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TO DIVIDE OR NOT TO DIVIDE; MicroRNAs AND SMALL COMPOUNDS AS MODULATORS OF MITOSIS

Jenni Mäki-Jouppila

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Faculty of Medicine
Institute of Biomedicine
Department of Pharmacology, Drug Development and Therapeutics
Drug Research Doctoral Programme (DRDP) and FinPharma Doctoral Program (FPDP)
VTT Health, VTT Technical Research Centre of Finland
Turku Centre for Biotechnology

Supervised by

Marko Kallio, Docent, Ph.D.
Department of Physiology,
Institute of Biomedicine, and
Turku Centre for Biotechnology
University of Turku
Turku, Finland

Reviewed by

Jorrit Enserink, Ph.D.
Department of Microbiology
Oslo University Hospital and University of Oslo
Oslo, Norway

Liisa Kauppi, Docent, Ph.D.
Genome-Scale Biology Research Program and
Institute of Biomedicine
University of Helsinki
Helsinki, Finland

Opponent

Jakob Nilsson, Associate Professor, Ph.D.
The Novo Nordisk Foundation Center for
Protein Research
The Faculty of Health and Medical Sciences
University of Copenhagen
Copenhagen, Denmark

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To my beloved family

“The important thing is not to stop questioning.
Curiosity has its own reason for existing.”
-Albert Einstein-

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Department of Pharmacology, Drug Development and Therapeutics, University of Turku; Drug Research Doctoral Programme (DRDP) and FinPharma Doctoral Program (FPDP); VTT Health, VTT Technical Research Centre of Finland; Turku Centre for Biotechnology, University of Turku
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ABSTRACT

Mitosis is under the stringent quality control of the spindle assembly checkpoint (SAC). However, in cancer cells this control can fail, leading to excessive cellular proliferation and ultimately to the formation of a tumor. Novel cancer cell selective therapies are needed to stop the uncontrolled cell proliferation and tumor growth. The aim of the research presented in this thesis was to identify microRNAs (miRNAs) that could play a role in cancer cell proliferation as well as low molecular weight (LMW) compounds that could interfere with cell division. The findings could be used to develop better cancer diagnostics and therapies in the future. First, a high-throughput screen (HTS) was performed to identify LMW compounds that possess a similar chemical interaction field as rigosertib, an anti-cancer compound undergoing clinical trials. A compound termed Centmitor-1 was discovered that phenocopied the cellular impact of rigosertib by affecting the microtubule dynamics. Next, another HTS aimed at identifying compounds that would target the Hec1 protein, which mediates the interaction between spindle microtubules and chromosomes. Perturbation of this connection should prevent cell division and induce cell death. A compound termed VTT-006 was discovered that abrogated mitosis in several cell line models and exhibited binding to Hec1 *in vitro*. Lastly, using a cell-based HTS two miRNAs were identified that affected cancer cell proliferation via Aurora B kinase, which is an important mitotic regulator. MiR-378a-5p was found to indirectly suppress the production of the kinase whereas let-7b showed direct binding to the 3'UTR of Aurora B mRNA and repressed its translation. The miRNA-mediated perturbation of Aurora B induced defects in mitosis leading to abnormal chromosome segregation and induction of aneuploidy. The results of this thesis provide new information on miRNA signaling in cancer, which could be utilized for diagnostic purposes. Moreover, the thesis introduces two small compounds that may benefit future drug research.

Keywords: mitosis, low molecular weight compound, microRNA, spindle assembly checkpoint, kinetochore

Jenni Mäki-Jouppila
MicroRNA-MOLEKYYLIT JA PIENYHDISTEET MITOOSIN SÄÄTELIJÖINÄ

Farmakologia, lääkekehitys ja lääkehoito, Turun yliopisto; Lääketutkimuksen tohtoriohjelma ja Suomen lääketutkimuksen tohtoriohjelma; VTT Health, Teknologian tutkimuskeskus VTT; Turun Biotekniikan Keskus, Turun yliopisto
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TIIVISTELMÄ

Solunjakautumisessa kromosomien jakaantumista muodostuviin tytärtumiin säätelee mitoottinen tarkastuspiste. Syöpäsolujen jakautumisen säätely voi kuitenkin olla vioittunut, mikä mahdollistaa solujen kontrolloimattoman jakautumisen ja kasvaimen muodostumisen. Yksi keino hillitä tätä prosessia on kehittää syöpäsoluspesifisiä lääkeaineita ja terapiamuotoja. Tässä väitöskirjassa esiteltävän tutkimuksen tarkoitus oli tunnistaa mikroRNA-molekyylejä ja pienyhdisteitä, jotka vaikuttavat solunjakautumiseen ja joita voidaan tulevaisuudessa käyttää syövän hoitomenetelmien ja diagnostiikan kehittämiseen. Tehoseulonnalla etsittiin pienyhdisteitä, joilla on samankaltainen kemiallinen vuorovaikutuskenttä kuin kliinisissä tutkimuksissa olevalla lääkeaineella rigosertib. Seulonnan tuloksena löydettiin yhdiste Centmitor-1, joka aiheutti soluilla samankaltaisen fenotyypin kuin rigosertib vaikuttamalla mikrotubulusten dynamiikkaan. Toisessa tehoseulonnassa etsittiin Hec1-proteiinin inhibiittoreita. Hec1 välittää mikrotubulusten ja kinetokorien vuorovaikutusta, minkä estyminen salpaa mitoosia ja aiheuttaa solukuolemaa. Löydetty yhdiste VTT-006 esti solunjakautumisen monessa eri solumallissa sekä sitoutui kohdeproteiiniinsa Hec1. Kolmannen tehoseulonnan avulla etsittiin syöpäsolujen solunjakautumiseen vaikuttavia mikroRNA-molekyylejä ja tunnistettiin kaksi mikroRNA-molekyyliä, jotka säätelevät mitoosin aikana toimivan Aurora B -kinaasin ilmentymistä. MiR-378a-5p alensi kinaasitasoja epäsuoran vaikutusmekanismin kautta, kun taas let-7b sitoutui suoraan Aurora B –kinaasin mRNA:han ja vähensi proteiinin ekspressiota. Aurora B -kinaasin häiriöt johtivat kromosomien epänormaaliin jakaantumiseen, mikä aiheuttaa aneuploidiaa. Väitöskirjan tulokset antavat uutta tietoa mikroRNA-molekyylien säätelystä, jota voidaan käyttää syövän diagnosoinnissa. Lisäksi väitöskirja esittelee kaksi uutta pienyhdistettä, joita voidaan hyödyntää lääketutkimuksessa.

Avainsanat: mitoosi, pienyhdiste, mikroRNA, mitoottinen tarkastuspiste, kinetokori

TABLE OF CONTENTS

ABBREVIATIONS

2’-MOE	2’-O-methoxyethyl
3D	three dimensional
Ago	Argonaute
APC/C	anaphase promoting complex, also called the cyclosome
ATP	adenosine-5’-triphosphate
AURKB	Aurora B
B56-PP2A	PP2A containing a B56 regulatory subunit
BIR	baculovirus IAP repeat
bp	base pair
Bub	Budding uninhibited by benomyl
BubR1	Bub1-related 1
CCAN	Constitutive centromere-associated network
Cdc	Cell division cycle protein
Cdh1	Cdc20 homologue 1
Cdk	Cyclin-dependent kinase
Cenp-E	Centromere-associated protein E
CHD	calponin homology domain
CIN	chromosomal instability
CK2	Casein Kinase II
CLASP	CLIP-associating protein
CLIP	Cytoplasmic linker protein
CLL	chronic lymphocytic leukemia
C-Mad2	closed Mad2
CPC	chromosomal passenger complex
Dam1	Duo1 and Mps1-interacting protein 1
D-box	destruction box
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
EB1	End-binding protein 1
EC_{50}	half maximal effective concentration
ECT2	Epithelial cell-transforming 2
EIF4E	Eukaryotic initiation factor 4E
ELISA	enzyme linked immunosorbent assay
EphA7	Ephrin type-A receptor 7
ER	Estrogen receptor-α
ErbB2	Erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-regulated kinase
ERR	Estrogen receptor-related receptor
FISH	fluorescence *in situ* hybridization

