at all. Obviously, more research work is required to clarify the specific function of the p38 MAPK isoforms in mitosis. What regulates the p38 MAPK pathway and what are the targets of the p38 MAPKs in mitosis remain to be elucidated.

2.6 Mitosis and cancer

One important goal for the research on chromosome segregation during mitosis is to contribute to the understanding of tumorigenesis and treatment of cancer. Cancer is one of the largest health problems in our society. It causes a heavy burden, in terms of both human suffering and medical costs. Cancer affects people at all ages and the risk for the most cancer types increases with age. Cancers are caused by abnormalities in the genetic material of the transformed cells. One potential mechanism by which the malignant cell growth can be induced is loss or gain of chromosomes due to mitotic errors. This aneuploidy is an irreversible consequence of chromosome mis-segregation during mitosis and a hallmark of many human tumours. Moreover, genetic and biochemical analysis of human tumours often indicate a mutation, overexpression, or haplodeficiency of certain key molecules that normally participate in the regulation of cell division (Jallepalli and Lengauer 2001). Many cancer cells mis-segregate their chromosomes at very high rates in a phenomenon called the chromosomal instability (CIN). Recently, it has also been suggested that the loss of mitotic fidelity can suppress tumorigenesis depending on the tissue context (Weaver and Cleveland 2007). For

example, the examination of mice with half of the normal levels of CENP-E, causing errors in chromosome segregation due to the weakened SAC and impaired kinetochore-microtubule interactions, showed increased incidence of certain tumor types in spleen and lung. Interestingly, a decreased incidence of spontaneous liver tumors, a carcinogen induced tumors, and tumors caused by the loss of a tumor suppressor were observed in these animals (Weaver et al. 2007). Moreover, induction of massive aneuploidy via inhibition of the SAC in cells with normal cytokinesis was found to cause loss of viability of cancer cells (Kops et al. 2004, Michel et al. 2004). Antimitotic drugs which affect microtubule dynamics are utilized to treat cancer. However, targeting the SAC without the inhibition of microtubule functions may be a more potent way to kill cancer cells. At the end, the development of more cancer cell selective or otherwice improved cancer therapies is needed. Here the basic research on mitotic processes can provide information on new drug targets as well as on novel biomarkers for diagnostics of drug efficacy and disease progression.

2.6.1 Cohesion defects

Chromosome cohesion must be maintained through G2 and mitosis and be abrubtly disrupted at the onset of anaphase. Somatic mutations in genes whose products regulate chromosome cohesion including subunits of cohesin complex have been identified in some human tumors (Barber et al. 2008) although the mutations in cohesin genes appear to be rare (Greenman et al. 2007). The depletion of cohesins or the cohesin regulators Sgo1 and separase by RNAi as well as the overexpression of separase or non-degradable securin has been shown to increase the number of tetraploid cells (Barber et al. 2008, Iwaizumi et al. 2008, Wirth et al. 2006, Zhang et al. 2008). This indicates that disturbing cohesion leads to global effects of chromosome segregation rather than inducing mis-segregation of individual chromosomes. High levels of separase have been detected in breast cancer samples (Zhang et al. 2008). Excessive separase may induce premature sister cromatid separation leading to chromosome mis-segregation, aneuploidy and tumorigenesis.

2.6.2 SAC defects

The failure of the SAC results in premature separation of sister chromatids even in the presence of unaligned chromosomes. Total ablation of the SAC is lethal in cultured cells and in mouse models (Ricke et al. 2008, Weaver and Cleveland 2005). The weakened SAC, however, can lead to aneuploidy. Indeed, in many tumor cells the checkpoint signal is not sustained allowing missegregation of chromosomes. For example, it has been observed that cancer cells often fail to maintain the SAC when exposed to spindle poisons for long times unlike normal cells indicating the weakened SAC. Also, the downregulation of the SAC genes are reported in human carcinomas (Weaver and Cleveland 2006). Experiments using mouse models have shown that heterozygous mutations in the SAC genes induce CIN and aneuploidy and can increase the incidence of tumor formation later in life (Iwanaga et al. 2007, Michel et al. 2001, Ricke et al. 2008). On the other hand, overexpression of Mad2 has been shown to trigger tumorigenesis (Sotillo et al. 2007). Mutations in the SAC genes, however, are

quite rare in human tumors which exhibit CIN. For example, the mutational inactivation of Bub1 in some cancers with CIN was suggested to be connected with the SAC inactivation (Cahill et al. 1998). In contrast, the studies of mutational status of human Mad2 in breast, lung and digestive tract tumors revealed that Mad2 was not commonly mutated in these cancer types (Gemma et al. 2001, Imai et al. 1999). In fact, the most cancer cells with CIN appear to have the functional SAC. There is evidence from cancer cells exhibiting CIN showing no difference in the ability to arrest in mitosis in response to spindle poisons (Tighe et al. 2001). Moreover, it has been observed that in many cell lines with CIN anaphase did not take place before proper chromosome alignment (Thompson and Compton 2008).

Despite the apparently normal SAC response, many cancer cells show prolonged activation of the checkpoint (Therman and Kuhn 1989, Yang et al. 2008). Thus, it seems that cancer cells commonly possess defects which prolong mitosis delaying the satisfaction of the SAC. Several causes for this have been described including the presence of extra chromosomes or extra poles, both of which are common features for cancer cells (Yang et al. 2008). One consequence of the prolonged SAC activation which may contribute to tumorigenesis is an increased frequency of chromosome missegregation. Interestingly, the extended activation of the SAC has been found to be important not only for the tumor formation but also for cancer treatment since antimitotic drugs can induce prolonged activation of the SAC and thereby elicit significant cytotoxicity (Jackson et al. 2007, Rieder and Maiato 2004). Precisely how mitotic arrest contributes to the formation and treatment of cancer is still incompletely understood.

2.6.3 Kinetochore-microtubule mis-attachments

Erroneous kinetochore-microtubule attachments are a natural event in early mitosis. However, most of them are detected and corrected by the correction machinery in a normal cell. The presence of monotelically attached chromosomes in the onset of anaphase unavoidably leads to the segregation of the two sister chromatids to the same daughter cell and causes aneuploidy. Also syntelic chromosomes localizes close to the attached pole. Thus, the presence of syntelic orientation at the anaphase onset can lead to the formation of aneuploid daughter cells. However, monotelic and syntelic attachments activate the SAC and are an unlikely source of aneuploidy in a cell with functional SAC. Merotely, in contrast, is not detected by the SAC and it occurs often in early mitosis. Therefore, it is a likely mitotic defect to cause aneuploidy and drive transformation and cancer in cells with the functional SAC (Cimini 2008).

Merotelic attachments, if not corrected, increase the frequencies of anaphase lagging chromosomes, which are very common in human tumors. At cytokinesis, the cleavage furrow can push the lagging chromosome to either one of the forming daughter cells inducing aneuploidy in 50% of the cases (Cimini et al. 2002). In a cell with merotelically attached chromosomes at the anaphase onset, lagging chromosomes form micronuclei during the nuclear envelope reformation. These micronuclei are very common in cancer cells and are believed to be a biomarker for high risk of cancer

(Fenech 2002). It has been shown that multipolar spindles in cancer cells increase the incidence of merotelic attacments (Ganem et al. 2009, Silkworth et al. 2009). This provides an explanation for the correlation between extra centrosomes and CIN in many cancer cells. Since merotely is the main cause of chromosome missegregation in chromosomally unstable human cells (Thompson and Compton 2008), the malfunction of the correction mechanism is one candidate driving the formation of aneuploid cells and tumorigenesis.

2.6.4 CPC in cancer

The Aurora B kinase is overexpressed in many human tumor types (Araki et al. 2004, Kurai et al. 2005, Ota et al. 2002, Smith et al. 2005). In some cancers increased levels of the kinase have even been associated with a poor patient prognosis (Kurai et al. 2005, Ota et al. 2002). Aurora B normally phosphorylates histone H3 at Ser10 which is important for the chromatin stability. However, excessive H3 phosphorylation has been linked to CIN and increased tumor invasiveness (Ota et al. 2002). The phosphorylated histone H3 is nowadays a useful biomarker in clinical studies. Despite the reports of overexpression in certain cancers, the role of Aurora B in tumorigenesis is not clear. Mitotic products are often upregulated during rapid cell division and overexpression may only reflect hyperproliferation rather than cell malignancy (Katayama et al. 2003). However, it has been suggested that Aurora B takes part on malignant transformation in cooperation with additional factors (Kanda et al. 2005, Meraldi et al. 2002). In contrast, Aurora A has been more consistently implicated in tumorigenesis (Lok et al. 2010). In addition to the CPC member Aurora B, INCENP has been found highly expressed in several colorectal cancer cell lines (Adams et al. 2001) and overexpression of Survivin has been detected in most of the malignancies (Duffy et al. 2007).

2.6.5 Sgo in cancer

Many chromosome segregation-regulating proteins, which are known to function in the same pathways with Sgo1 (such as Aurora B, Plk1 and Bub1) are overexpressed or downregulated in cancers (Eckerdt et al. 2005, Holtrich et al. 1994, Shichiri et al. 2002, Smith et al. 2005). However, the role of Sgo1 in cancers is not as extensively studied. Sgo1 was observed to be overexpressed in many breast cancer tissues (Scanlan et al. 2001). Recently, it was shown that Sgo1 is downregulated at the transcriptional and protein level in human colorectal cancers and that the knock-down of Sgo1 in these cancer cell lines led to CIN (Iwaizumi et al. 2008).

It is believed that a tumor suppressor gene resides to a locus where human *sgo1* gene is located. Loss of heterozygosity or homozygous deletion at this locus is detected in many cancers (Dahiya et al. 1997, Shao et al. 2000, Yang et al. 2001). Several known genes are proposed as potential candidates, one of them being *sgo1* which is critical for regulating chromosomal stability. However, the identity of this possible tumor suppressor remains to be discovered.

2.6.6 p38 MAPK in cancer

Aneuploidy puts a significant stress on cells. In response to this, cell proliferation defects and metabolic changes have been observed in trisomic MEFs and yeast strains (Williams et al. 2008, Torres et al. 2007). It has been suggested that in these cells the general stress pathways activate due to protein imbalances (Torres et al. 2007). Typically, this leads to inhibition of cell proliferation of the aneuploid cells. Some of the same effects have been observed also in cancer cells (Williams and Amon 2009). However, different cancer cells possibly possess a selection of mutations that protect the cells from aneuploidy-induced harmful effects (Williams and Amon 2009, Torres et al. 2010). The pathways that are required for aneuploid cell survival could be a possible target for the cancer therapy development.

The ERK pathways are the best studied MAPK pathways and they are known to be deregulated in approximately one-third of all human cancers. Cancer cells are exposed to many types of stress and thus an important role for stress-activated pathways in cancer is emerging related to their modulatory functions on inflammation, DNA damage response and apoptosis for example. In general, their effect is anti-proliferative and proapoptotic, but depending on the cellular context they may contribute to cell survival and tumorigenesis (Dhillon et al. 2007). The p38 MAPK has been suggested to function as a tumor suppressor based on the analysis of the phenotype of the mice disrupted in MKK3 and MKK6 or p38α genes (Bulavin and Fornace 2004). The tumor suppressive effects of p38 MAPK may be mediated through several different mechanisms. The p38 MAPK negatively regulates the cell cycle and activates p53 inducing p53-mediated apoptosis (Bulavin and Fornace 2004, Kummer et al. 1997). The p38α MAPK also activates in response to oncogenic stress and have been shown to play a role in senescence in mouse embryo fibroblasts (Bulavin et al. 2003, Molnar et al. 1997). Therefore, it appears that the decrease of the p38 MAPK activity may play an essential role in cancer. In addition, the activity of the p38 MAPKs has been shown to be reduced in some cancers for example hepatocellular carcinomas in comparison to normal adjacent tissue (Iyoda et al. 2003).

Mitosis is an important research topic in the attempt to understand tumorigenesis and to develop new more effective and cancer cell specific therapies. Basic research on regulatory pathways of mitosis may provide candidate proteins as targets for the cancer drug discovery. In this thesis, the tasks of selected mitotic proteins were studied aiming to expand the knowledge about the processes of cell division and the involvement of these proteins of interest in mitotic regulation.

3 AIMS OF THE STUDY

Mitosis is known to proceed under strict regulation of the SAC involving a highly organized signaling network of proteins. However, the exact mechanisms of how each pair of sister kinetochores achieve a stable bipolar microtubule attachment and how unbalanced attachments generate and transmit the mitosis-inhibiting signals are not completely understood. In addition, the function of many mitotic proteins is only partially known and various proteins related to mitotic progression remains to be completely characterized. The purpose of this study was to increase the knowledge about mitotic regulation and clarify how the selected proteins of our interest, Sgo1, INCENP and p38 MAPK participitate in mitotic signaling. Project was divided into three individual subprojects and the specific objectives of these subprojects were:

1. To study the role of Sgo1 in the kinetochore assembly and SAC signaling

2. To investigate the mitotic tasks of INCENP and study the dynamic properties of CPC

3. To study the function of individual p38 MAPK isoforms in mitotic progression and SAC signaling

4 MATERIALS AND METHODS

Cell lines and reagents (I, II, III)
Cell culture media, supplements and chemicals were purchased from Sigma if not stated otherwise. HeLa and LLCPK cells were maintained at +37˚C with 5% CO_2 in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 20 mM HEPES. The medium of stable H2B-GFP HeLa cell line (Kanda et al. 1998) included 2 ng/ml blasticidin. Xeno S3 cells were grown at room temperature (RT) in Leibovitz's L-15 medium supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin and 15% H_2O. The Aurora kinase inhibitor ZM447439 was a generous gift from AstraZeneca and Plk1 inhibitor ZK-Thiazolidinone (TAL) was received from Bayer Schering Pharma AG.

Plasmids (I, II)
hSgo1 was fused to the N-terminus of YFP (YFP-hSgo1) by cloning it into pEYFP-C1 (BD Biosciences). The coding sequence of hSgo1 was amplified from Human Small Intestine QUICK-Clone cDNA (BD Biosciences) using primers that introduced specific restriction sites for XhoI and BamHI at the 5' and 3'-ends for cloning. GFP-hIncenp was a kind gift from Erich Nigg and was described previously (Klein et al. 2006). GFP-xIncenp was constructed from pCS2-YFP-xIncenp by amplification of xIncenp using primers that introduced restriction sites for Xho1 and BamH1 and cloning into pEGFP-C1 (BD Biosciences). pCS2-YFP-xIncenp plasmid was generated from Pet28B-xINCENP (Bolton et al. 2002) by cloning into Sal1 and Xba1 sites into PCS2 + YFP, which was a generous gift from David Wotton (Kagey et al. 2003). xAurora B-YFP was constructed by amplifying the coding sequence of xAurora B (xAIRK2) from cDNA of Xeno S3 cells and cloning it into into pEYFP-N1 (BD Biosciences). Primers that introduce specific restriction sites for HindIII and BamHI at the 5′ and 3′-ends were used. pEGFP-tubulin (BD Biosciences) was used to generate Xeno S3 GFP-tubulin cell line.

Transfections (I, II, III)
To transfect YFP-hSgo plasmid into HeLa cells Lipofectamin 2000 (Invitrogen) was used according to the manufacturer's protocol, with an exception that only 20% of the recommended Lipofectamine 2000 was used. GFP-hIncenp was transfected into HeLa and LLC-PK cells using Effectene (Qiagen) according to the manufacturer's protocol. Xeno S3 cells were transfected with GFP-xIncenp or xAurora B-YFP by electroporation using the ECM 830 electroporator (BTX, Holliston, MA, USA). Oligofectamine (Invitrogen) was used to transfect siRNAs according to the manufacturer´s protocol. Used siRNAs are listed in Table 1. All stars negative control siRNA (Qiagen) was used in scrambled siRNA transfections and AllStars Hs Cell Death siRNA as a positive cell death phenotype control.

Table 3. siRNAs

siRNA	Sequence	Source	Reference
Incenp	5'-AAGAAGCAGATTGAGCAGAAG-3'	Dharmacon	(Honda et al. 2003)
Sgo1	5'-AAGAUAUCAUCCUACAGCUGA-3'	Dharmacon	(Salic et al. 2004)
Plk1	5'-AAGATCACCCTCCTTAAATAT-3'	Dharmacon	(Spankuch-Schmitt et al. 2002)
Bub1	5'-AAAUACCACAAUGACCCAAGA-3'	Dharmacon	-
CENP-F	5'-AAACAACCUCCUUAAGAGUCA-3'	Dharmacon	-
p38α siRNA-1	5'-AACTGCGGTTACTTAAACATA-3'	Qiagen	-
p38α siRNA-2	5'-CAGAGAACTGCGGTTACTTAA-3'	Qiagen	-
p38β	5'-CAGGATGGAGCTGATCCAGTA-3'	Qiagen	-
p38γ siRNA-1	5'-CTGGACGTATTCACTCCTGAT-3'	Qiagen	-
p38γ siRNA-2	5'-TGGAAGCGTGTTACTTACAAA-3'	Qiagen	-
p38δ	5'-CCGGAGTGGCATGAAGCTGTA-3'	Qiagen	-
Aurora A	5'-CACCTTCGGCATCCTAATATT-3'	Qiagen	-

Immunofluorescence labeling (I, II, III)

Cells on coverslips were fixed and permeabilizised simultaneously in 2-4% paraformaldehyde in 60 mM Pipes, 25 mM Hepes (pH 7.0), 10 mM EGTA, 4 mM MgSO4 (PHEM) containing 0,5% triton-X-100 for 15 min. Microcystin-LR (400 nM) or Phosstop phosphatase inhibitor cocktail (Roche) was added into fixative for phospho-antibodies and 0.2 % glutaraldehyde for microtubule staining. Coverslips were rinsed in 10 mM MOPS (pH 7.4), 150 mM NaCl and 0.05 % Tween 20 (MBST) and blocked with 20% boiled normal goat serum (BNGS) for 1 h at RT. Cells were stained with primary antibodies (Table 2) for 1 h at RT or over night at +4°C. Coverslips were washed with MBST and treated with FITC-(1:600), CY3-(1:1000) or Cy5-conjugated (1:400) anti-rabbit, anti-mouse, anti-rat or anti-human secondary antibodies (Jackson Immuno Research) or corresponding AlexaFluor antibodies (Invitrogen) for 1 h at RT. Antibodies were diluted to MBST containing 5% BNGS. Coverslips were washed in MBST and DNA was stained with DAPI (4',6-diamidino-2-phenylindole, 10 ng/ml). Coverslips were washed with H_20 and mounted to microscope slides with Vectashield mounting medium (Vector laboratories). The edges of the coverslips were sealed with nail polish.

Lysed cell assay (III)

HeLa cells on coverslips were rinsed briefly in rinse buffer (50 mM Tris-HCl, pH 7.5, 4 mM MgSO4 (TM), 5 µg/ml protease inhibitor cocktail) and extracted in extraction buffer (TM, 0.5% Triton-X-100, 1 mM DTT, 5 µg/ml protease inhibitor cocktail) for 5 min. Samples were dephosphorylated in dephosphorylation buffer (TM, 1 mM DTT, 5 µg/ml protease inhibitor cocktail) for 7 min (sometimes λ-phosphatase in dephosphorylation buffer), rinsed twice in rinse buffer and treated with either rephosphorylation buffer (TM, 1 mM DTT, 400 nM Microcystin-LR or PhosSTOP, 1 mM ATP, 5 µg/ml protease inhibitor cocktail) for 20 min or with buffer without ATP (TM, 1 mM DTT, 400 nM Microcystin-LR or PhosSTOP, 5 µg/ml protease inhibitor cocktail). In some assays 10 µM ZK-Thiazolidinone (TAL) or 20 µM ZM447439 was added to the rephosphorylation buffer before incubation of the samples for 20 min. In exogenous rephosphorylation assay cells were treated with N-ethylmaleimide (NEM) buffer (5 nM NEM in TM, 5 µg/ml protease inhibitor cocktail) for 10 min and washed in rinse buffer. Rephosphorylation buffer +/- ATP was added together with mitotic or S

phase Hela extract or mitotic Plk1 depleted extact (5 x 10^6 cells/ml in extraction buffer, 1:20 in rephosphorylation buffer) for 40 min at RT. In some samples extracts were added in rephosphorylation buffer without ATP for 40 min, washed out in presence of phosphatase inhibitor followed by ATP addition for 40 min. Samples were fixed and stained with phospho-antibodies using immunofluorescence protocol.

Table 4. Primary antibodies

Antibody	Species	IF dilution	Source	Reference
anti-Bub1	mouse	1:125	Upstate	-
anti-INCENP	rabbit	1:1000	gift from Dr. E. Nigg	(Honda et al. 2003)
anti-Aurora B	rabbit	1:1000	Abcam	-
anti-Sgo1	rabbit	1:200	gift from Dr. W. Dai	(Wang et al. 2006)
anti-Plk1	rabbit	1:125	Abcam	-
anti-Cenp-F	mouse	1:400	BD Biosciences	-
anti-Hec1	mouse	1:500	Abcam	-
CREST auto-immune serum	human	1:200	Antibodies Inc.	-
anti-tubulin	rat	1:1000	Abcam	-
Cy3-conjugated T95-pMCAK		1:300	gift from Dr. T. Stukenberg	(Zhang et al. 2007)
anti-phospho-p38	rabbit	1:500	Cell signaling	-
anti-Plk1	mouse	1:100	Abcam	-
anti-phospho-CENP-A	rabbit	1:200	Upstate	-
anti-phospho-Plk1 (Ser326)	rabbit	1:200	gift from Dr. X. Liu	(Tang et al. 2008)
anti-α-tubulin	mouse	1:200	Abcam	-
anti-pericentrin	rabbit	1:2000	Abcam	-
anti-p38γ	rabbit	1:100	Cell signaling	-

Cold calsium lysis (III)

Non-perturbed and siRNA treated cells on coverslips were rinsed in 0.1 M Pipes (pH 6.95) and placed in ice cold (0 - 2˚C) 0.1 M Pipes, 80 uM CaCl2, 1% Triton X-100 for 5 min on ice bath. After lysis, cells were rinsed in Pipes as above. Cells were fixed for microtubule staining.

Monastrol washout experiment (III)

Non-perturbed and siRNA treated cells were treated with 100 μM Monastrol for 4 h. Cells were released from monastrol into 20 μM MG132 for 1 h followed by glutaraldehyde fix. Some samples were fixed directly after monastrol treatment before washout. Microtubules and spindle poles were stained according to immunofluorescence protocol.

Microscopy of fixed samples and quantification of fluorescence (I, II, III)

Fixed samples were imaged with a Zeiss Axiovert microscope equipped with 63× (N.A. 1.4) and 100× (N.A. 1.4) objective, Hamamatsu Orca-ER camera (Hamamatsu Photonics) and Metamorph imaging software (Universal imaging). For quantification of kinetochore protein amounts, fluorescence intensities at the kinetochore were normalized against the anti-CREST signals of the same kinetochore. For each kinetochore analysis approximately fifty kinetochores were quantified. For quantification the amount of phospho-MCAK (Thr95) the signal was measured on chromosomes and amount of phospho-Plk1 (Ser326) was quantified on spindle poles.

In lysed cell assay and in Plk1 depleted cells followed by mitotic wash-out the intensity of phospho-p38 MAPK was measured from the whole cells. At least 5 cells were quantified for each analysis and all values were corrected with background deduction.

Antibody injection and imaging (II)

Polyclonal rabbit Incenp^{-ab} antibody was raised against His-tagged INCENP$_{677-874}$ corresponding to C-terminal sequence of *Xenopus* INCENP and has been characterized previously (Bolton et al. 2002). For microinjections and live cell analysis XenoS3, HeLa and LLC-PK cells were grown on 35 mm class bottom chambers (MatTek, Corp.), and for fixed cell analysis, cells were grown on coverslips. Incenp^{-ab} microinjections were performed using Narishige MN-151 micromanipulator and Narishige PN-30 needle puller (World Precision Instruments, Inc.) at 5.0 mg/ml needle concentration. The average injected volume was 0.1 pL which equals to a mean intracellular concentration of 0.125 mg/ml of Incenp^{-ab} in a *Xenopus* cell with volume of approximately 4 pL. Control cells were injected with unspecific rabbit IgG (Jackson Immuno research) at 5 mg/ml or 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). In experiments where nocodazole, taxol, or MG132 were used, cells were preincubated with the drugs for 20–60 min before microinjection. Fixed cells on coverslips were incubated in FITC-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) to detect Incenp^{-ab} injected cells, or antibody was injected together with FITC-dextran (Invitrogen). Xeno S3 cells were imaged at RT using phase-contrast and/or fluorescence illumination. Images were captured as described above.

Fluorescence recovery after photobleaching (II)

Confocal images were captured using Zeiss LSM510 META confocal microscope with 63× (N.A. 1.4) objective using LSM5 3.2 software with Physiology option (Carl Zeiss Corporation, Jena, Germany). Experiments with HeLa and LLC-PK cells were performed at 37°C and 5% CO_2 in an incubation chamber, and experiments with Xeno S3 cells at RT. Three images were captured prior to photobleaching, after which a region of interest covering one or two centromeres was bleached with 488 nm laser. Fluorescence recovery was followed by scanning the sample with 488 nm low intensity laser irradiation at regular intervals. The frame capture interval was 5–20 s and total time-lapse duration 5–10 min. 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 protein turnover half-time ($t_{1/2}$) and total recovery of fluorescence (recf).