FOXM1	Forkhead box protein M1
FRET	Förster resonance energy transfer
GAP	GTPase activating protein
GABP	GA binding protein transcription factor
GDP	guanosine-5'-diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine-5'-triphosphate
GW182	Glycine-tryptophan protein of 182 kDa
HCV	Hepatitis C virus
Hec1	Highly expressed in cancer 1
HER2	Human epidermal growth factor receptor 2
HP1	Heterochromatin protein 1
HTS	high-throughput screen
ICIS	Inner centromere KinI stimulator
INCENP	Inner centromere protein
Kif	kinesin family member
KMN	Knl1 Mis12 Ndc80
Knl1	Kinetochore null 1
LMW	low molecular weight
LNA	locked nucleic acid
Loqs	Loquacious
Lys	lysine
Mad	Mitotic arrest deficient
MAP	microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MCAK	Mitotic centromere-associated kinesin
MCC	mitotic checkpoint complex
miRNA	microRNA
Mis12	Missegregation 12
MKLP	Mitotic kinesin-like protein
Mps1	Monopolar spindle protein 1
mRNA	messenger RNA
MTOC	microtubule-organizing center
Ndc80	Nuclear division cycle 80
NEBD	nuclear envelope breakdown
Nek2	Never in mitosis A-related kinase 2
Nuf2	Nuclear filamentous 2
NuMA	Nuclear mitotic apparatus protein 1
O-Mad2	open Mad2
PABP	Poly(A) binding protein
PACT	Protein kinase R-activating protein
PBD	polo box domain

PCM	pericentriolar material
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphoinositide 3-kinase
Plk1	Polo-like kinase-1
PP	protein phosphatase
PR	progesterone receptor
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA transcript
RISC	RNA-induced silencing complex
RNA Pol	RNA polymerase
RNA	ribonucleic acid
RNAi	RNA interference
RTK	receptor tyrosine kinase
RT-PCR	real-time polymerase chain reaction
RZZ	Rod-ZW10-Zwilch
SAC	spindle assembly checkpoint
Ser	serine
Sgo	Shugoshin
Ska	Spindle and kinetochore-associated protein
Spc	Spindle pole component
STAG2	Stromal antigen 2
TAR	Transactivation-response
Thr	threonine
TIRF	total internal reflection fluorescence
TRBP, TARBP2	TAR RNA-binding protein
TUT	terminal uridylyltransferase
Tyr	tyrosine
U	uridylyl
UTR	untranslated region
VEGF-A	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
Zwint1	ZW10-interacting protein 1
γTuRC	γ-tubulin ring complex
γTuSC	γ-tubulin small complex

LIST OF ORIGINAL PUBLICATIONS

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

I **Mäki-Jouppila JHE***, Laine LJ*, Rehnberg J, Narvi E, Tiikkainen P, Hukasova E, Halonen P, Lindqvist A, Kallio L, Poso A and Kallio MJ. Centmitor-1, a novel acridinyl-acetohydrazide, possesses similar molecule interaction field and anti-mitotic cellular phenotype as rigosertib, ON 01910.Na. Mol Cancer Ther. 2014. 13(5): 1054–1066.

II Laine LJ*, **Mäki-Jouppila JHE***, Kutvonen E, Tiikkainen P, Nyholm TKM, Tien J, Umbreit N, Härmä V, Kukkonen-Macchi A, Kallio L, Davis T, Asbury CL, Poso A and Kallio MJ. VTT-006, a novel anti-mitotic compound, binds to the Ndc80 complex and suppresses cancer cell growth *in vitro*. Manuscript.

III Winsel S*, **Mäki-Jouppila J***, Tambe M*, Aure MR, Pruikkonen S, Salmela A-L, Halonen T, Leivonen S-K, Kallio L, Børresen-Dale A-L and Kallio MJ. Excess of miRNA-378a-5p perturbs mitotic fidelity and correlates with breast cancer tumorigenesis *in vivo*. Br J Cancer. 2014. 111(11): 2142-2151.

IV **Mäki-Jouppila JHE**, Pruikkonen S, Tambe MB, Aure MR, Halonen T, Salmela A-L, Laine L, Børresen-Dale A-L and Kallio MJ. MicroRNA let-7b regulates genomic balance by targeting Aurora B kinase. Mol Oncol. 2015. doi:10.1016/j.molonc.2015.01.005 [Epub ahead of print].

* Equal contribution

1. INTRODUCTION

Cell division and mitosis in a somatic cell aim at producing two identical daughter cells with a normal chromosome set characteristic of the species. The accurate chromosome segregation into daughter cells is necessary to maintain genomic stability and is thus under stringent quality control. The spindle assembly checkpoint (SAC) is a conserved mitotic surveillance mechanism that involves several proteins (Musacchio and Salmon, 2007). Normally, the SAC allows a cell to segregate its chromosomes only when they are correctly aligned to the equator of a bipolar spindle. Errors in the congression of chromosomes or in their attachment to spindle microtubules can give rise to mitotic mistakes that contribute to genomic imbalance, a phenomenon associated with cancer initiation and progression.

Cancer cells can acquire the ability to divide in an uncontrolled way as opposed to non-transformed cells that are subjected to normal growth restriction and quality control mechanisms. Many properties of cancer cells and tumors, such as the ability to escape cell death signals, to promote angiogenesis, to develop resistance to drugs, and to metastasize, make growth suppression a challenging task (Hanahan and Weinberg, 2011). Although many efforts have been taken to tackle this challenge, cancer still kills millions of people every year. There is a clear need to develop more cancer cell specific and less toxic therapeutics, and mitosis is considered as one putative target for these new therapies. Targeting mitosis could be one way to direct the therapeutic drug effect against excessively dividing cancer cells. This approach could cause fewer side effects than the current microtubule targeting chemotherapeutics, such as taxanes and vinca alkaloids that affect all of the cells in a human body.

Microtubules are however a very effective target in the treatment of cancer (Jordan and Wilson, 2004). Microtubule targeting drugs are widely recognized as being efficacious anti-mitotics since the treated cells undergo a transient mitotic arrest, which suppresses cell viability. Even though these drugs are very effective, they are unspecific and they damage normal proliferating and differentiated cells. Their clinical use is associated with harmful side effects, such as neurotoxicity, and tumors resistant to microtubule targeting drugs are encountered in the clinics. The new classes of anti-mitotic drugs for example targeting motor proteins and kinases are hoped to overcome the problems that are associated with microtubule targeting drugs (Salmela and Kallio, 2013).

MicroRNAs (miRNAs) are non-coding RNA molecules of 18-25 nucleotides in length and post-transcriptional regulators of gene expression (He and Hannon,

2004). One gene can be regulated by several miRNAs and one miRNA can have several target genes, which makes miRNA signaling a part of a complex regulatory network. Although miRNAs have been implicated in almost all cellular processes, our understanding of their biogenesis and regulation is very limited. After the biosynthesis of the miRNA, the mature form associates with the RNA-induced silencing complex (RISC) that guides the miRNA to its target mRNAs via complementary base pairing. The target mRNAs are then degraded or translationally inhibited. Many miRNAs have been associated with tumorigenesis, either in tumor suppression or in promoting malignant growth. It is hoped that the expression profiling of miRNAs will contribute to the diagnostics of cancer in the future.

The research presented in this thesis aimed at discovering novel ways to interfere with the division and proliferation of cancer cells. Two new low molecular weight (LMW) compounds with anti-mitotic properties were characterized and these compounds may be of benefit in drug development and basic research in the future. Moreover, two miRNAs were discovered that take part in cancer cell signaling and could be used in cancer diagnostics as novel biomarkers.

2. REVIEW OF THE LITERATURE

2.1. Cell cycle and mitosis

2.1.1. Phases of the cell cycle

When a cell divides, it produces new daughter cells; this process is essential for the maintenance of tissue growth and repair, development, health, and reproduction of organisms. The series of events leading to cell division are an entity called the cell cycle. The purpose of the cell cycle is to copy the hereditary material and separate the replicated chromosomes into two daughter cells (Nurse, 2000). The proliferation capacity and the cell cycle rate depend on the cell type. For example, skin cells need to renew themselves constantly whereas nerve cells do not normally divide after differentiation. The cell cycle of a somatic cell can be divided into two main phases, interphase and the cell division phase, also called the M phase. Furthermore, the M phase can be divided into the division of the nucleus called mitosis and subsequently cytokinesis, where the cytosol and the cell organelles are re-organized into two daughter cells. Interphase is partitioned into gap1 (G1), replication (S) and gap2 (G2) phases (Fig. 1). During the gap phases, a cell grows and undergoes very active metabolism. In the S phase, the genome of a cell is replicated so that it can later be divided into two identical daughter cells in the M phase. A cell can also enter quiescence, the so-called G0 phase, from G1 if the conditions are not favorable for continuing the cell cycle or in the case when a cell permanently exits the cell cycle in order to differentiate into a specialized cell type. The nuclear division that produces haploid gametes (eggs or sperm) is termed meiosis.

The cell monitors the progression of the cell cycle at three main checkpoints (Hartwell and Weinert, 1989). The first checkpoint surveys the external environment of the cell, cell size, and DNA damage in late G1 when the cell will commit itself to enter the S phase to duplicate its genome. Next, at the G2/M checkpoint in late G2, the cell can prevent the onset of the M phase if there is damaged or erroneously replicated DNA. The third main checkpoint, the SAC, monitors the fidelity of chromosome segregation during mitosis in order to maintain genomic balance. In addition to these three main checkpoints, DNA replication can be delayed during the S phase in response to DNA damage. Cyclin dependent kinases (Cdk) and Cyclins are essential regulators of the cell cycle. The Cdks are activated upon binding to Cyclins and the activity of different Cdk-Cyclin complexes is characteristic of specific cell cycle phases

(Fig. 1). Moreover, the Cdks phosphorylate the substrates that regulate the cell cycle progression.

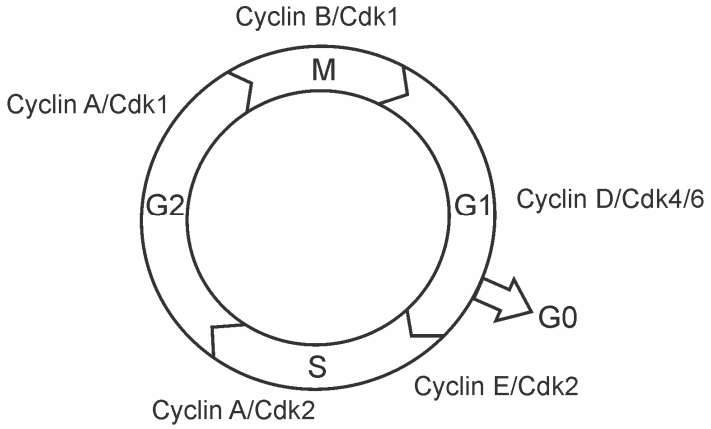

Figure 1. The cell cycle. The phases of the cell cycle and the Cyclin/Cdk complexes are shown.

2.1.2. Phases of mitosis

Mitosis can be divided into five phases (Fig. 2; reviewed in Gadde and Heald, 2004). In prophase, the duplicated chromatids condense to form tightly packed mitotic chromosomes. The centrosomes, also called spindle poles, separate and move towards the opposite sides of the nucleus. The nuclear envelope breakdown (NEBD) marks the beginning of the next phase, prometaphase, where the mitotic spindle starts to form when the microtubules emanating from spindle poles become attached to the chromosomes. The microtubule attachment occurs via kinetochores, which are large protein complexes located at the centromeres of chromosomes (Foley and Kapoor, 2013). The microtubules start to move chromosomes to the spindle equator in concert with several motor proteins and microtubule-associated proteins (MAPs). A mitotic cell is in metaphase when all the chromosomes have congressed at the spindle equator (Cheeseman and Desai, 2008), at the metaphase plate. In this phase, the sister chromatids, formed in the S phase, are held together by the Cohesin complex at the centromere region. During anaphase, Cohesin is cleaved and the daughter chromosomes are pulled apart by the microtubules. In early anaphase, anaphase A, the daughter chromosomes are moved towards the spindle poles as the microtubules depolymerize. In late anaphase, anaphase B, also the spindle poles move apart when the microtubules slide next to each other, powered by motor proteins, at the midzone to elongate the spindle. In telophase, the

chromosomes reach the spindle poles, start to decondense, and the nuclear envelopes start to reform. During cytokinesis, the cell organelles and cytoplasm are divided between the two daughter cells as a contractile ring of actin and myosin pinches the mother cell into two parts. Finally, the daughter cells are detached from each other through abscission of the midbody, a thin bridge connecting the progeny cells. Stringent regulation of mitosis is needed in order to ensure the proper chromosome segregation to daughter cells.

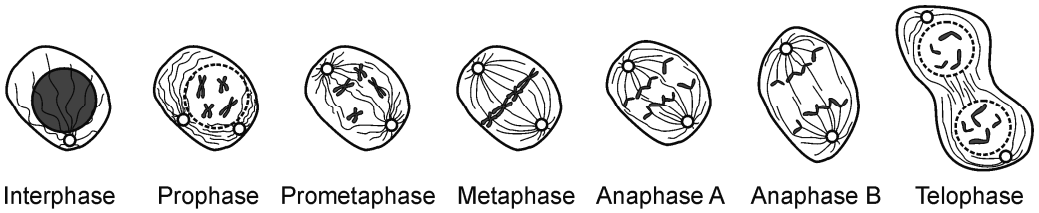

Figure 2. The phases of mitosis.

2.2. Mitotic spindle

2.2.1. Centrosomes

Centrosomes are the nucleation centers of microtubules and they form the opposite ends of the mitotic spindle. Therefore, they are called microtubule-organizing centers (MTOCs) or the spindle poles (reviewed in Bettencourt-Dias and Glover, 2007). Centrosomes have several important functions for example in cellular transportation, cell polarity, organization of the cytoskeleton, as well as in mitosis. During interphase, before centrosome duplication, a single centrosome is located near to the nucleus. Centrosome duplication takes place in the S phase and the two centrosomes start to move to the opposite sides of the nucleus in late G2 and in prophase. The mammalian centrosome contains two tubular structures called centrioles that are situated perpendicular to each other. Centrioles are composed of nine microtubule triplets and are about 0.5 μm in length and 0.2 μm in diameter (Bettencourt-Dias and Glover, 2007). They are surrounded by an electron-dense matrix called the pericentriolar material (PCM), which is essential for anchoring and nucleating the microtubules. In addition, the centrioles serve as basal bodies for cilia and flagella (Nigg et al., 2014).

An abnormal centrosome number can give rise to erroneous cell division (Sluder and Nordberg, 2004). Moreover, defects in MTOC have been associated

with genomic instability, which is a characteristic of cancer cells (Nigg, 2002). However, although centrosomes are usually present in most animal cells, they are generally absent during female meiosis. In addition, higher plant cells do not have centrosomes. Thus, centrosomes are not absolutely necessary for spindle formation and cells without centrioles are able to proliferate (Basto et al., 2006; Bettencourt-Dias et al., 2005). Spindle microtubules can be nucleated from chromosomes instead of centrosomes, and the minus ends of microtubules can be focused possibly via minus end directed motor proteins and MAPs (Wadsworth and Khodjakov, 2004). The Nuclear mitotic apparatus protein 1 (NuMA) is a MAP taking part in the formation and the maintenance of spindle poles (Silk et al., 2009) and also participates in centrosome-independent spindle pole formation (Khodjakov et al., 2000; Merdes et al., 2000). Together with dynein, NuMA is involved in spindle assembly by focusing the microtubules to the spindle poles (Khodjakov et al., 2003; Merdes et al., 1996). Even though cell division is possible without centrioles, cells might divide asymmetrically in their absence (Basto et al., 2006). Moreover, centrioles are essential for basal body formation and for male meiotic divisions (Bettencourt-Dias et al., 2005).

In mitosis, Polo-like kinase-1 (Plk1) -mediated phosphorylation of pericentrin initiates centrosome maturation (Lee and Rhee, 2011), during which the PCM becomes thicker as the cell prepares for division. The PCM contains members of the pericentrin and AKAP450 family of proteins (Bornens, 2002). They attach to the regulatory components essential for microtubule nucleation, such as γ-tubulin (Zheng et al., 1995), which serves as the basis of the structure needed for nucleation. Two γ-tubulin molecules, GCP2 and GCP3 (in mammals), form the γ-tubulin small complex (γTuSC). These subcomplexes form a larger ring-like complex, termed the γ-tubulin ring complex (γTuRC). Microtubule nucleation during the cell cycle is regulated by several factors that either promote or restrict the recruitment of required proteins. For instance, mitotic kinases Plk1 and Aurora A promote microtubule nucleation whereas protein phosphatase-1 (PP1), PP4 and other protein phosphatases oppose this process (Blagden and Glover, 2003; Trinkle-Mulcahy and Lamond, 2006).

In addition to microtubule nucleation and anchorage, centrosomes regulate other aspects of cell division (Doxsey et al., 2005) as they are involved in cytokinesis and in the G1/S phase transition (Hinchcliffe et al., 2001; Piel et al., 2001). It has been shown that the mother centriole moves towards the midbody at telophase at the same time as abscission, the separation of two cells, happens (Piel et al., 2000; Piel et al., 2001). If the centrosome has been removed by microsurgery or laser removal, it was found that many cells were not able to complete cytokinesis (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). In addition, when several centrosome-associated proteins have been silenced or

centrioles removed followed by exposure to light, the cell cycle is arrested in G1 (Bettencourt-Dias and Glover, 2007; Doxsey et al., 2005).