Live cell imaging (III)

Cells were grown on a well multiplate or µ-Slide 8 well (Ibidi). Experiment was done with or without synchronization (double thymidine block). 48 h after siRNA transfection cells were imaged for 16 h using a Zeiss Axiovert microscope equipped with a 40x or 63x objective, Hamamatsu Orca-ER camera (Hamamatsu Photonics) and Metamorph imaging software (Universal imaging) or with the 3I-spinning disc confocal unit Yokogawa CSU22 and Slidebook imaging software (Intelligent Imaging Innovations, Inc). Cell populations on 384-well dish were imaged with incucyte imaging system (Essen instruments Ltd.) immediately after siRNA transfection for 66

h. The length of mitosis was measured from NEB to end of telophase, or to the time of mitotic cell death. For calculating the cell death indices morphological criteria (Hacker 2000) was used and 1000 cells were scored per each time point from three replicate assays.

Statistical analysis (I, II, III)
Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc.) and Excel softwares using student's t-test or two factor ANOVA test.

Western blot (I, III)
Cycling HeLa cells or cells arrested into mitosis with 3 μM nocodazole or 0.6 μM taxol for 16 h were harvested by trypsinizing or by mitotic shake-off and centrifugation. For the preparation of 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 5 mg/ml protease inhibitor cocktail. Cells were incubated on ice for 10 min with vortexing and extracts were cleared by centrifugation at 14,000 x g for 10 min followed by addition of sample buffer. In some assays cells were lysed directly in SDS sample buffer. Proteins were separated by SDS-PAGE with 4–12% gradient gel (Lonza) and transferred onto nitrocellulose membrane. Membranes were blocked in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 (TBST) containing 5% nonfat dry milk. The membranes were incubated with rabbit anti-Plk1, mouse anti-Cenp-F, mouse anti-Aurora B (all 1 mg/ml) and rabbit anti-phospho-p38 (1:1000). After washing three times with TBST the membrane was incubated in IRDye® Conjugated 680 nm goat anti-mouse or anti-rabbit IgG (Rockland) diluted 1:5000 in TBST. Incubations were carried out for 1-2 h at RT or over night at +4°C. The Odyssey Infrared Imaging System (LI-COR Biotechnology) was used for detection.

Immunoprecipitation (III)
Dynabeads (Invitrogen) were used according to manufacturer´s protocol to bind p38γ antibody in a citrate-phosphate buffer (pH 5) for 40 min at RT. After washing the beads with buffer, taxol arrested mitotic cell lysate was added and immunoprecipitation reaction was incubated at +4°C for 3 h. Dynabeads-Ab-Ag complexes were washed with PBS-based IP-buffer (PBS, 1 mM EDTA, 2 mM MgCl , 0.2 mM CaCl, 0.5% Triton-X-100, 150 mM NaCl) and proteins were eluted from the beads with SDS sample buffer. Proteins from supernatant were precipitated with acetone. Samples were prepared and western blot was performed as described above. The membrane was blotted with rabbit anti-phospho-p38 (1:1000), rabbit anti-p38α (1:20000) and rat anti-α-tubulin (1:1000).

***In vitro* phosphorylation assays and chromosome isolation (I, II)**
Sgo1 *in vitro* phosphorylation assay was performed as described previously (Lan et al. 2004). Four overlapping fragments of *Xenopus* Sgo1 were used as substrates (amino acids 1-200, 200-350, 350-500, 500-664) which were subcloned into pET-30 (Novagen) by PCR from full length Sgo1 cDNA. Casein or MCAK was used as a control substrate. The recombinant Plk1 and AuroraB/IN box were expressed, purified and used as described earlier (Kumagai and Dunphy 1996, Lan et al. 2004, Rosasco-

Nitcher et al. 2008). To study the *in vitro* effect of INCENP antibody on Aurora-B kinase activity, recombinant Aurora B/IN-box was incubated with INCENP antibody for 10 min at RT prior to *in vitro* kinase assay. In the kinase assay GST-Histone H3 (1–40) was used as a substrate. Kinase assays were started upon addition of substrates and γ-^{32}P-ATP. SDS sample buffer was added to the samples and analyzed by SDS-PAGE. PO_{-4} incorporation on substrate was quantified on a phosphoimager. Chromosome isolation and anti-3F3/2 immunofluorescence were performed as described previously (Ahonen et al. 2005). These assays were done in collaboration with Dr. T. Stukenberg.

FACS (III)
Silenced HeLa cells were harvested after 48 or 72 h after transfection. Cells were fixed in -20˚C EtOH followed by an incubation in -20˚C. Cells were centrifuged with 1300 rpm 4 min. The cell pellet was washed and rehydrated in PBS. RNAse (100 µg/ml) and propidium iodide (20 µg/ml) in PBS was added to the cell pellet and cells were transferred to a round-bottom 96-well plate followed by 30 min incubation with agitation at RT protected from light. Samples were measured with LSR II system (BD Biosciences) and results were analysed with FCS Express –program.

Reverse-Transciptase-PCR (RT-PCR) and Quantitative PCR (Q-PCR) (III)
For both PCR assays nonsilenced and p38 silenced HeLa cells were harvested after 48 h of transfection. RNA was isolated according to the instruction of the RNeasy Midi Kit (Qiagen). For Q-PCR RNA was treated with DNAse I (Invitrogen) before cDNA production. cDNA was made according to the manufacturer´s protocol using iScript™ cDNA Synthesis Kit (Biorad). In RT-PCR different isoforms of p38 were amplified from cDNAs using specific primers for different isoforms and the products were separated on 1% agarose gel including 0.5 µg/ml etidium bromide and imaged with UV light (Gene Genius Bio Imaging System, Syngene). Band intensities were quantified using Metamorph imaging software. In Q-PCR p38 isoforms from cDNA were amplified by specific primers for different isoforms using Q-PCR (TaqMan®, Applied Biosystems).

Caspase activity and inhibition assays (III)
The Apo-ONE homogeneous caspase 3/7 assay (Promega, Madison, WI) was utilized to evaluate the activities of caspase 3 and -7. HeLa cells were silenced with negative control, All Death control, p38α and p38γ siRNAs in 384-well format using oligofectamin reagent in a reverse transfection according to the manufacturer's instructions. After 24, 48 or 72 h of incubation in +37°C, part of the medium was aspirated and Apo-ONE substrate buffer mix was added to each well. The plate was incubated for 30-60 min in agitation protected from light. EnVision 2100 multilabel reader (Perkin Elmer) was used to measure the fluorescence (excitation wavelength 485 nm, emission wavelength 535 nm). Pan-caspase inhibitor zVAD.fmk (20 µM) was used to inhibit caspases in p38γ silenced cells. Caspase inhibitor was added 40 h post-transfection.

5 RESULTS

5.1 Sgo1 in kinetochore assembly (I)

5.1.1 Regulation of Sgo1 kinetochore localization

Previously, Sgo1 has been suggested to localize either at the inner centromere region (Kitajima et al. 2005, Tang et al. 2004a) or at the inner and outer kinetochore (Salic et al. 2004) or both the inner centromere and inner and outer kinetochores (McGuinness et al. 2005). We aimed to clarify this contradiction by both expressing YFP-Sgo1 fusion protein in Hela cells and studying the localization of endogenous Sgo1 by antibody labeling. Sgo1 localization was compared to the outer kinetochore proteins Hec1 and CENP-F (DeLuca et al. 2002, Rattner et al. 1993), inner centromere protein Aurora B (Cooke et al. 1987) and kinetochore marker CREST in different stages of mitosis (I, Fig. 1). Both YFP-Sgo1 and endogenous Sgo1 localized in two dot-like pattern between the two Hec1 and two CENP-F signals from sister kinetochores and outside but partially overlapping with Aurora B signal (I, Fig. 1A-D). This data suggests that Sgo1 localizes at the inner kinetochore region. Interestingly, more Sgo1 was present at unaligned kinetochores and at the kinetochores in monopolar cells compared to metaphase kinetochores (I, Fig. 1-2). Similarly, Aurora B is present in high quantities at unaligned kinetochores (I, Fig. 1, Lan et al. 2004). Thus, the reduction of Sgo1 and Aurora B in aligned chromosomes could be explained by the presence of interkinetochore tension. Occasionally, brighter Sgo1 signal was observed at kinetochores at the metaphase plate (I, Fig. 2). Temporal changes in the interkinetochore tension due to local microtubule dynamics may result in Sgo1 recruitment to metaphase kinetochores. On the other hand, it has been shown that merotelic attachments of the metaphase kinetochores exhibit bright Aurora B signals (Knowlton et al. 2006). Thus, the bright Sgo1 signals at the metaphase plate could mark the same phenomenon.

Sgo1 protein levels were quantified at the kinetochores of Bub1, INCENP, Plk1 and CENP-F depleted Hela cells to determine whether these mitotic proteins were required for the kinetochore binding of Sgo1 (I, Fig. 2, Table 1). Efficacy and specificity of siRNA depletions was confirmed by western blotting (I, Fig. S1) and/or immunostaining the individual kinetochores. Sgo1 levels were similar to nonsilenced controls in mitotic cells depleted for Plk1 and CENP-F (I, Fig. 2, Table 1). As reported earlier (Kitajima et al. 2005, Tang et al. 2004a), Sgo1 levels at the kinetochores were significantly reduced when Bub1 was silenced (I, Fig. 2, Table 1). However, we did not observe Sgo1 accumulation at chromosome arms (Kitajima et al. 2005). In accordance to other reports (Boyarchuk et al. 2007, Dai et al. 2006, Kueng et al. 2006) depletion of the CPC by knocking down INCENP caused Sgo1 accumulation at chromosome arms (I, Fig. 2). Moreover, the Aurora B kinase activity was discovered to be required for the kinetochore localization of Sgo1 based on the result from Aurora kinase inhibitor ZM447439 treated cells where Sgo1 accumulated on chromosome arms in a similar manner as in INCENP depleted cells (I, Fig. 2). Importantly, Aurora

B has been suggested as the main target of ZM447439 (Ditchfield et al. 2003, Girdler et al. 2006, Hannak et al. 2001, Hauf et al. 2003).

5.1.2 Kinetochore hierarchy downstream of Sgo1

Kinetochore binding of Bub1, INCENP, Plk1, CENP-F and Hec1 was studied in mitotic Hela cells after the depletion of Sgo1 by RNAi (I, Fig. 3). Sgo1 silencing efficacy was confirmed with immunofluorescent labeling. Phenotypically Sgo1 silenced cells were easy to recognize because they arrest the cell cycle in so called pseudo-metaphase stage with many unaligned chromosomes (I, Fig. 3) as also reported earlier (McGuinness et al. 2005, Salic et al. 2004). Quantification of kinetochore signals revealed that the binding of Plk1 and CENP-F to the prometaphase kinetochores was significantly reduced due to Sgo1 depletion compared to nonsilenced control cells (I, Fig. 3, Table 1). In contrast, Hec1 and INCENP signal intensities were not affected by Sgo1 depletion (I, Fig. 3, Table 1). Moreover, the levels of Bub1 were significantly higher in Sgo1 depleted cells compared to control cells (I, Fig. 3, Table 1). Interestingly, in nocodazole treated cells Bub1 signal intensity was approximately two times higher compared to controls ($P<0.001$) while in taxol treated cells no enhancement of Bub1 signal was detected ($P<0.015$). Since nocodazole is known to induce unattached kinetochores by depolymerising microtubules while taxol causes microtubule hyperstabilization that preserves the attachments but reduces interkinetochore tension (Waters et al. 1999) the result suggested that Bub1 is enriched at the unattached kinetochores. However, we cannot exclude the possibility that microtubule attachment is the factor that reduces the Bub1 staining due to epitope masking. Importantly, Bub1 was not significantly increased in Sgo1 depleted cells which were treated with nocodazole ($P=0.18$).

Our results showed that Sgo1 is required for Plk1 localization to the kinetochores (I, Fig. 3) that might implicate Sgo1 in tension-sensing mechanism of the SAC. A tension-sensing phosphoepitope 3F3/2 (Gorbsky and Ricketts 1993, Li and Nicklas 1995, Nicklas et al. 1995, Nicklas et al. 1998) is created by Plk1 (Ahonen et al. 2005, Wong and Fang 2005) at the kinetochores in the absence of interkinetochore tension. Interestingly, this phosphoepitope was reduced also at the kinetochores of Sgo1 depleted cells (I, Fig. 3). This data suggests that 3F3/2 phosphoepitope at the kinetochores is dephosphorylated in Sgo1 depleted cells since the phosphorylating kinase Plk1 is also lost in the absence of Sgo1.