2.2.2. Microtubules and motor proteins

Microtubules are hollow tubular structures 25 nm in diameter, consisting of 12-15 tubulin protofilaments, which are made up of several polymerized α- and β-tubulin dimers (Cheeseman & Desai, 2008). The orientation of tubulin dimers makes the microtubules polar, defining their minus and plus ends by orientation of the α- and β-tubulin, respectively. This also defines the directions for the motor proteins. The minus ends of microtubules become attached to the centrosome, whereas the more dynamic plus ends reach outwards from the spindle pole. During prophase, microtubule nucleation increases and microtubules become more dynamic as the cell prepares for chromosome segregation. Microtubules are organized in an antiparallel structure in the mitotic spindle and microtubules outside the spindle body form two asters, one around each spindle pole. In vertebrate cells undergoing mitosis, a bundle of 20-25 kinetochore microtubules form one kinetochore fiber (k-fiber), which attaches to a kinetochore (McEwen et al., 1997). Dynamic microtubules are constantly interchanging between the polymerization and depolymerization states at both ends and the polymerizing and depolymerizing polymers coexist in a steady state called dynamic instability (reviewed in Cheeseman and Desai, 2008; Desai and Mitchison, 1997). The change from the polymerization to depolymerization state is termed a catastrophe whereas the opposite transition is called a rescue (Fig. 3). The spindle microtubules and the kinetochore microtubules exhibit microtubule flux, which is characterized by a net addition of tubulin at the plus ends and a net loss of tubulin at the minus ends. During polymerization, GTP is hydrolyzed on β-tubulin, which provides the energy for the microtubule dynamics. GDP remains in the polymer lattice until the depolymerization begins and GDP is released. The energy from GTP hydrolysis is stored in the polymer lattice, which results in an over 1000-fold higher dissociation of GDP-tubulin upon depolymerization compared to the dissociation of GTP-tubulin at a polymerizing end. GTP-tubulin forms a stabilizing cap in the polymerizing end possibly due to a lag between tubulin dimer addition and GTP hydrolysis. If the cap is lost, rapid depolymerization begins. The released tubulin dimers exchange nucleotides from GDP to GTP and are able to bind again to the polymerizing microtubule end (Desai and Mitchison, 1997).

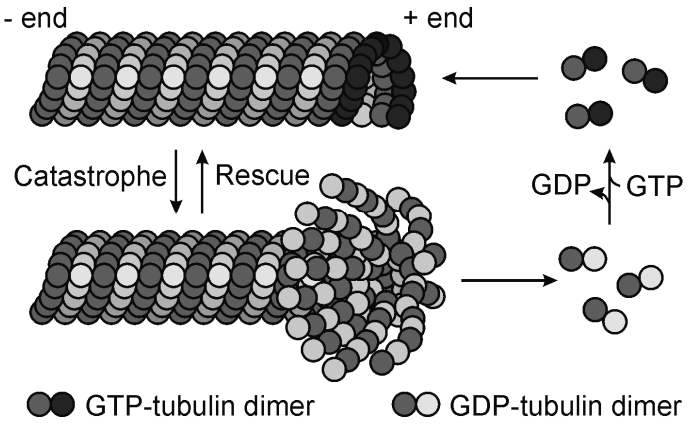

Figure 3. Dynamic instability of microtubules. Adapted from Cheeseman and Desai (2008).

In addition to microtubules, motor proteins contribute to the movements of chromosomes towards or away from the spindle equator. Motor proteins fuel their work through ATP hydrolysis enabling sliding of microtubules relative to each other or to other structures when transporting cargoes along spindle microtubules and regulating microtubule assembly dynamics (Sharp et al., 2000b). There are multiple motor proteins, which work in harmony during spindle assembly and it is changes in the balance of their forces that contribute to spindle pole separation (Gadde and Heald, 2004). The two main types of motor proteins taking part in mitosis progression are dyneins and kinesins. Dyneins are minus end directed motor proteins whereas the majority of kinesins are plus end directed. There are 14 families of kinesins coded for by a total of 45 genes in mammals (reviewed in Wordeman, 2010). Many of kinesins participate in mitotic spindle assembly and chromosome segregation. In addition, kinesin families 8 and 13 are implicated in microtubule depolymerization and are enriched at the centromeres and centrosomes where the assembly and disassembly of microtubules occur. Mitotic centromere-associated kinesin (MCAK), a member of the kinesin family 13, depolymerizes microtubules and promotes catastrophes (Hunter et al., 2003; Newton et al., 2004). It is involved in chromosome congression (Walczak et al., 2002), k-fiber turnover (Wordeman et al., 2007) and corrections of erroneous attachments (Lan et al., 2004). The Inner centromere KinI stimulator (ICIS) stimulates MCAK (Ohi et al., 2003). Phosphorylation by Aurora B localizes MCAK to the centromere, which suppresses the microtubule depolymerizing activity (Lan et al., 2004; Tanenbaum et al., 2011). Kinesin Eg5 belongs to the kinesin family 5 and moves towards the plus ends of microtubules, sliding the microtubules relative to each other (Kapitein et al., 2008; Kwok et al., 2006). It participates in spindle assembly (Kapoor et al., 2000), congression of chromosomes (Gardner

et al., 2008), spindle pole separation and spindle bipolarity (Sawin et al., 1992). Mitotic kinesin-like protein 1 (MKLP1) and MKLP2 from the kinesin family 6 take part in spindle assembly and elongation (Cesario et al., 2006; Fu et al., 2009) and cytokinesis (Kuriyama et al., 2002). The kinesin Centromere-associated protein E (Cenp-E) associates with unaligned chromosomes (Chan et al., 1998) and is involved in the movement of mono-oriented chromosomes to the spindle equator (Cai et al., 2009; Kapoor et al., 2006; Schaar et al., 1997) and SAC signaling (Ditchfield et al., 2003; Guo et al., 2012; Yao et al., 2000). The minus end directed motor protein dynein is involved in chromosome movements, spindle organization, spindle positioning, and checkpoint silencing (Howell et al., 2001; Sharp et al., 2000a; Varma et al., 2008). However, neither Cenp-E nor dynein is essential for chromosome alignment: knockdown of Cenp-E allows the majority of the chromosomes to align and depletion of dynein does not have a major impact on the rates of chromosome motility (Kapoor et al., 2006; McEwen et al., 2001; Yang et al., 2007).

2.2.3. Kinetochores and centromeres

Kinetochores are protein complexes of about 100 proteins that are localized in the centromeric region of chromosomes (Foley and Kapoor, 2013). In a metaphase chromosome, the centromeric region called the inner centromere connects the two sister chromatids and there are two kinetochores, one for each sister chromatid. Kinetochores have three layers (Fig. 4): the inner region (inner plate) connecting to chromatin, the 50-60 nm thick outer region (outer plate) interacting with spindle microtubules, and the less dense central region (interzone) (Cheeseman and Desai, 2008). Moreover, the fibrous corona extends outwards from the outer kinetochore. Kinetochore proteins are responsible for the attachment between chromosomes and spindle microtubules. For example, the Nuclear division cycle 80 (Ndc80) complex mediates the physical kinetochore-microtubule connections and is involved in the regulation of the interaction stability via its phosphorylation status (DeLuca et al., 2006; DeLuca et al., 2011). Furthermore, several centromeric proteins interact with kinetochore proteins to ensure the correct attachments. In addition to connecting chromosomes to microtubules, the kinetochores contribute to the forces required for chromosome segregation (Cheeseman and Desai, 2008).

In humans, the centromeres contain tandem repeats of a 171-base pair (bp) α–satellite DNA sequence. In this sequence, a 17-bp motif, the Cenp-B box (Masumoto et al., 1989), can bind to the inner centromere protein Cenp-B. However, it seems that established centromeric loci can be maintained even in the absence of the Cenp-B-α-satellite DNA interaction. Therefore, epigenetics

is thought to have a more essential role in determining the site for kinetochore assembly (Karpen and Allshire, 1997). The centromeric chromatin contains regions where histone H3 is replaced by the H3 variant Cenp-A (Blower et al., 2002). The constitutive centromere-associated network (CCAN) consisting of Cenp-C and 15 interacting proteins called Cenp-H, Cenp-I, Cenp-K-U, Cenp-W, and Cenp-X (Foltz et al., 2006; Izuta et al., 2006; McAinsh and Meraldi, 2011; Obuse et al., 2004; Okada et al., 2006) colocalizes with Cenp-A in vertebrates throughout the cell cycle (Foltz et al., 2006; Okada et al., 2006). Cenp-A and CCAN together with the KMN complex, containing Kinetochore null 1 (Knl1), and the complexes Missegregation 12 (Mis12) and Ndc80, all contribute to the kinetochore assembly (Fig. 4). Furthermore, the KMN associates with ZW10-interacting protein 1 (Zwint1) (Obuse et al., 2004), which is a receptor for the Rod-ZW10-Zwilch (RZZ) complex (Starr et al., 2000). The RZZ complex recruits the minus end directed motor protein dynein to kinetochores (Karess, 2005). In addition, several microtubule-associated and SAC signaling related proteins, which will be described later, localize to kinetochores.

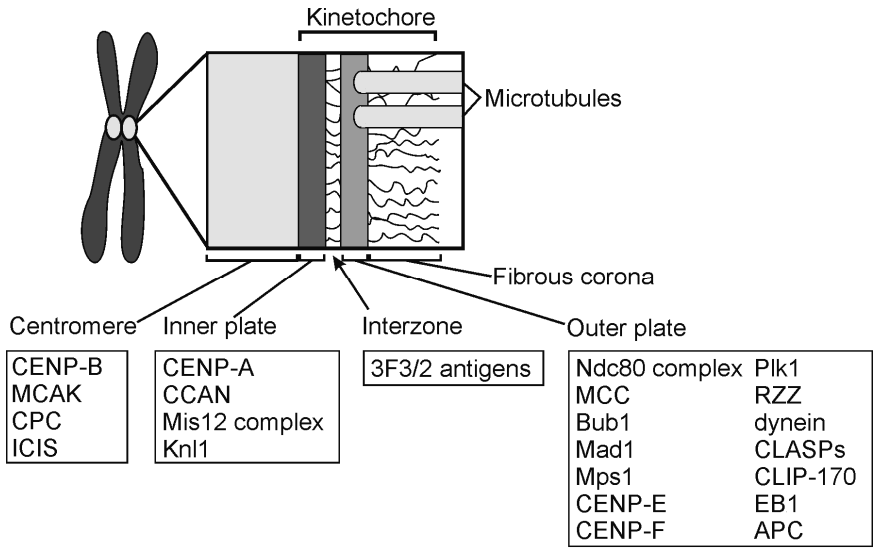

Figure 4. The kinetochore structure. The localization of representative proteins and protein complexes is shown (CPC = chromosomal passenger complex, MCC = mitotic checkpoint complex, CLIP = Cytoplasmic linker protein, CLASP = CLIP associating protein, APC = anaphase promoting complex). Modified from Maiato et al. (2004).

2.2.4. Kinetochore-microtubule attachment types

Correct chromosome segregation requires bi-orientation of chromosomes where sister chromosomes are connected to opposite spindle poles via the microtubules (Walczak et al., 2010). Abnormal attachments (Fig. 5) between kinetochores and microtubules need to be corrected in order to maintain genomic stability. Erroneous attachments may lead to aberrant chromosome segregation and consequently aneuploidy, which in turn can promote tumorigenesis (Cimini et al., 2003; Ganem et al., 2009; Thompson and Compton, 2008). Amphitelic bi-oriented attachments are a prerequisite for normal chromosome segregation as the sister kinetochores are connected to opposite spindle poles and thus can be pulled in opposite directions (Walczak et al., 2010). In monotelic attachment, only one of the sister kinetochores is attached to one spindle pole. Conversely, when both kinetochores are attached to the same spindle pole, the attachment is called syntelic. In the merotelic attachment, one kinetochore has become attached to both spindle poles while the other is attached to one pole. This attachment still creates tension across the kinetochores similar to amphitelic attachments and is thus unrecognizable for the SAC (Cimini, 2008).

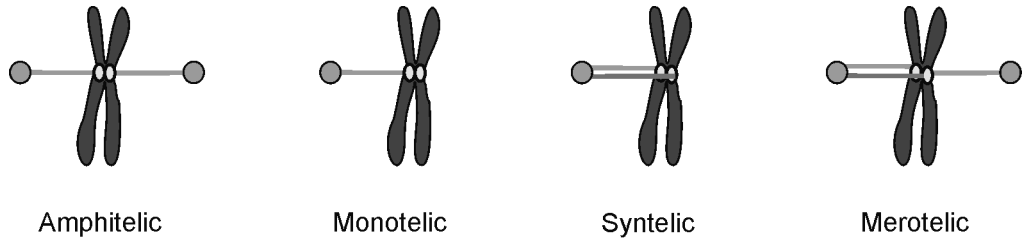

Figure 5. Kinetochore-microtubule attachment types.

During early mitosis, kinetochores attach to the microtubule lattice to form lateral attachments followed by the formation of end-on attachments (Magidson et al., 2011). The lateral kinetochore-microtubule attachments are mediated by motor proteins, minus end directed dynein and plus end directed Cenp-E (Kapoor et al., 2006; Yang et al., 2007). In prometaphase, the laterally attached chromosomes exhibit rapid poleward movements, which are motored by dynein (Rieder and Alexander, 1990; Yang et al., 2007). This is thought to facilitate end-on attachments at the kinetochore outer plate by moving the chromosomes near to the poles where there are more microtubules. On the other hand, Cenp-E facilitates the alignment of laterally attached chromosomes that are in the proximity of a spindle pole (Cai et al., 2009; Kapoor et al., 2006). Lateral attachments are usually further matured into end-on attachments (Walczak et al., 2010). The bi-oriented chromosomes then undergo poleward and anti-

poleward movements called oscillations (Skibbens et al., 1993). Both microtubule attachments and dynamics seem to contribute to oscillations (Walczak et al., 2010). The Ndc80 complex is necessary for the formation of the stable end-on attachments and is required for oscillatory movements (DeLuca et al., 2011; Vorozhko et al., 2008). However, the complex is not involved in the formation of lateral attachments (Vorozhko et al., 2008).

2.2.5. Spindle assembly and chromosome congression

The alignment of chromosomes at the spindle equator (also called the metaphase plate) is termed chromosome congression (reviewed in Walczak et al., 2010). In the "search and capture model" (Kirschner and Mitchison, 1986; Walczak et al., 2010), monotelic chromosomes are thought to stay near to one centrosome until microtubules from the opposite centrosome attach to one of the sister kinetochores. This microtubule bundle would then exhibit depolymerization leading to shortening and at the same time pulling the chromosome to the spindle equator. This process can be assisted by motor proteins such as Cenp-E (Schaar et al., 1997; Wood et al., 1997) and dynein (Yang et al., 2007). However, estimated by mathematical modelling this congression mechanism alone would take too much time (Wollman et al., 2005) as chromosomes congress to the spindle equator normally in 10-15 minutes (Walczak et al., 2010). It is thus likely that there are other additional mechanisms involved.

The "k-fiber nucleation model" suggests that kinetochore microtubules nucleate at kinetochores instead of microtubules emanating from spindle poles followed by capture of the chromosomes. This model is supported by the assembly of microtubules onto the isolated mitotic chromosomes (Telzer et al., 1975). In addition, kinetochore-mediated formation of k-fibers and their incorporation into the spindle has been seen in live cells (Khodjakov et al., 2003; Maiato et al., 2004). GTPase-Ran is likely to be involved in the kinetochore nucleation of microtubules (Tulu et al., 2006) as its activity gradient is needed for nucleating microtubules near to the centromere region. A kinetochore may capture the short microtubule, after which the plus end of the microtubule begins to polymerize pushing the more stable minus end onwards. Moreover, kinetochore nucleated microtubules can later become attached to the spindle microtubules followed by positioning of the chromosomes by microtubule depolymerization and motor proteins. Thus, the two models are not exclusive of each other and it is very likely that several processes are utilized when chromosomes are being moved to the spindle equator. However, the kinetochore-mediated nucleation is supported by the concept that k-fibers are formed also in cells lacking

centrosomes, for example in female meiosis in flies (McKim and Hawley, 1995).

2.3. Kinetochore-microtubule interface

2.3.1. The KMN complex

The kinetochore-microtubule interface (reviewed in Cheeseman and Desai, 2008) comprises of several protein complexes aiming to maintain kinetochore-microtubule attachments in the presence of dynamic microtubules. Many of these proteins take part in the SAC signaling and error correction of the incorrect attachments. The kinetochore-localized KMN network (Fig. 6), composed of Knl1, the four-subunit Mis12 complex and the four subunit Ndc80 complex, has functions in both microtubule binding and in SAC signaling (Caldas et al., 2013; Cheeseman et al., 2006; Welburn et al., 2010). It contains two low-affinity microtubule-binding sites, one in the Ndc80 complex and the other in the N-terminus of Knl1 (Cheeseman et al., 2006). Knl1 acts as a scaffold protein for many kinetochore localized proteins important for the SAC signaling (Caldas and DeLuca, 2014). It interacts directly with Mis12, Zwint-1, PP1, Budding uninhibited by benomyl 1 (Bub1), Bub3 and the Bub1-related 1 (BubR1) (Kiyomitsu et al., 2007; Liu et al., 2010; London et al., 2012; Petrovic et al., 2010; Shepperd et al., 2012). In addition, Knl1 indirectly recruits many SAC components to the kinetochores (Caldas and DeLuca, 2014). Depletion of Knl1 results in partial unalignment of chromosomes due to kinetochore-microtubule attachment defects (Caldas et al., 2013). The Mis12 complex, composed of Mis12, Nsl1, Dsn1, and Nnf1 (Petrovic et al., 2010), directly binds to Knl1, the Ndc80 complex, and Heterochromatin protein 1 (HP1). Moreover, Nsl1 C-terminus is required for kinetochore localization of Knl1 and Ndc80 (Petrovic et al., 2010). Interestingly, Knl1, the Mis12 complex, and the Ndc80 complex work synergistically to bind microtubules, probably by reinforcing the binding affinity of the Ncd80 complex (Cheeseman et al., 2006).