Interdependencies between Bub1, INCENP, Plk1 and CENP-F were further studied (I, Table 1) the results being consistent with the previous data (Goto et al. 2006, Johnson et al. 2004, Qi et al. 2006, van Vugt et al. 2004, Yang et al. 2005). It was concluded that these proteins are bound to the kinetochore in a hierarchical manner; Bub1 and the CPC are independently required for Sgo1 binding, Sgo1 attracts Plk1 to the kinetochores which is further required for CENP-F binding (I, Fig. 5).

5.1.3 Sgo1 is directly phosphorylated by Aurora B and Plk1

Sgo1 and Aurora B disappear from kinetochores when chromosomes align to metaphase plate (Lan et al. 2004). In addition, Aurora B activity is required for the kinetochore localization of Sgo1 in prometaphase. These observations raised a question whether Sgo1 is a substrate for the Aurora B kinase. To answer this question *in vitro* kinase assay was performed using recombinant *Xenopus* Aurora B/INCENP and four overlapping recombinant fragments of *Xenopus* Sgo1, purified from E. coli, as substrates (I, Fig. 4). The fragment containing amino acids (aa) 1-200 and the full length Sgo1 were not soluble and not included in the assay. Proven Aurora B substrate, MCAK, was used as a positive control (Lan et al. 2004). Conserved and basic C-terminal region of Sgo1 (aa 500-664) was highly phosphorylated by Aurora B (I, Fig. 4). In addition, the fragment containing aa 200-350 was phosphorylated by Aurora B with lesser extend (I, Fig. 4). However, this fragment is not conserved between human and *Xenopus*. Plk1 homolog, Polo, has been shown to bind and phosphorylate Sgo1 homolog, MEI-S332 in *Drosophila in vitro* (Clarke et al. 2005). To test if Plk1 phosphorylates Sgo1 also in vertebrates, the fragments of Sgo1 were used as a substrate for Plx1 (*Xenopus* Plk1) in an *in vitro* kinase assay (I, Fig. 4). Casein was used as a positive control (Fenton and Glover 1993). The result revealed that Sgo1 fragment aa 350-500 was efficiently phosphorylated by Plx1 and also the fragment aa 200-350 but to a lesser extend. A mobility shift of Sgo1 fragment aa 350-500 compared to the Aurora B/INCENP treated fragment was observed suggesting that Plx1 phosphorylates multiple Ser/Thr residues on Sgo1.

5.2 Role of INCENP during mitosis (II)

5.2.1 Early mitotic effects of INCENP^{-ab}

To temporally analyze the mitotic tasks of the CPC, we used function blocking antibody against INCENP (INCENP^{-ab}) which is suggested to target INCENP in its natural localizations based on its immunostaining pattern (II, Fig. S1A, (Cooke et al. 1987)) and western blot analysis in *Xenopus* (Bolton et al. 2002). When INCENP^{-ab} was injected into metaphase or anaphase Xeno S3 cells (II, Fig. S1B-S1C), it located rapidly to the endogenous INCENP locations.

INCENP^{-ab} was injected into prophase *Xenopus* cells and they were followed with time-lapse microscopy. Rapid poleward chromosome movements were observed upon NEB indicating that the chromosomes made initial attachments with microtubules (II, Fig. 1A-B, Supplemental material). Nevertheless, most chromosomes of the antibody injected cells were unable to align to metaphase plate within the same time as the chromosomes of the control IgG injected cells (II, Fig. 1C). Also chromosome movements appeared slower in INCENP^{-ab} injected cells. These results agree with the previous data that showed defects in chromosome alignment when INCENP was silenced or genetically perturbed (Adams et al. 2001b, Mackay et al. 1998). These data suggest that INCENP^{-ab} does not prevent formation of kinetochore-microtubule

attachments but interferes with the correction of erroneous connections leading to abnormal chromosome congression.

Injection of INCENP^{-ab} to Xeno S3 cells between prophase and metaphase caused premature decondensation of the chromosomes followed by exit from M-phase without cytokinesis in 75% of the injected cells resulting in polyploidy (II, Fig. 1A-B, Supplemental material). However, the time between NEB and premature chromosome decondensation in the INCENP^{-ab} injected cells did not statistically differ from the time from NEB to anaphase in control cells. The rest 25% of the INCENP^{-ab} injected early mitotic cells underwent cytokinesis with abnormal sister chromatid movements (data not shown). The inability of the INCENP^{-ab} injected cells to delay mitosis despite of the unaligned chromosomes indicated weakened or inactived SAC. Thus, we analyzed if INCENP^{-ab} causes an override of the SAC induced by microtubule interfering drugs nocodazole or taxol or a proteasome inhibitor MG132 that causes metaphase block downstream of the SAC. After INCENP^{-ab} injection cells were incubated for two hours in the continuous presence of the drugs and then fixed and categorized into interphase or mitotic cells based on their nuclear morphology (II, Fig 1D). The cells with normal SAC arrest in mitosis for several hours upon a microtubule drug treatment (Stukenberg and Burke 2004). The majority of the cells treated with microtubule drugs prior to INCENP^{-ab} injection exhibited forced mitotic exit without completion of cytokinesis as well as the INCENP^{-ab} injected cells without any drug treatment (II, Fig. S1D-F). Interestingly, most cells treated with MG132 prior to INCENP^{-ab} injection arrested in mitosis (II, Fig. S1D-F). This indicates that the SAC is prematurely inactivated in INCENP^{-ab} injected cells resulting in proteasome-dependent forced mitotic exit.

The cell cycle defects caused by INCENP^{-ab} resemble the defects of Aurora B inhibition with small molecule inhibitors (Gadea and Ruderman 2005, Hauf et al. 2003). The *in vitro* kinase assay showed that INCENP^{-ab} inhibited Aurora B activity by ~40% (II, Fig. 1E). *In vivo*, INCENP^{-ab} reduced the integrated signal intensity of Aurora B target phosphoepitope Thr95 on MCAK (Zhang et al. 2007) at the chromosome arms and centromeres by 28% compared to control IgG injected cells (II, Fig. 1F-H, $P=0.01$). As expected, the signal of pThr95 was reduced almost to background levels in cells cotreated with Aurora B inhibitor ZM447439 and MG132 (II, Fig 1F and H, $P<0.0001$). Because INCENP^{-ab} causes partial inhibition of Aurora B, the kinase most probably contributes to the observed early mitotic defects.

In order to study if the observed chromosome alignment defects could be explained by the spindle damage, the microtubule network of INCENP^{-ab} injected cells was analysed. Prophase cells were pre-incubated with MG132 prior to INCENP^{-ab} injection to prevent precocious exit from mitosis followed by microtubule staining (II, Fig. 2A). In MG132 treated control cells all chromosomes were aligned to metaphase plate and thick bundles of microtubules were attached to chromosomes (II, Fig. 2A). In contrast, in INCENP^{-ab} injected cells the spindle morphology was abnormal and fewer and thinner kinetochore-microtubule bundles were detected (II, Fig. 2A). These cells were unable to align their chromosomes to metaphase plate, as expected (II, Fig. 2A). When cells were pretreated with nocodazole to depolymerize the microtubules followed by

INCENP^{-ab} injection and incubation in MG132-containing medium to allow the spindle reformation, the spindle effects were even stronger (II, Fig. 2B). Importantly, the amount of microtubules emanating from the spindle poles was significantly reduced and the microtubules were without clear connections with chromosomes (II, Fig. 2B). In sharp contrast, in control cells bipolar spindles had reformed with thick kinetochore-microtubule bundles, spread astral microtubule sheets and all chromosomes in metaphase configuration (II, Fig. 2B).

INCENP^{-ab} or control buffer was injected into metaphase Xeno S3 cells stably expressing GFP-tubulin in presence of stable kinetochore-microtubule attachments and followed with time-lapse microscopy (II, Fig. 2C-D). In INCENP^{-ab} injected cells reduced chromosome oscillations were observed. Later, the chromosomes started to decondence maintaining the chromosomes in metaphase configuration. This indicated that their connections with microtubules were not lost (II, Fig. 2C). However, the shortening of the spindle and extension of astral microtubules was observed compared to control cells (II, Fig. 2C-E, Supplemental material). Moreover, the kinetochore-microtubule bundles appeared to depolymerise from their minus ends during the forced mitotic exit (Supplemental material). The average metaphase spindle length in MG132 pretreated cells was observed to be significantly shorter due to INCENP^{-ab} injection compared to controls (II, Fig. 2F, $P<0.001$). These data indicates that when INCENP^{-ab} is injected into prophase cells it interferes with the assembly of the mitotic spindle and when injected into metaphase cells it perturbs the maintenance of the normal spindle.

5.2.2 Dynamics of INCENP and Aurora B

Rapid turnover of the regulatory centromere and kinetochore proteins is thought to be essential for the SAC signaling (Howell et al. 2004, Kallio et al. 2002a, Shah et al. 2004). The protein turnover halftime ($t_{½}$) and the mobile protein fraction (recf) of GFP-tagged x/hINCENP was studied in *Xenopus*, LLC-PK and Hela cells using fluorescence recovery after photobleaching (FRAP) technique. The average $t_{½}$ of GFP-INCENP at the metaphase centromeres of Xeno S3 was 53 ± 19 s (II, Table 1) and of LLC-PK 100 ± 37 s (II, Table S1) . Similar values compared to Xeno S3 were measured in Hela cells (data not shown). High recf value (83 ± 8%) of GFP-xINCENP suggested that immobile fraction of the protein was very small. When microtubules were depolymerized with nocodazole, no significant change in GFP-INCENP turnover or mobile fraction in Xeno S3 or in LLC-PK cells was observed (II, Table 1, Table S1). Thus, INCENP is exchanging at the inner centromeres with a constant rate independently from the kinetochore-microtubule attachments and SAC activity. Dynamics of Aurora B kinase was also measured. The values for Aurora B-YFP in Xeno S3 (II, Table 1) and Hela (data not shown) were consistent with each other and demonstrated that Aurora B is turning over at the centromeres with moderate dynamics similarly as INCENP in early mitotic cells. Our observation supports the previous data suggesting moderate dynamics for Aurora B kinase at the centromeres (Murata-Hori et al. 2002) instead of the other results indicating that Aurora B is stably associated with the centromeres (Delacour-Larose et al. 2004).

Due to the fast override of the SAC by INCENP^{-ab} we asked whether INCENP^{-ab} has an effect on the binding dynamics of the CPC members with the inner centromeres. To answer this $t_{½}$ and recf values were measured for GFP-xINCENP and xAurora B-YFP in uninjected control and INCENP^{-ab} injected Xeno S3 pretreated with MG132. MG132 slightly increased the turnover of INCENP (II, Table 1, Fig. 3A) compared to untreated metaphase cells (II, Table 1). The normal centromere association of GFP proteins was confirmed in early mitotic cells that were injected into nucleus with INCENP^{-ab} at prophase (data not shown). In MG132-treated control cells, GFP-xINCENP and xAurora B-YFP showed similar dynamics (II, Fig. 3A and C, Table 1, Supplemental material) and INCENP^{-ab} significantly reduced their recovery at the centromeres (II, Fig. 3B and D, Table 1, Supplemental material, $P<0.0001$). Protein half lives could not be measured in INCENP^{-ab} injected cells because of the very low recf values. Based on these results, INCENP^{-ab} strongly inhibited the turnover of both INCENP and Aurora B preventing INCENP and Aurora B dissociation from the centromeres.

5.2.3 Late mitotic effects of INCENP^{-ab}

To perturb INCENP/CPC in anaphase without disturbing the early mitotic stages INCENP^{-ab} was introduced into anaphase cells at one min intervals after the first signs of sister chromatid separation. Blocking INCENP function within 3 min after anaphase onset (n=6 cells) prevented normal sister chromatid movements (II, Fig. 4A-B). When anaphase cells were injected around the onset of anaphase, sister chromatids became almost immobile (II, Fig. 4A, Supplemental material). Normally, incomplete sister chromatid separation led to failure in cytokinesis but some cells underwent cytokinesis despite of the erratic sister separation (II, Fig. 5A). Typically, ingression of cleavage furrow was initiated but the furrow regressed (II, Fig. 4B, Supplemental material). Injection after 3-4 min after anaphase onset (n=3 cells) resulted in a failure in separation of the daughter cells and abnormal midbody formation (II, Fig. 4C, Supplemental material). When INCENP^{-ab} was introduced 5 or more min after anaphase onset (n=4 cells) sister chromatid separation and cytokinesis were normal (II, Fig. 4D, Supplemental material). Control IgG was injected into cells 1-2 min after anaphase onset (n=5 cells) and all the cells went through normal anaphase and cytokinesis within 15 min (II, Fig. 4E).