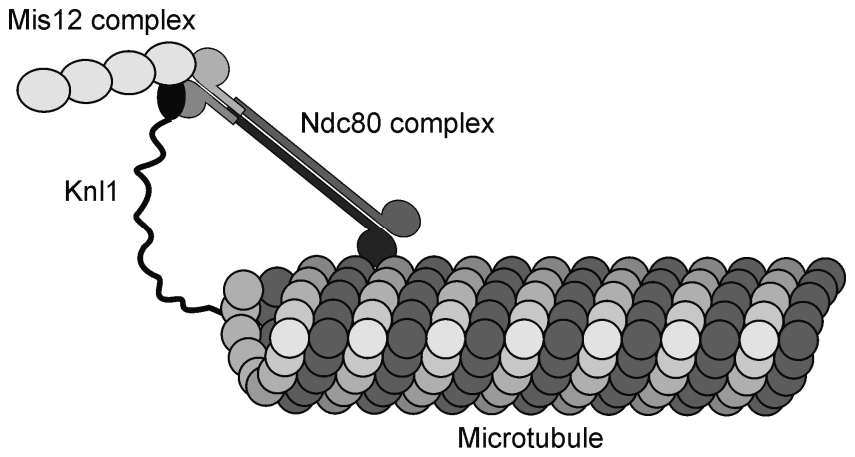

Figure 6. The KMN complex mediates kinetochore-microtubule attachments. Modified from Foley and Kapoor (2013).

The Ndc80 complex physically mediates the kinetochore-microtubule interactions (DeLuca et al., 2002; McCleland et al., 2003; Wigge and Kilmartin, 2001). The complex contains four proteins, Nuclear filamentous 2 (Nuf2), Ndc80 (called Hec1 in humans), Spindle pole component 24 (Spc24) and Scp25, and forms a rod-like structure where its two globular heads are separated by a long coiled-coil domain (Fig. 7; Ciferri et al., 2005; Wei et al., 2005). This domain is truncated in the recombinant Ndc80 complex, Bonsai (Ciferri et al., 2008), which was used in this thesis (II) to determine the binding of a small compound to the complex. The dimer of Ndc80 and Nuf2 on the other end of the Ndc80 complex binds directly to microtubules at the outer kinetochore (Cheeseman and Desai, 2008; DeLuca et al., 2005; Wei et al., 2007). The N terminus of Ndc80 contains the microtubule binding calponin homology domain (CHD) similar to that of the plus end tracking protein End-binding protein 1 (EB1) (Wei et al., 2007). The other end is composed of Spc24 and Spc25 and it faces towards the inner kinetochore. In *Caenorhabditis elegans*, Spc24 and Spc25 are required for the interaction between the Ndc80 complex, the Mis12 complex and Knl1 (Cheeseman et al., 2006). This association increases the microtubule binding affinity. In vertebrates, the Mis12 complex bridges Knl1 and the Ndc80 complex to the CCAN, which is missing in *Caenorhabditis elegans* (Kline et al., 2006; Okada et al., 2006). Aurora B phosphorylates nine phosphoresidues (DeLuca et al., 2011; Guimaraes et al., 2008) on the Ndc80 N-terminal tail, which destabilizes wrong kinetochore-microtubule connections allowing more time for error correction. Knl1 and Mis12 complex are also phosphorylated by Aurora B, which results in a decrease in the microtubule binding affinity (Welburn et al., 2010). The Knl1 and Ndc80 complexes are organized in such a way that several low-affinity

interactions per single microtubule form together stronger kinetochore-microtubule connections. In budding yeast, there is eight Ndc80 complexes per microtubule (Joglekar et al., 2006), and in vertebrates several fibrillar structures have been found to interact with microtubules at the outer kinetochore (Dong et al., 2007). The many low-affinity interactions allow the dynamic instability in the microtubules without them detaching from kinetochores.

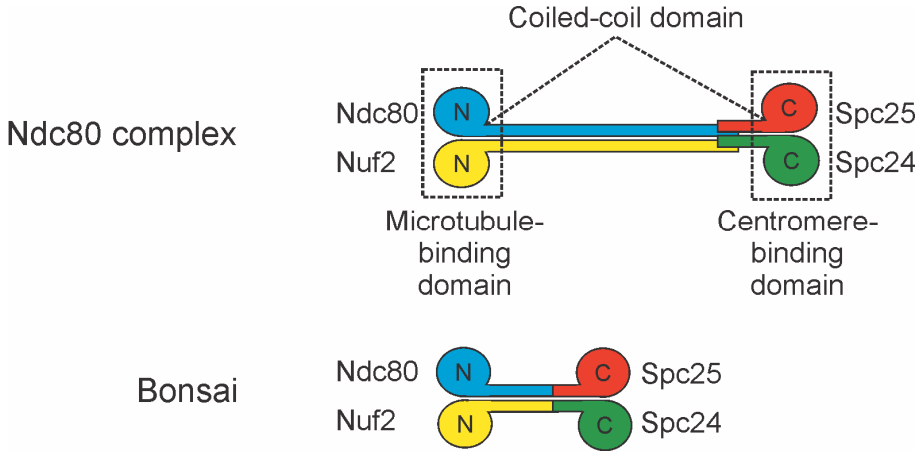

Figure 7. Simplified structures of the Ndc80 complex and its recombinant form Bonsai (Ciferri et al., 2008). Adapted from Santaguida and Musacchio (2009).

2.3.1.1. Ndc80/Hec1

Ndc80 is one of the four members of the Ndc80 complex and is called Highly expressed in cancer 1 (Hec1) in humans. Hec1 mediates the attachments between kinetochores and microtubules, and is essential for correct chromosome congression and SAC signaling (Ciferri et al., 2007; DeLuca et al., 2005; DeLuca et al., 2006; McCleland et al., 2003; McCleland et al., 2004). The kinetochore-microtubule interactions need to be strong enough but not excessively strong to prevent microtubule dynamics. The N-terminal CHDs of Hec1 and Nuf2 and also the 80 amino acid long N-terminal tail of Hec1 all contribute to the binding of microtubules to the Ndc80 complex (Alushin et al., 2012; Ciferri et al., 2008; Guimaraes et al., 2008; Sundin et al., 2011). The binding is facilitated by the opposite charges of the binding partners: CHD and the N-terminal tail of Hec1 are positively charged whereas C-terminal E-hook tails of tubulin are negatively charged (Ciferri et al., 2008). Moreover, the Ndc80 complex recognizes both α-tubulin and β-tubulin at the inter- and intra-tubulin dimer interfaces (Alushin et al., 2010). This allows the Ndc80 complex to bind microtubules at every 4 nm (the spacing of tubulin monomers) whereas

most other microtubule binding proteins bind at every 8 nm (the spacing of tubulin dimers). This ability of the Ndc80 complex facilitates oligomerization of the complex on microtubules. Furthermore, it allows the Ndc80 complex to sense the tubulin conformation as the microtubules depolymerize and bend. Thus, the Ndc80 complex bound near to the depolymerizing microtubule end can become detached from the filament. However, the oligomerization of the Ndc80 complexes also allows their co-operation in order to maintain persistent interactions with dynamic microtubules (Foley and Kapoor, 2013). Mutations of the Hec1 N-terminal tail or CHD impair binding stability and induce errors in chromosome congression (DeLuca et al., 2006; DeLuca et al., 2011; Sundin et al., 2011; Tooley et al., 2011; Umbreit et al., 2012). Furthermore, the Hec1 protein structure contains an internal loop in its coiled coil domain, which creates a kink in the structure increasing flexibility. The loop is needed for proper kinetochore-microtubule binding and chromosome segregation (Nilsson, 2012). It acts as a binding site for the proteins needed for establishing kinetochore-microtubule interactions (Hsu and Toda, 2011; Maure et al., 2011; Tang et al., 2013; Varma et al., 2012; Zhang et al., 2012).

Phosphoresidues serine (Ser) 4, Ser5, Ser8, Ser15, Ser44, threonine (Thr) 49, Ser55, Ser62 and Ser69 on Hec1 are putative Aurora B kinase phosphorylation sites (Ciferri et al., 2008; DeLuca et al., 2006). Their phosphorylation destabilizes kinetochore-microtubule connections allowing more time for error correction. Phosphomimetic mutations in Hec1 do not support stable kinetochore-microtubule attachments (Guimaraes et al., 2008) whereas nonphosphorylatable mutants hyperstabilize the attachments (DeLuca et al., 2006; DeLuca et al., 2011). Never in mitosis A-related kinase 2 (Nek2) phosphorylates Ser165 in the CHD of Hec1 regulating chromosome alignment and the SAC signaling (Chen et al., 2002; Wei et al., 2011). Moreover, Hec1 is needed for the localization of the SAC proteins Mitotic arrest deficient 1 (Mad1) and Mad2 to kinetochores (DeLuca et al., 2003; McCleland et al., 2003) ensuring the quality control function of the SAC.

The high expression of Hec1 associates with both the early and late stages of breast cancer tumorigenesis and is linked to poor prognosis (Bieche et al., 2011; Glinsky et al., 2005; Mo et al., 2013; Qu et al., 2014). In mice, overexpression of Hec1 induces hyperactivation of the SAC and tumorigenesis (Diaz-Rodriguez et al., 2008). The possibility to target specifically mitotic cancer cells makes Hec1 an attractive target for future cancer interventions by LMW compounds. The Hec1 binding compound INH1 and its analogues have been reported to inhibit Hec1 by halting its interaction with Nek2. INH1 induces mitotic arrest and cell death after mitotic slippage (Huang et al., 2014; Qiu et al., 2009; Wu et al., 2008). Depletion of Hec1 by RNA interference (RNAi)

results in SAC-dependent mitotic arrest with unaligned chromosomes and finally cell death (Gurzov and Izquierdo, 2006; Martin-Lluesma et al., 2002). Hec1 depletion by viral vectors has been shown to inhibit tumor growth in xenograft mouse models (Gurzov and Izquierdo, 2006; Li et al., 2007). Moreover, an RNAi lethality screen identified Hec1 and Nuf2 as putative targets and their silencing was able to suppress ovarian cancer cell viability (Sethi et al., 2012). Another RNAi screen discovered Hec1 as a potential therapeutic target in malignant pleural mesothelioma (Linton et al., 2014). Interestingly, Hec1 depletion sensitizes ovarian cancer cells to the microtubule drug taxol (Mo et al., 2013).

2.3.2. Other proteins at the kinetochore-microtubule interface

The essential feature of the microtubule attachment site at the kinetochore is to be able to couple binding with microtubule polymerization and depolymerization dynamics (Maiato et al., 2004). Many kinetochore proteins and MAPs control the polymerization state of microtubules to make this possible. Depolymerization is promoted by the kinesin-13 family of depolymerases, such as MCAK, and the kinesin-8 family members, such as Kif18A, which act as motors and microtubule depolymerases (Howard and Hyman, 2007). The microtubule binding protein, Cytoplasmic linker protein (CLIP) associating protein (CLASP), localizes to kinetochores and promotes polymerization of kinetochore microtubules (Maiato et al., 2003; Maiato et al., 2005). CLIP170 is implicated in creating the initial kinetochore-microtubule interactions (Tanenbaum et al., 2006), and MAP215 promotes microtubule polymerization and functions during spindle assembly and chromosome segregation (Gard et al., 2004). EB1 interacts with the microtubule plus end but also binds and stabilizes the microtubule lattice (Sandblad et al., 2006).

Several Ndc80 complexes bind to a single microtubule to ensure the binding of kinetochores to dynamic microtubules as described above. In addition, kinesin Cenp-E has been postulated to have a role in this process (Lombillo et al., 1995) even though Cenp-E loss-of-function does not induce major chromosome misalignment (McEwen et al., 2001; Weaver et al., 2003). In budding yeast, the 10-subunit Dam1 (Duo1 and Mps1-interacting protein 1) complexes form a ring, containing about 16 complexes, around microtubules (Wang et al., 2007). The Dam1 complex promotes polymerization of microtubules in response to tension and couples the movement of the cargo to depolymerization (Franck et al., 2007; Westermann et al., 2006). No homologue of the Dam1 complex has yet been found in organisms outside the class of fungi. In metazoans, the Spindle and kinetochore-associated protein (Ska) complexes localize to outer

kinetochores and spindle microtubules (Hanisch et al., 2006). Even though the Dam1 complex and the three-subunit Ska complex are structurally unrelated, they both bind to dynamic microtubules (Welburn et al., 2009; Westermann et al., 2006). They also retain the Ndc80 complex at the depolymerizing microtubule ends (Schmidt et al., 2012; Tien et al., 2010). The recombinant Ndc80 complexes become attached to dynamic microtubules only as oligomers (Powers et al., 2009), but when present with the Dam1 or Ska complex then even the Ndc80 complex monomers can stay attached (Schmidt et al., 2012; Tien et al., 2010). Thus, the Dam1 or Ska complexes are needed for proper kinetochore-microtubule interactions. The depletion of the Ska complex induces mitotic arrest and leads to diminished sister chromatid cohesion (Daum et al., 2009). Aurora B-dependent phosphorylation negatively regulates the association of the Dam1 and Ska complexes with microtubules and the Ndc80 complex (Chan et al., 2012b; Cheeseman et al., 2002; Tien et al., 2010).

2.4. Spindle assembly checkpoint (SAC)

2.4.1. Introduction to the SAC

The SAC is the primary surveillance mechanism of chromosome segregation (Fig. 8) and involves a regulatory network of several proteins (reviewed in Foley and Kapoor, 2013; Musacchio and Salmon, 2007). The SAC prevents the transition from metaphase to anaphase in the presence of defective kinetochore-microtubule attachments until each chromosome is congressed to the spindle equator in a bipolar manner. The SAC includes the Ser/Thr kinases Monopolar spindle protein 1 (Mps1) and Bub1 and also the proteins Mad1, Mad2, Bub3, and BubR1 (also called Mad3 in yeast), which is referred to as a pseudo-kinase (Foley and Kapoor, 2013). Several SAC proteins such as Mad1, Mad2, Bub1 and BubR1 transiently localize to unattached kinetochores and, upon microtubule attachment, become removed from the kinetochores (Cheeseman and Desai, 2008; Taylor et al., 2001). The SAC inactivates Cell division cycle protein 20 (Cdc20), a cofactor of the E3 ubiquitin ligase called the anaphase promoting complex (APC/C; also called the cyclosome) (Hwang et al., 1998; Kramer et al., 1998). The formation of the Cdc20 inhibitory complex called the mitotic checkpoint complex (MCC) is catalyzed by the SAC. The complex contains the proteins Cdc20, Mad2, BubR1/Mad3 and Bub3 (Sudakin et al., 2001). The SAC normally becomes inactivated by correct chromosome alignment at the metaphase plate but the inactivation can be disrupted by spindle poisons, interference with the kinetochore assembly, impairment of microtubule motor proteins and alterations in microtubule dynamics (Musacchio and Salmon, 2007; Rieder and Maiato, 2004). The subsequent

mitotic arrest can eventually result in cell death or aberrant exit from mitosis. On the other hand, inhibition of the proteins necessary for the correct function of the SAC may induce a premature forced exit from mitosis leading to aberrant chromosome number in cells (Hauf et al., 2003; Kallio et al., 2002; Santaguida et al., 2010).

2.4.2. Anaphase promoting complex/cyclosome (APC/C)

Sister chromatids are held together by the Cohesin protein complex. The enzyme, Separase, proteolytically cleaves Cohesin complexes but is kept in check until anaphase onset by Securin (Sudakin et al., 1995). The APC/C triggers the exit from mitosis by ubiquitylation and subsequent 26S proteasome-mediated destruction of Cyclin B (Glotzer et al., 1991) and Securin (Yamamoto et al., 1996). As a consequence, Cohesin is cleaved by Separase leading to the separation of the sister chromatids. The proteolysis of Cyclin B inactivates Cdk1, which drives cells out of mitosis (Peters, 2006). The activation of the APC/C requires dissociation of Cdc20 from the MCC. The dissociation happens upon proper attachment of chromosomes to spindle microtubules. The ubiquitylation of Cyclin B and Securin is mediated via the destruction box (D-box) (Glotzer et al., 1991), a sequence that is present in both proteins and recognized by the APC/C. The D-box recognition site is formed by the APC/C cofactors, Cdc20 and Cdc20 homologue 1 (Cdh1), and the APC/C component APC10 (da Fonseca et al., 2011).

2.4.3. Mitotic checkpoint complex (MCC)

The MCC, including the proteins Cdc20, Mad2, BubR1/Mad3 and Bub3, binds the APC/C (Fig. 8) and inhibits its ubiquitin ligase activity (Sudakin et al., 2001). The APC/C activator Cdc20 is kept inactive by the MCC. BubR1/Mad3 binding to Cdc20 masks the D-box recognition site in the protein and disrupts the interaction between Cdc20 and APC10 (Chao et al., 2012). Finally, binding of Cdc20 to the MCC promotes APC/C-dependent autoubiquitylation of Cdc20, which diminishes the Cdc20 protein levels and thus allows efficient inhibition of the remaining pool of Cdc20 by the SAC (Foley and Kapoor, 2013; Foster and Morgan, 2012; Nilsson et al., 2008). The details of how the MCC binds to the APC/C to inhibit its activity are still not fully known. Possibly the binding is mediated by a KEN-box motif of BubR1 (Lara-Gonzalez et al., 2011). A KEN-box motif is a sequence present in many substrates of the APC/C (Musacchio and Salmon, 2007). BubR1 binds to Cdc20 directly and exerts a synergistic effect on APC/C inhibition with Mad2 (Fang, 2002; Lara-Gonzalez

et al., 2011). In humans, binding of the N terminus of BubR1 to Cdc20 requires prior binding of Mad2 to Cdc20 (Davenport et al., 2006). However, the Mad2-Cdc20 subcomplex is not sufficient to sustain mitotic arrest as BubR1 is also needed (Chen, 2002). Bub1, Mitogen-activated protein kinase (MAPK) and Cdk1 phosphorylate Cdc20 to affect its binding to Mad2 and BubR1 (Chung and Chen, 2003; Kramer et al., 2000; Tang et al., 2004). Bub3 is required for the kinetochore localization of Bub1 and BubR1 (Taylor et al., 1998) and for the formation of the MCC.