Observed mistakes in anaphase sister chromatid separation could be explained by spindle malfunction. Therefore, INCENP^{-ab} or control buffer was introduced into early anaphase Xeno S3 cells expressing GFP-tubulin and followed using time-lapse microscopy until the mitotic exit. The result revealed that INCENP^{-ab} disturbed spindle elongation and separation of the spindle poles (II, Fig. 5A-D, Supplemental material) since spindles were notably shorter after the first 5 min of anaphase in INCENP^{-ab} injected cells (2.9 ± 2.9 µm, n=5 cells) compared to controls (6.3 ± 2.0 µm, n=5 cells, $P=0.03$). Microtubule bundles at the spindle midzone were also thicker (II, Fig. 5E-F) and their number was reduced in INCENP^{-ab} injected cells (13 ± 2, n=5 cells) compared to controls (22 ± 2, n=5 cells, $P<0.001$). Moreover, astral microtubules (II, Fig, 5G) were significantly longer in INCENP^{-ab} injected cells (22.8 ± 3.9 µm, n=5 cells) compared to controls (14.2 ± 6 µm, n=5 cells, $P<0.001$). These data imply that

perturbation of INCENP function in early anaphase prevents normal sister chromatid and spindle pole separation. Our results support the previous data reporting that INCENP function is required for cytokinesis and abscission of the midbody (Ruchaud et al. 2007).

5.3 Novel role of p38γ MAPK in mitosis (III)

5.3.1 Effect of p38γ depletion on mitotic progression and survival

All four p38 isoforms were depleted individually from Hela cells using RNAi to study their roles in mitotic progression. The efficacy and specificity of siRNAs were determined using both reverse transcriptase PCR (RT-PCR) and quantitative PCR (Q-PCR). RT-PCR revealed that p38α and p38γ were the most abundant isoforms in Hela cells (III, Fig 1A-B) and their depletion by RNAi was more effective compared to p38β and p38δ (III, Fig. 1B-C). The depletion of p38α and p38γ with second siRNAs with different targeting sequence was not as effective as with the first siRNAs tested (III, Fig 1C). For this reason the first siRNAs were chosen for further studies. Q-PCR confirmed that p38α and p38γ siRNAs did not affect the mRNA quantities of the other p38 isoforms (III, Fig. 1D).

The cell cycle progression of control, p38α and p38γ depleted Hela cells were monitored. Live cell imaging (III, Fig. 2A-C) and FACS (III, Fig. 2E) showed normal proliferation rates, mitotic indices (4.1 ± 1.8% and 6.2 ± 2.3%), cell cycle progression and low cell death indices (4.8% and 4.0% at 72 h) for control and p38α silenced populations. In contrast, silencing of p38γ with first siRNA caused accumulation of mitotic cells (27.4 ± 8.3%, $P<0.001$) resulting in increased cell death (31.6% at 72 h, III, Fig. 2E). Importantly, the second siRNA for p38γ produced the same phenotype (III, Fig. 2A-B), but milder as seen with the first siRNA. Thus, the first siRNA was selected for further studies. In protein level p38γ was reduced 39 % in the p38γ silenced cycling population after 30 h of transfection (III, Fig. 2D). The cell death induced by p38γ depletion was confirmed with light microscopy, because FACS may underestimate cell death due to the loss of fragmented nuclei. Cell populations were filmed for 72 h starting immediately after transfection and cells were categorized into living or dead cells using criteria defined by Häcker 2000 (n=500 cells per each time point). Result confirmed that more p38γ silenced cells died by 72 h (69.0 ± 13.0%) compared to control (16.4 ± 1.0%) or p38α (19 ± 3%) silenced populations (III, Fig. 2F, $P<0.001$). Apo-ONE® assay indicated the elevation of caspase 3 and -7 activities in p38γ and cell death control siRNA transfected cells after 48 and 72 h of transfection compared to control and p38α silenced cells (III, Fig. 2G, $P<0.01$ at 48 h and $P<0.001$ at 72 h post-transfection for p38γ siRNA cells). In addition, the cell death in p38γ silenced population was observed to be reduced in response to pan-caspase inhibitor zVAD.fmk treatment compared to DMSO treated population (III, Fig.S1, $P<0.05$). These data suggested that the cell death induced by the depletion of p38γ is caspase-dependent. Time-lapse filming indicated that the majority of the cells died during mitosis or immediately after. Thus, we followed the fate of mitotic cells of siRNA transfected cells starting time-lapse filming 48 h after transfection. The viability of

mitotic cells in p38γ silenced population decreaced significantly during the mitotic arrest. Seven hours after the cells were entered mitosis an average of 50% of the cells was dead and after 12 h the maximum death index (96%) was achieved (III, Fig. 2H). The difference is dramatically different ($P<0.001$) between p38γ silenced and p38α or control silenced populations in which less than 5% of mitotic cells died during filming (III, Fig. 2H). Majority of the mitotic cells (72%) died without exit from mitosis while 24% exited mitosis. However, all the formed daughter cells died within four hours after exit from M phase.

5.3.2 Chromosome alignment and anaphase defects caused by p38γ depletion

To reveal the details of chromosome segregation in p38γ silenced cells Hela cells stably expressing H2B-GFP were transfected with p38γ, p38α or control siRNA and their progression was monitored with time-lapse microscopy (III, Fig. 3, supplemental material). As expected, the depletion of p38γ caused mitotic arrest followed by cell death in most cells (III, Fig 3A-B). The average length of mitosis in the p38γ depleted cell population was significantly longer (275 ± 130 min, range 49 - 910 min, $P<0.001$) than in p38α (54 ± 28 min) or control silenced populations (59 ± 19 min, III, Fig. 3C) and many misaligned chromosomes were observed. The majority of the cells had problems in aligning the chromosomes to metaphase plate while in some cells the alignment was successful but the cells failed to maintain the alignment (III, Fig. 3B). The amount of prometaphase cells was increased while less anaphase cells were observed in the p38γ silenced population compared to p38α or control silenced populations ($P<0.001$, III, Fig 3B). Some mitotic cells which were able to enter anaphase exhibited unaligned chromosomes and chromatid bridges (III, Fig. 3C). No significant differences were observed in the duration of mitotic arrest or in the number of escaping cells from taxol block indicating that the depletion of p38γ did not cause an override of the mitotic arrest induced by microtubule interfering drugs suggesting a functional SAC in these cells.

5.3.3 The effect of p38γ depletion on mitotic spindle and interkinetochore tension

To study whether the defective chromosome alignment in p38γ depleted cells could be explained by the changes in mitotic spindle morphology, control and p38γ silenced cells were stained with anti-pericentrin and anti-tubulin antibodies 48 h after siRNA transfection. In p38γ silenced cells multipolarity was observed to be remarkably increased (65.5 ± 4%, $P<0.001$) compared to control cells (3 ± 3%) while monopolarity was not detected in either populations (III, Fig. 4A). Extra microtubule emanating foci was typically formed near the main spindle pole in p38γ silenced cells. In addition, the spindle length was significantly increased ($P<0.01$) in p38γ silenced cells compared to controls indicating the elongation of the spindle. Other disturbance of the spindle structure was not detected (III, Fig 4A). Interkinetochore distance in metaphase cells was significantly reduced due to p38γ depletion compared to controls ($P<0.001$) being almost identical to the distance between the sister kinetochores in nocodazole treated cells (III, Fig. 4B). This indicates strong reduction of tension between sister kinetochores in p38γ silenced cells. However, the cold calcium buffer treatment which

removes unstable microtubules revealed that kinetochore-microtubules were stable in p38γ silenced cells (III, Fig 4C).

A mitotic kinesin, Eg5, is required for the bipolar spindle formation (Kapoor et al. 2000). To test if this kinesin is required for the generation of multipolar spindles in p38γ depleted cells, we treated the p38γ and control cells with Eg5 inhibitor, monastrol, washed it out and allowed the spindles to reform in MG132 containing medium to maintain the cells in mitosis. The majority of the control cells arrested in mitosis with monopolar spindles as expected while only 37 ± 8% of p38γ silenced cells exhibited monopolar spindles after four hours treatment with monastrol (III, Fig. 4D). The difference can be explained by the fact that many p38γ silenced cells were already arrested in mitosis with bi- or multipolar spindles at the time of monastrol addition and remained in this stage (III, Fig 4D). The majority of control cells formed bipolar spindles within one hour after removal of monastrol. In contrast, most of the p38γ silenced cells formed multi-polar spindles and only 24 ± 4% formed the bipolar spindles with unaligned chromosomes (III, Fig. 4D-F). This indicates that Eg5 reactivation was required for multipolar spindle formation in p38γ silenced cells.

5.3.4 Regulation of p38 MAPKs at the kinetochores and poles

Previously, it has been reported that active phosphorylated p38 localizes to cytoplasm (Fan et al. 2005) or spindle poles during mitosis (Cha et al. 2007, Lee et al. 2010, Tang et al. 2008). We wanted to confirm the mitotic location of the active kinase by immunostaining the cells with anti-phospho-p38 antibody (p-p38) that recognizes the Thr180/Tyr182 phosphorylated p38 MAPK isoforms (III, Fig. 5 A-E). The active p38 MAPKs were detected at the kinetochores from prophase to telophase, at the spindle poles from prometaphase to late telophase and at the midbody at telophase. The cytoplasmic fraction was observed as well throughout mitosis. Immunoprecipitation of p38γ from taxol arrested mitotic cells and detection with p-p38 antibody in western blot confirmed that the p-p38 antibody also recognized p38γ isoform (III, Fig. 5F). Detection with p38α antibody in western blot showed that p38γ antibody did not recognize or pull down p38α isoform. This result also indicated that p38γ and p38α isoforms do not interact with each other during mitosis.

We depleted many mitotic proteins in Hela cells using RNAi and immunostained the cells with p-p38 antibody to examine what may regulate the activation of p38 MAPKs at the kinetochores and spindle poles. Surprisingly, depletion of Plk1, Sgo1 and Aurora A caused remarkable upregulation of p-p38 signal as the signal intensity became very intense and it also appeared to mislocalize from the kinetochores to chromosome arms while the depletion of INCENP did not have the same effect on p-p38 signal (Fig. 11A, unpublished results). We also treated the cells with nocodazole, monastrol and Plk1 inhibitor ZK-thiazolidinone (TAL, Santamaria et al. 2007) and discovered that these inhibitors had similar effect on p-p38 compared to Plk1, Sgo1 and Aurora A depletion (Fig. 11A, unpublished results). However, p-p38 did not spread as widely to the chromosome arms due to the treatment with monastrol and TAL than due to Plk1, Sgo1 and Aurora A depletion or nocodazole treatment. Depletion of Plk1, Sgo1 and

Aurora A as well as the treatment with the used inhibitors all cause mitotic arrest in response to the continuous SAC activation (Ducat and Zheng 2004, Salic et al. 2004, Santamaria et al. 2007, Sumara et al. 2004, Tang et al. 2004a, van Vugt et al. 2004) while depletion of INCENP causes an override of the SAC and forced exit from mitosis (Adams et al. 2001b, Honda et al. 2003). The result implied that p38 MAPKs accumulate or hyperphosphorylate and mislocalize in response to the hyperactivated SAC rather than to loss of function of one specific protein silenced/inhibited here. To examine the time dependency of p-p38 signal upregulation during prolonged mitosis, we depleted cells of Plk1, which caused mitotic arrest due to monopolar spindle structure. We washed out the mitotic cells 24 h after Plk1 siRNA transfection. We fixed and stained the cells with anti-p-p38 antibody one, three and six hours after the mitotic wash-out. Interestingly, p-p38 signal intensity increased during the prolonged mitosis and was 2-fold after three hours and 2.5-fold after six hours of mitotic arrest compared to p-p38 intensity in cells which had spent only one hour in mitosis (n=26 cells at each time point). In addition, p-p38 staining spread more to chromosome arms longer the cell was arrested to mitosis (Fig. 11B, unpublished results). This result supports the hypothesis that the kinetochore and spindle pole localized p38 MAPKs are regulated by SAC pathway and suggest that p38 MAPKs may be involved in the maintenance of SAC signaling.

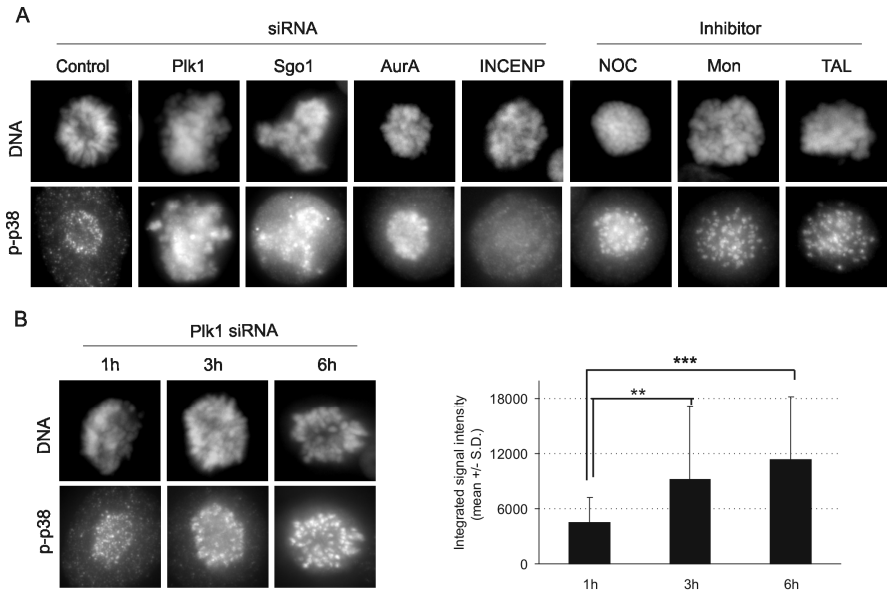

Figure 11. The p38 MAPKs are upregulated and mislocalized in response to the SAC hyperactivation. (A) Representative Hela cells depleted for control, Plk1, Sgo1, Aurora A or INCENP, or treated with nocodazole, monastrol or TAL showing DNA and p-p38 staining. (B) Plk1 depleted cells which have spent 1, 3 or 6 h in mitosis showing DNA and p-p38 staining. Integrated signal intensity increases in time being 4.5 ± 2.7 after 1 h, 9.1 ± 8.0 after 3 h and 11.3 ± 6.9 after 6 h in mitosis (values x 10^6). Data are mean ± s.d. The asterisks point to a statistical significance (** $P<0.01$, *** $P<0.001$).

Next, we utilized lysed cell assay to examine which kinases might be responsible for p38 MAPK activation during mitosis (Ahonen et al. 2005, Daum and Gorbsky 2006). This method allowed us to study the p38 MAPK activation in stressless conditions in normally cycling cells. In this assay, nontreated cells are extracted with a detergent followed by a treatment with buffer lacking phosphatase inhibitors. During this step, the cytoplasm is removed and phosphoepitopes on subcellular structures such as kinetochores are dephosphorylated by active phosphatases. An addition of ATP and phosphatase inhibitors results in reactivation of bound kinases and rephosphorylation of their substrates. As expected p-p38 signal was removed from kinetochores and poles after dephosphorylation (III, Fig. 6A) and addition of ATP and phosphatase inhibitor was able to reactivate it in these structures without any external source of kinase activity (III, Fig. 6B). This indicates that the kinase(s) which activate p38 MAPK are bound to mitotic structures. We tested many inhibitors to discover which specific kinase was responsible for the activation of p38 MAPK. Interestingly, Plk1 inhibitor TAL reduced the p-p38 signal intensity during rephosphorylation step (III, Fig 6B) while Aurora B inhibitor ZM447439 (Ditchfield et al. 2003) had no effect on the p-p38 signal. The involvement of Plk1 in phosphorylation of p38 MAPK was confirmed with exogenous rephosphorylation step. All endogenous kinase activity was first inactivated with N-ethylmaleimide (NEM) followed by addition of exogenous kinases present in S phase extract, M phase extract or Plk1 depleted M phase extract. Addition of M phase extract, but not S phase extract, regenerated p-p38 signal indicating that kinase required for p38 MAPK activation was only present during mitosis (III, Fig 7A and C). Expectedly, the depletion of Plk1 from M phase extract appeared to remove the ability of M phase extract to reactivate p38 MAPK at kinetochores and spindle poles (III, Fig. 7B-C). This confirms that Plk1 is indeed required for p38 MAPK activation during mitosis.

5.3.5 p38γ is required for kinetochore targeting of Plk1

Recently, it was reported that p38 MAPK substrate MK2 phosphorylates Plk1 on Ser326 indicating that Plk1 is a target of p38 MAPK pathway during mitosis (Tang et al. 2008). To study the effect of p38γ depletion on Plk1 subcellular localization we stained the cells with Plk1 antibody after p38γ siRNA treatment. The kinetochore accumulation of Plk1 was dramatically reduced due to p38γ depletion compared to control ($P<0.001$) while cytoplasmic fraction was increased (III, Fig 8A). Plk1 at the main spindle poles was also diminished in p38γ silenced cells compared to control cells ($P<0.01$). However, signal intensity of phospho-Plk1 (Ser326) at the poles was not affected by p38γ depletion (III, Fig. 8B). This suggests that other p38 MAPK isoforms are responsible for pole localized Plk1 phosphorylation on Ser326. Moreover, the phosphorylation of MK2 on Thr334 was not affected by p38γ depletion (data not shown).

6 DISCUSSION

6.1 The central role of Sgo1 in kinetochore assembly (I)

In many species, Sgo1 is best characterized in the protection of centromeric cohesion during both mitosis and meiosis (Kerrebrock et al. 1995, Kitajima et al. 2004, Salic et al. 2004). Futhermore, it has been described in tension-sensing mechanism of the SAC and in regulation of kinetochore-microtubule stability (Salic et al. 2004). In human, Sgo1 is also suggested in kinetochore-driven microtubule formation (Suzuki et al. 2006). Interestingly, many tasks of Sgo1 appear to be coupled with the function of two important mitotic regulators, the CPC and Plk1 (Ahonen et al. 2005, Gorbsky and Ricketts 1993, Goto et al. 2006, van Vugt et al. 2004). The CPC and Plk1 are recognized as cancer relevant mitotic factors but the role of Sgo1 in cancer is not extensively studied. Although some links between Sgo1 and cancer has been established (Iwaizumi et al. 2008, Scanlan et al. 2001), very little is known how it may be involved in tumorigenesis. Therefore, more research on the function of Sgo1 in mitotic progression as well as in cancer cells is required.

6.1.1 Bub1 and Aurora B independently regulate Sgo1 binding to the kinetochores

Based on our results, Sgo1 localizes to the inner kinetochore of the mitotic chromosome from prophase to early metaphase. Earlier, Sgo1 has been detected at the inner centromere and inner and outer kinetochore regions (Kitajima et al. 2005, McGuinness et al. 2005, Salic et al. 2004, Tang et al. 2004a). Since many alternative splice variants have been found for Sgo1 (McGuinness et al. 2005), the discrepancy in localization results could be explained by the used antibodies, which may recognize different isoforms. The levels of Sgo1 at the kinetohores follow the levels of mitotic kinases Bub1 and Aurora B kinases (Lan et al. 2004, Taylor and McKeon 1997) suggesting a relationship between Sgo1 and these kinases. Indeed, it has been shown by us and the others (Kitajima et al. 2005, Resnick et al. 2006, Tang et al. 2004a) that the kinetochore localization of Sgo1 depends on the presence of Bub1 and the members of CPC as well as the Aurora B kinase activity. Our results disagree with previous studies which suggest that Aurora B inactivation decreases Bub1 levels at the kinetochore (Hauf et al. 2003). Instead, our data supports the independent roles for Bub1 and Aurora B in the regulation of Sgo1 binding at the kinetochores (Meraldi and Sorger 2005). Depletion of Sgo1 caused an increase of Bub1 levels at the kinetochore. Importantly, Bub1 signal was increased at the kinetochores in taxol treated cells which suggest that Bub1 is enriched at the kinetochores that lack microtubule attachments. This observation strengthens the notion that Sgo1 participates on kinetochore-microtubule interactions (Salic et al. 2004).

Sgo1 kinetochore signals were brighter at the unaligned chromosomes and the intensity diminished when chromosomes aligned to metaphase plate resembling the labeling pattern of Aurora B kinase. Thus, the affinity of both Sgo1 and Aurora B binding may be regulated by interkinetochore tension across the centromere created by bipolar

microtubule attachments. Occasionally, changes in the interkinetochore tension occur at the chromosomes that are already aligned to the metaphase plate, for example, due to erroneous microtubule attachments. It has been shown that metaphase kinetochores possessing merotelic attachments show bright Aurora B signals (Knowlton et al. 2006). Accordingly, bright Sgo1 signals were occasionally detected at the metaphase plate. Thus, it is possible that Sgo1 is recruited to the merotelically attached kinetochores by the other SAC proteins such as the CPC subunits. The accumulation of Sgo1 to merotelically attached kinetochores could be tested in an experiment where the cells are co-stained with anti-Sgo1 and anti-Ndc80 antibodies after nocodazole treatment followed by wash-out. This treatment has been shown to increase the number of merotelic attachments in cells (Cimini et al. 2001). Moreover, it has been demonstrated that misshapen Ndc80 foci can be used as a visual marker of merotelically attached kinetochores (Knowlton et al. 2006). Thus, the increased accumulation of Sgo1 on metaphase kinetochores of nocodazole wash-out cells compared to nontreated control cells as well as the colocalization of bright Sgo1 signal with distorted Ndc80 signal would provide evidence on Sgo1 accumulation on merotelically attached kinetochores. Nevertheless, this experiments remains to be performed.

It was shown previously that *Drosophila* Mei-S332 is phosphorylated by Aurora B *in vitro* (Resnick et al. 2006). Moreover, the CPC was involved in regulating Mei-S332 localization and in controling the sister chromatid cohesion in meiosis. Our *in vitro* kinase assay provided evidence that Sgo1 is a direct substrate of Aurora B kinase also in vertebrates. This suggests that Sgo1 phosphorylation by Aurora B possibly leads to Sgo1 recruitment to the kinetochores. In the future, this could be confirmed with studies using phospho-site mutated Sgo1 fusion proteins. Sgo1 was observed along chromosome arms in Aurora B inhibited or depleted cells as also shown previously (Boyarchuk et al. 2007, Dai et al. 2006, Kueng et al. 2006). This might explain the function of Aurora B in removing cohesion from chromosome arms during early mitosis (Gimenez-Abian et al. 2004, Losada et al. 2002). However, Sgo1 was not accumulated along chromosome arms in INCENP depleted cells in *Drosophila*, but it was absent from both kinetochores and chromosomes (Resnick et al. 2006). The discrepancy might be simply explained by the differences between mitosis and meiosis or between different species. However, the link between the CPC and Sgo1 appears evolutionary conserved and to be involved both in mitotic and meiotic regulation.

6.1.2 Sgo1 regulates the kinetochore binding of Plk1 and is linked to tension sensing mechanism of the SAC

Plk1 is a multifunctional mitotic kinase regulating centrosome maturation and duplication, mitotic entry, bipolar spindle assembly, removal of cohesin from chromosome arms as well as cytokinesis (Barr et al. 2004). We observed that Plk1 localization at the kinetochores, but not at spindle poles, was dependent on the presence of Sgo1. Furhermore, CENP-F lost its kinetochore localization in the absence of Sgo1. The study of interdependencies between many kinetochore proteins showed that in fact Plk1 was responsible for CENP-F localization at the kinetochores.

The observation that Sgo1 is required for Plk1 kinetochore accumulation suggests a role for Sgo1 in tension sensing mechanism. The phosphorylation status of a kinetochore protein(s) can be identified by labeling with an antibody against the 3F3/2 phosphoepitope. The kinetochores that are not under tension are specifically phosphorylated at an epitope recognized by this monoclonal antibody. When sister kinetochores become fully saturated with microtubules and equal pulling forces are exerted upon them, 3F3/2 phosphoepitope is lost (Gorbsky and Ricketts 1993, Li and Nicklas 1995, Nicklas et al. 1995, Nicklas et al. 1998). Plk1 is known to create this tension-sensing 3F3/2 phosphoepitope at the tensionless kinetochores (Ahonen et al. 2005, Wong and Fang 2007). Importantly, the 3F3/2 phosphoepitope was reduced in Sgo1 depleted cells. This suggests that 3F3/2 phosphoepitope was lost or dephosphorylated at the kinetochores of Sgo1 depleted cells. Since Plk1 was also depleted from kinetochores in Sgo1 silenced cells the latter option appears to be the more likely reason.

Interestingly, xSgo1 was observed to be directly phosphorylated by Plx1 possibly on multiple Ser/Thr residues *in vitro*. Previously, it was shown that Plk1 binds and phosphorylates Mei-S332 *in vitro* (Clarke et al. 2005) and that Plk1 activity was required to remove Mei-S332 and human Sgo1 from kinetochores during metaphase-anaphase transition (Clarke et al. 2005, Tang et al. 2006). Collectively, our results and other published data suggest that Plk1 is targeted to kinetochores after recognition of a priming phosphorylation on Sgo1 or in some other kinetochore protein, which is dependent on Sgo1 for its localization. It has been shown that Plk1 phosphorylates Bub1, which was suggested to target Plk1 to the kinetochores (Qi et al. 2006). However, based on the observations that Sgo1 depletion removes Plk1 but enhances Bub1 at the kinetochores, alternative model can be proposed. According to this model, Sgo1 intially recruits Plk1 to the kinetochores where it phosphorylates Bub1 and possibly its other targets. Our results disagree with the notion that INCENP is required for Plk1 kinetochore recruitment (Goto et al. 2006). In our studies Plk1 dimished from the kinetochores in Sgo1 depleted cells despite the normal levels of INCENP.

In summary, our data together with published results reveal a central role for Sgo1 and Plk1 in kinetochore assembly (Fig. 9. I, Fig. 5). Based on our results, kinetochore accumulation of Sgo1 is independently regulated by the presence of Bub1 and the phosphorylation by Aurora B. Presence of Sgo1 is required for Plk1 recruitment to the kinetochore that in turn is responsible for CENP-F recruitment. Sgo1 is known to protect centromeric cohesion in cooperation with PP2A, which is thought to counteract cohesin phosphorylation by Plk1 (Kitajima et al. 2006). We speculated that when tension is established across the centromere Plk1 phosphorylates Sgo1 leading to the removal of Sgo1 from the kinetochores. Thus, also the protection of centromeric cohesion ceases. It has been shown in yeast, that the removal of centromeric cohesin requires Scc1 phosphorylation by Cdc5, a polo-like kinase before separase cleavage (Alexandru et al. 2001). Removal of Sgo1 from kinetochores results in Plk1 and CENP-F dissosiation from the kinetochores as well. This event may contribute to the SAC inactivation in metaphase cells since Plk1 is a central kinase which is required for proper subcellular localization and activation of several important proteins regulating

mitotic progression. The events leading to Sgo1 phosphorylation by Plk1 still remain to be discovered. Our conclusions combined with previous data by the others are summarized in fig. 12.

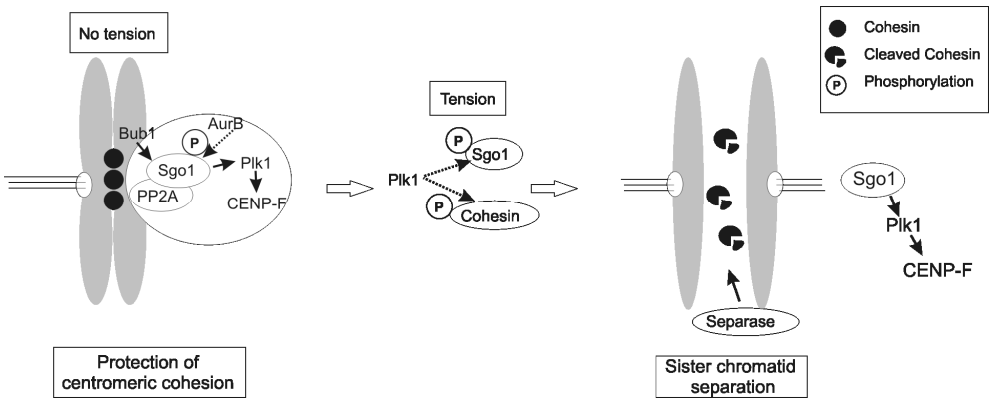

Figure 12. Sgo1 is a central kinetochore protein. Model is based on our results and previously published data by others.

6.2 Mitotic tasks of INCENP (II)

The CPC has various tasks during cell division from early mitotic phases until completion of cytokinesis (Ruchaud et al. 2007). The Aurora B kinase is the enzymatic member of the complex and has multiple mitotic substrates. Other members of the core CPC (INCENP, Survivin and Borealin) are also essential for the proper function of the complex and mitotic progression. They are thought to be responsible for the targeting of the complex to its subcellular locations (Adams et al. 2001b, Honda et al. 2003). Moreover, INCENP is required for the full activity of Aurora B (Bishop and Schumacher 2002, Honda et al. 2003, Sessa et al. 2005). The CPC is a potential target for cancer treatment as the dysfunction of CPC can lead to significant aneuploidy in daughter cells followed by their elimination via apoptosis. Aurora B, as well as INCENP and Survivin, are observed to be overexpressed in human tumors suggesting a role for the CPC in tumorigenesis (Adams et al. 2001, Duffy et al. 2007, Katayama et al. 2003). Thus, the inhibition of the CPC has raised an interest as a potential target for cancer therapies. In fact, many Aurora B inhibitors for medical use are currently under development by the pharmaceutical industry.

6.2.1 Perturbation of INCENP in early mitosis interferes with chromosome alignment and SAC signaling

Inhibition of INCENP with the function blocking antibody in early mitosis resulted in chromosome misalignment followed by premature inactivation of the SAC and forced mitotic exit. This resembles the cellular phenotype observed after inhibition of Aurora B kinase activity (Harrington et al. 2004, Hauf et al. 2003, Kallio et al. 2002b). Aurora B is thought to participate in tension-sensing mechanism of the SAC since Aurora B

inhibition (or loss from centromere by Survivin depletion) caused exit from mitosis in taxol treated cells but not in nocodazole treated cells (Carvalho et al. 2003, Ditchfield et al. 2003, Hauf et al. 2003, Lens et al. 2003). However, in experiments where Aurora B is depleted using RNAi or its function is blocked by antibodies, the cells escape also from nocodazole induced mitotic block (Ditchfield et al. 2003, Kallio et al. 2002b). Consistently, INCENP^{-ab} caused exit from nocodazole arrest. It is possible that the antibody has an effect on CPC function(s) which is important for the SAC activity but independent of Aurora B kinase activity. INCENP^{-ab} binds to C-terminus of INCENP (Bolton et al. 2002) where Aurora B also binds. Thus, there is a possibility that INCENP^{-ab} interferes with normal binding between Aurora B and INCENP, and with full activation of the kinase (Bishop and Schumacher 2002, Honda et al. 2003, Sessa et al. 2005). Indeed, INCENP^{-ab} partially inhibited Aurora B *in vivo* and *in vitro*.

After NEB, the initial kinetochore-microtubule attachments formed in INCENP^{-ab} injected cells. Often the initial kinetochore-microtubule attachments are monotelic or syntelic and they are later converted to stable bipolar attachments (Hauf et al. 2003, Rieder and Salmon 1998). In INCENP^{-ab} injected cells chromosomes failed to align to metaphase plate implying defective correction mechanism of erroneous kinetochore-microtubule attachments. Dysfunction of MCAK, a microtubule depolymerizing kinesin, could explain this defect in INCENP^{-ab} injected cells with lowered Aurora B activity as MCAK is an Aurora B substrate, which has a central role in converting the erroneous attachments to stable bipolar attachments (Lan et al. 2004).

Defects caused by the INCENP^{-ab} antibody resemble the errors seen after anti-Hec1 antibody injection targeting the N-terminus of Hec1, which is phosphorylated by Aurora B. Hec1 is suggested to mediate the establishment of proper kinetochore-microtubule attachments (DeLuca et al. 2006). Blocking the function of either INCENP or Hec1 by the antibodies causes the shorthening of the spindle and reduced chromosome oscillations. In addition, the minus-ends of kinetocore-microtubules at the poles appeared to depolymerize during the exit from mitosis. Plus-end microtubule dynamics at the kinetochore, but not the minus-end depolymerization at the pole, was blocked by the Hec1-antibody injection affecting to the spindle length (DeLuca et al. 2006). INCENP^{-ab} may block the regulation of Hec1 N-terminus by Aurora B explaining the decrease of spindle length in the injected cells.

6.2.2 Dynamic turnover of INCENP and Aurora B is prevented by INCENP^{-ab}

Our FRAP data on different cells lines provided evidence that INCENP and Aurora B recover at the photobleached centromeres of mitotic cells with moderately fast turnover (30-100 s). These observations are contradicting with some earlier reports suggesting that Survivin is the only mobile member of the CPC at the centromeres (Delacour-Larose et al. 2004, Delacour-Larose et al. 2007). INCENP^{-ab} injection resulted in stably bound centromeric INCENP and Aurora B. Thus, it can be speculated that the members of the CPC exchange dynamically at the centromeres and this process is required for proper SAC signaling and Aurora B activity. Furthermore, INCENP^{-ab} induced exit from mitotic arrest caused by microtubule interfering drugs could be

explained by INCENP and Aurora B immobilization. Our data proposes that Aurora B and INCENP turnover at the centromeres as a complex while at least part of Survivin pool exchanges independently since the turnover of Survivin is faster compared to INCENP and Aurora B.

6.2.3 Perturbation of INCENP in late mitosis disrupts sister chromatid separation and cytokinesis

Studying the anaphase function of a mitotic protein is near to impossible using RNAi approach or chemical inhibition due to the perturbation of earlier mitotic tasks of a protein that can lead to masking of the anaphase functions. Microinjection of function blocking INCENP antibody that took action within seconds after its introduction to a cell allowed us to investigate the tasks of the CPC in anaphase without disturbing the earlier functions of the complex. In cells injected with INCENP^{-ab} shortly after the onset of anaphase the poleward movements of sister chromatids stopped completely. Importantly, kinetochore-microtubule assosiations were found to be preserved and sister chromatids remained oriented towards the poles during the mitotic exit. The situation appeared to be caused by the inability of microtubules to promote poleward sister chromatid movements and suggested that INCENP^{-ab} prevented normal kinetochore-microtubule depolymerization which is required for sister chromatid separation during anaphase A (Sharp and Rogers 2004). Thus, it can be speculated that the normal functions of microtubule depolymerizing motor proteins participating on the events of anaphase A are perturbed in INCENP^{-ab} injected cells. However, it is not known whether the CPC controls such motors during mitosis. Consistently with the data from vertebrates, it has been reported previously that depletion of INCENP related protein in *C. elegans* caused defects in chromosome segregation and cytokinesis. These defects were explained by improper dissolution of sister chromatid cohesion (Kaitna et al. 2000). In our experiments, INCENP^{-ab} was injected into cells after removal of cohesion and the injected antibody did not disturb the INCENP function before anaphase onset as happens after siRNA treatment. Thus, dissolution of cohesion could not play a role in the failure of sister chromatid separation.

At anaphase B spindle elongates driving the separation of spindle poles and the poleward chromatid movements. Interestingly, in INCENP^{-ab} injected cells spindles were shorter compared to control anaphase cells. Possibly, the midzone microtubule bundles were unable to undergo normal elongation and/or sliding movements. Many motor proteins are described in these processes during anaphase B (Brust-Mascher et al. 2004, Kwon and Scholey 2004, Kwon et al. 2004, Sharp et al. 2000). Also, the function of the CPC in central spindle has been well characterized (Giet and Glover 2001, Kaitna et al. 2000, Wheatley et al. 2001). The anaphase B defects in INCENP^{-ab} injected cells could be explained by the defective motor protein function. However, possible direct links between CPC and these motor proteins remain to be explored. It has been proposed earlier that Aurora B regulates cytokinesis through astral microtubule dynamics (Miyauchi et al. 2007). This suggestion is supported by our results as the astral microtubules were elongated in INCENP^{-ab} injected cells.

In addition, cytokinesis was incomplete due to the failure of the cleavage furrow in INCENP^{-ab} injected cells. Our result describing defective cytokinesis in INCENP^{-ab} injected cells supports the data presented previously (Adams et al. 2001b, Kaitna et al. 2000). MKLP1 is a known substrate of Aurora B and it forms a complex with MgcRacGAP. This complex is thought to take part in the completion of cytokinesis (Guse et al. 2005). Cytokinesis defects in INCENP^{-ab} injected may be related to problems in association or function of MKLP1-MgcRacGAP at the midzone due to the perturbation of the CPC. Aurora B phosphorylation on vimentin is required for the modulation of vimentin filament network and cleavage furrow formation (Goto et al. 2003, Yasui et al. 2004). Thus, the interference with vimentin by INCENP^{-ab} injection may be one explanation for the cleavage furrow failure during cytokinesis. Importantly, when INCENP^{-ab} was injected 5 min, or more, after the onset of anaphase the cytokinesis appeared normal. This proposes the existence of a time point after which the CPC is no longer essential for completion of late mitotic events. Effects of the INCENP^{-ab} in cells are summarized in fig. 13.

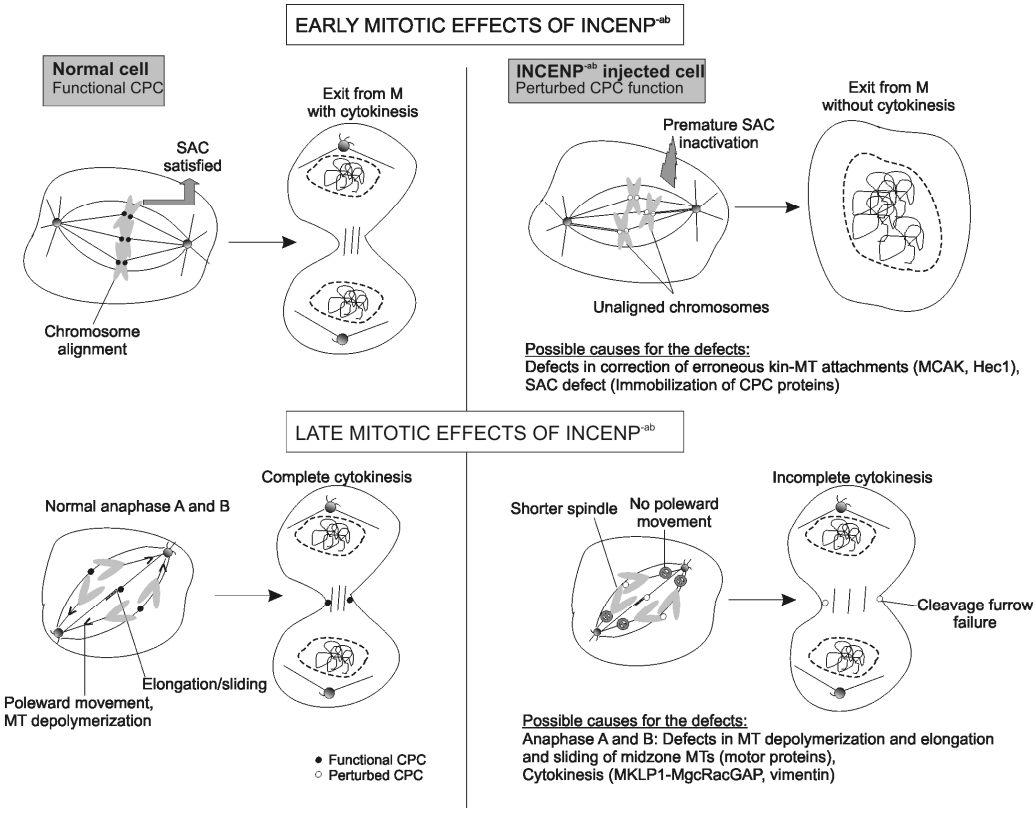

Figure 13. Summary of early and late mitotic effects caused by INCENP^{-ab}.

Taken together, our results show that INCENP is an important member required for proper CPC function and the interference with INCENP by the C-terminus targeting function blocking antibody inhibits the activity of the CPC. Our data extends the

knowledge about the CPC function in regulation of SAC. Importantly, the data revealed a new role for the CPC during anaphase. It is very likely that the observed errors induced by INCENP^{-ab} in late mitosis are consequences of targeting more than one CPC dependent protein function.

6.3 A novel role of p38γ MAPK in mitosis (III)

The p38 MAPKs are described in regulation of many signaling events in a cell such as those implicated in inflammation, cell growth, cell cycle and cell death. The family of p38 MAPKs consist of four members (p38α, p38β, p38γ and p38δ), the p38α being the best characterized isoform. The p38 MAPKs are known to play a role during cell cycle checkpoints G1/S and G2/M (Thornton and Rincon 2009). Moreover, the function of p38 MAPKs in mitotic entry has been well described both in response to stress and in stressless conditions (Bulavin et al. 2002, Cha et al. 2007, Mikhailov et al. 2005). The p38 MAPKs have been described in cell survival or tumorigenesis depending on the cellular context (Dhillon et al. 2007). Previously, it has been suggested that the function of p38 MAPKs is tumor suppressive causing negative regulation of the cell cycle and inducing p53-mediated apoptosis (Bulavin and Fornace 2004). Thus, it appears that the activity of p38 MAPKs is important for inhibiting the survival of cancer cells. The p38 MAPKs have been described during mitosis in regulation of the SAC, but the data regarding this function has remained controversial. In addition, the specific mitotic tasks of the p38 MAPK isoforms have remained unknown.

6.3.1 p38γ MAPK is required for mitotic progression and survival

It has been reported that the p38 MAPK isoforms are differently expressed in tissues. It has been suggested that p38α is a ubiquitous protein while p38γ is predominantly found in skeletal muscle (Jiang et al. 1996, Lechner et al. 1996, Li et al. 1996). Unexpectedly, we discovered p38γ as the most abundant isoform in Hela cells together with p38α. A specific depletion of the p38 MAPK isoforms revealed an interesting mitotic phenotype when p38γ was knocked down. Importantly, similar phenotypes were not observed in cells depleted with other three p38 MAPK isoforms. p38γ depleted cells accumulated in mitosis with defects in chromosome alignment due to incorrect spindle organization. Indeed, multiple microtubule nucleating foci were observed in these cells. Multipolarity was found to be dependent on the mitotic kinesin Eg5 activity. In studies where Eg5 was reactivated after inhibition in p38γ depleted cells, the chromosomes failed to align normally also in cells with bipolar spindles. However, kinetochore-microtubule attachments were discovered to be stable, but reduced interkinetochore tension was observed in p38γ silenced cells resembling the effects of microtubule stabilizing drug taxol. Therefore, these results suggest that p38γ is required for normal microtubule dynamics. It may also regulate motor proteins required for chromosome movements. In fact, the p38 MAPK pathway has been connected to cytoplasmic dynein (Whyte et al. 2008). This microtubule minus-end directed motor has been described in spindle organization and chromosome movements (Karki and Holzbaur 1999). However, the functional link between p38γ and dynein remains to be determined. Furthermore, loss of p38γ prevented exit from mitosis. The

p38γ silenced cells appeared to have a functional SAC as no significant escape was detected from microtubule-interfering drug induced mitotic block. Finally, the majority of mitotic cells in the p38γ depleted population underwent the caspase-dependent apoptosis. The cells which survived through mitosis and divided died shortly after cell division. As the loss of p38γ results in multiple mitotic defects the kinase possibly has several substrates whose disturbance contributes to the observed phenotype.

6.3.2 p38 MAPK pathway is connected with Plk1

Recently, a substrate of p38 MAPK, MK2, was reported to directly phosphorylate Plk1 on Ser326 and that the phosphorylation was required for normal progression of mitosis (Tang et al. 2008). Moreover, MK2 and Plk1, as well as p38 MAPK, have been shown to colocalize at the spindle poles during mitosis (Tang et al. 2008). The phenotype of Plk1 depleted cells resembles the effects caused by the loss of p38γ. The Plk1 silenced cells arrest in mitosis and undergo mitotic cell death or die immediately after abnormal exit from mitosis. The cell death induced by the loss of Plk1 has been shown to involve p53 (Guan et al. 2005). However, the role of p53 in p38γ silenced cells remains unknown. The effect of p38γ loss-of-function on p53 mediated processes could be investigated using isogenic p53 wild-type and null/mutant cell lines. Whether Plk1 and p38γ function in the same pathway regarding the mitotic cell death remains to be determined. We observed that the kinetochore and spindle pole accumulation of Plk1 was dependent on p38γ suggesting a connection between Plk1 and p38 MAPK pathway in mitotic progression and survival.

Our observation that active p38 MAPKs localized to the kinetochores was a new finding as previously p38 MAPKs have been detected only at the spindle poles (Cha et al. 2007, Lee et al. 2010, Tang et al. 2008) and in the cytoplasm in mitotic cells (Fan et al. 2005). The antibodies used in our study (p-p38) and in the studies of the others recognized conserved phosphoepitope, which is present in all p38 MAPK isoforms. Moreover, the depletion of p38γ isoform did not abolish the p-p38 signals. This suggests that other isoform(s) are present at the same mitotic structures and may contribute to mitotic regulation. Moreover, even if we did not observe severe mitotic errors in p38α, p38β, or p38δ silenced cells, it is possible that functional overlap exists between the isoforms in mitotic processes. For these reasons, many isoform specific mitotic tasks may be masked making the dissection of the p38 MAPK signaling very difficult. Finally, we observed that the active p38 MAPKs at the kinetochores were upregulated and mislocalized due to the depletion/inhibition of the factors that arrest the cell cycle in M phase (unpublished results). This suggested that p38 MAPKs respond to the activation of SAC and may contribute to the SAC signaling event. Alternatively, p38 MAPK hyperactivation may protect the mitotic cells from apoptosis during the cell's attempts to correct the errors that keep the SAC activated. This issue should be further investigated in the future.

In order to dissect what creates the p38 MAPK phosphoepitope at the kinetochores and spindle poles lysed cell model was utilized (Ahonen et al. 2005, Daum and Gorbsky 2006). The results pointed to an upstream regulator, which was bound to kinetochores

and spindle poles. It should be noticed that p38 MAPKs can also be autophosphorylated in a TAB-1 dependent mechanism (Ge et al. 2002, Salvador et al. 2005). Thus, the result may also be explained by the presence of this mechanism at the mitotic structures. Recreation of p-p38 signals by mitotic extract, but not S phase extract, suggested that the regulator of p38 MAPK activity was present in the mitotic fraction. Interestingly, the inhibition of Plk1 or depletion of Plk1 from mitotic extract suppressed the p38 MAPK phosphorylation. This data provides another support for the link between p38 MAPK and Plk1 pathways and suggests that Plk1 regulates the activity of p38 MAPKs or stimulates their autophosphorylation mechanism during mitosis. In an experiment where Plk1 was silenced/chemically inhibited in cells causing long mitotic arrest followed by p-p38 staining the enhancement of p-p38 signal was observed. Importantly, this effect was seen also in other conditions that arrest cell cycle in M phase. Therefore, it can be speculated that the enhancement of p-p38 in response to the SAC hyperactivation masked the actual effect of the Plk1 depletion which could be observed using the lysed cell model. These data implies that there are other still unidentified factor(s) within the SAC pathway participating on p38 MAPK activation during mitosis. Identification of this yet unknown factor would be valuable for better understanding of mitotic signaling and cell fate after mitotic defects.

To summarize, our study extends the knowledge about the involvement of p38 MAPKs in mitosis and reveales a new player, p38γ, in mitotic regulation. We showed that p38γ MAPK is required for the normal progression of mitosis and mitotic cell survival. One potential task of p38γ during mitosis may be to protect cells from apoptosis. Therefore, it would be expected that the inhibition of p38γ in wildly dividing cancer cells may decrease their survival rate via activation of apoptosis. Thus, p38γ may be an interesting target molecule for the future cancer drug discovery. However, the story of p38γ as a mitotic protein has just begun to emerge and more detailed functional studies are needed before one can evaluate its anti-cancer potency. Finally, our data provides the first evidence for a possible connetion between the p38 MAPK and Plk1 pathways during mitosis. The detailed mechanisms of this cooperation however wait to be discovered.

7 CONCLUSIONS

Ordered mitotic progression and successful division of genes between the daughter cells is a strictly regulated multi-phased process. This process requires the function and cooperation of numerous mitotic proteins. The main principles of mitotic cell division including the conserved control mechanism, SAC, are known. However, the details of many regulatory mechanisms as well as the identity and function of various proteins participating in mitotic progression still remain to be discovered.

Kinetochores are the central structures in mitosis and many mitotic proteins reside at these structures. The ordered assembly of kinetochore proteins reflects the hierarchical relationship of the proteins. In this thesis, a central role for the multifunctional protein, Sgo1, together with a central mitotic kinase, Plk1, was described in a novel branch of kinetochore assembly. Two mitotic kinases, Bub1 and Aurora B, were shown to regulate the kinetochore binding of Sgo1. Furthermore, Sgo1 was responsible of Plk1 recruitment to the kinetochores, which in turn attracted CENP-F. It was speculated that the creation of tension between the sister kinetochores triggered the observed Sgo1 phosphorylation by Plk1 and led to the removal of Sgo1, Plk1 and CENP-F from the kinetochores. This event was suggested to participate in the SAC inactivation. Also, another protein, p38γ MAPK, was discovered to be linked with Plk1 during mitosis. Importantly, p38γ MAPK was characterized as a novel mitotic protein as the depletion of p38γ MAPK caused mitotic arrest due to spindle abnormalities possibly involving motor protein activity followed by apoptotic cell death. The Plk1 kinase was also discovered to be required for the activation of the kinetochore and pole localized p38 MAPKs. In addition, our results supported the previous suggestions that p38 MAPK activation may have a function in the SAC signaling. The CPC consisting of Aurora B, INCENP, Survivin and Borealin is responsible for the mechanism which destabilizes the erroneous kinetochore-microtubule attachment. This creates unattached kinetochores which are known to keep the SAC active. Introduction of the function blocking INCENP antibody during different stages of mitosis allowed us a temporal control over the CPC functions. In early mitosis, perturbation of INCENP led to defects in chromosome alignment, premature inactivation of the SAC and forced mitotic exit. Immobilization of INCENP and Aurora B at the centromeres was suggested to cause the premature SAC inactivation. In late mitosis, perturbation of INCENP prevented normal chromosome movements at anaphase A and elongation of the spindle at anaphase B followed by incomplete cytokinesis.

In summary, our data on Sgo1 and INCENP broadened the knowledge about their tasks during mitosis suggesting new aspects on their function. Moreover, we identified p38γ MAPK as a novel mitotic protein and linked its function with a known mitotic regulator, Plk1 in promotion of ordered mitotic processes and cell survival.

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