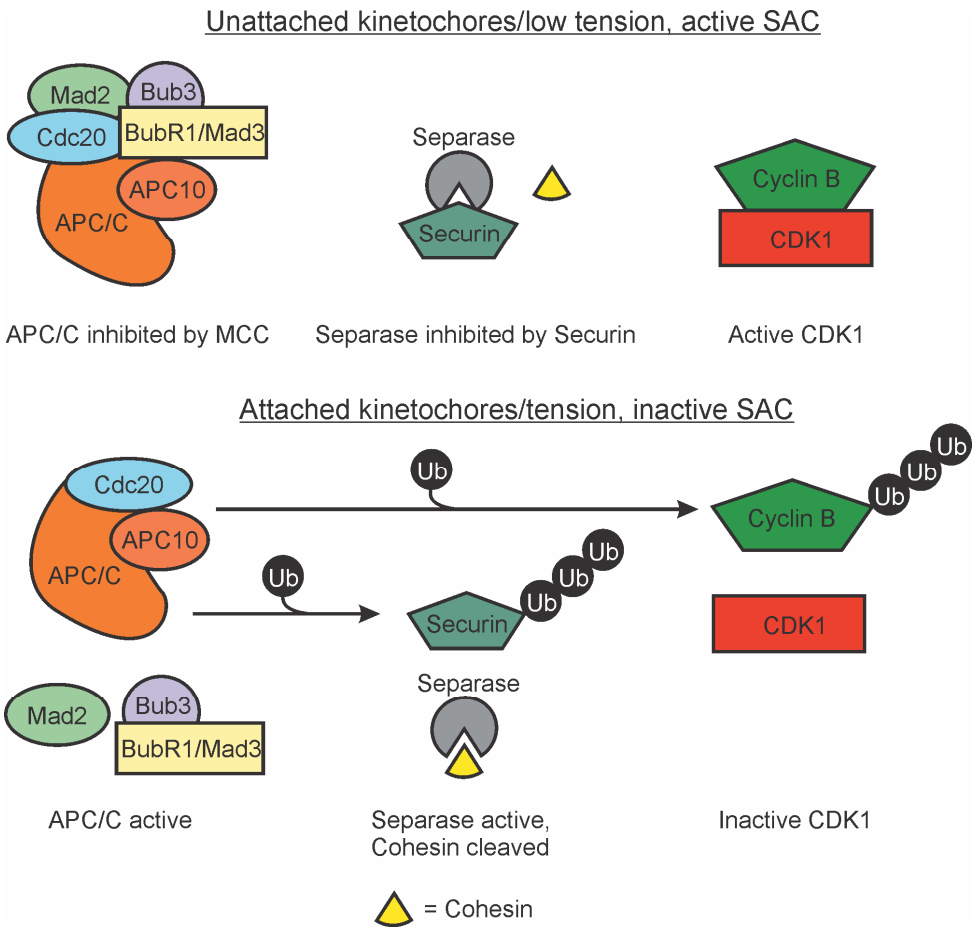

Figure 8. The principle of the SAC and the APC/C. Adapted from Foley and Kapoor (2013) and Musacchio and Hardwick (2002). Ub = Ubiquitin.

Mad2 is thought to bind Cdc20 according to "the Mad2-template model" (Mapelli et al., 2007; Musacchio and Salmon, 2007). Mad2 can exist in two different conformations: a closed Mad2 (C-Mad2) that can bind to Cdc20 and

Mad1 and the open Mad2 (O-Mad2) that does not bind to these binding partners. C-Mad2 is bound to Mad1 when the SAC is active and thus creates a more stable pool of Mad2 compared to the O-Mad2 pool. According to "the Mad2-template model", a heterodimer of C-Mad2 and Mad1 at the kinetochore acts as a "template", which catalyzes the change of cytosolic O-Mad2 to C-Mad2. C-Mad2 can then bind to Cdc20 (Musacchio and Salmon, 2007).

Most SAC proteins localize to unattached kinetochores until chromosomes attach to microtubules (Musacchio and Salmon, 2007). Kinetochore localization of SAC proteins is considered to be a marker for SAC activity. For example, Mps1 kinetochore localization is needed to arrest cells in mitosis (Maciejowski et al., 2010), and targeting of Mad1 to attached kinetochores can maintain the activity of the SAC (Maldonado and Kapoor, 2011). However, kinetochores are not essential for the MCC formation as the complex is present also in interphase cells (Sudakin et al., 2001). This kinetochore-independent pool of the MCC is not sufficient to delay mitosis in the presence of erroneous attachments. Instead, it can inhibit APC/C function in prometaphase when the kinetochores are still recruiting SAC proteins (Meraldi et al., 2004).

2.4.4. Regulation of the SAC activity

2.4.4.1. SAC activation

Unattached kinetochores activate the SAC even to such an extent that the cell remains in the M phase for several hours (Rieder et al., 1995). The activity of Mps1 kinase is required for the recruitment of other SAC components to the kinetochore (Maciejowski et al., 2010; Santaguida et al., 2010). Knl1, a part of the KMN complex, is a substrate of Mps1 that phosphorylates Thr residues within conserved MELT repeats of Knl1 at unattached kinetochores (London et al., 2012; Shepperd et al., 2012). Mutation of phosphorylation sites in Spc7 (the fission yeast homologue of Knl1) is able to halt SAC signaling (Shepperd et al., 2012). The phosphorylation of Knl1 by Mps1 creates a binding site for Bub1, (London et al., 2012; Shepperd et al., 2012), which is able to recruit Bub3, BubR1/Mad3 and Mad1 (Rischitor et al., 2007; Sharp-Baker and Chen, 2001). The detailed mechanism for the association of Mad1 and Bub1 is still unknown. Another SAC activation pathway involving Knl1 depends on Zwint1. It associates with Knl1 and the RZZ complex, which in addition to Bub1 is required for Mad1 and C-Mad2 localization to kinetochores (Kiyomitsu et al., 2007; Kops et al., 2005). The presence of Mps1 activity is needed for the recruitment of the RZZ complex to kinetochores (Maciejowski et al., 2010; Santaguida et al., 2010). Aurora B kinase also contributes to SAC activation by

destabilizing erroneous kinetochore-microtubule attachments (DeLuca et al., 2011) in the presence of low interkinetochore tension.

2.4.4.2. SAC inactivation by attachment and tension

The inactivation of SAC is dependent on the status of the kinetochore-microtubule attachment and the interkinetochore tension. For example, Mad1 and Mad2 are localized to unattached kinetochores in prometaphase but become reduced at attached metaphase kinetochores when the SAC is inactivated (Howell et al, 2001; Musacchio and Salmon, 2007; Waters et al, 1998). Therefore, kinetochore localization of Mad1 and Mad2 is considered to represent a lack of attachment. Additionally, Securin and Cyclin B start to be degraded after all of the chromosomes have aligned in a bipolar manner (Clute and Pines, 1999; Hagting et al., 2002). Upon correct bi-orientation of the chromosomes, the centromeric chromatin becomes stretched increasing the distance of sister kinetochores, the so-called interkinetochore distance, and the kinetochore tension (Musacchio and Salmon, 2007). The lack of tension indicates that there are erroneous attachments, which need to be destabilized to allow for error correction (Hauf et al., 2003; Tanaka et al., 2002). Phosphorylation by Aurora B kinase (Ipl1 in yeast) can destabilize these attachments (Welburn et al., 2010). For example, syntelic attachment does not create enough tension to promote the SAC inactivation. On the other hand, bi-orientation is achieved with normal amphitelic attachments or merotelic attachments, which produce sufficient attachment and tension to turn off the SAC. Accordingly, merotelic attachments are not sensed by the SAC, which is why it is critical that they are corrected by the functions of Aurora B kinase. If merotelic attachments are not corrected, lagging chromosomes and chromosome missegregation may occur (Cimini et al., 2001; Cimini et al., 2003).

Tension and attachment contribute to the extinction of the SAC by affecting the association of SAC proteins with kinetochores. For example, the SAC is activated by taxol that decreases the tension by stabilizing microtubules and diminishing microtubule dynamics while attachments are preserved. In taxol treated cells, SAC activation correlates with the increased concentration of BubR1 and Bub1 at the kinetochores (Johnson et al., 2004; Taylor et al., 2001) and the phosphorylation of a phosphoepitope 3F3/2 by Plk1 kinase (Ahonen et al., 2005). In vinblastine (microtubule depolymerizing drug) treated cells, Bub1 and BubR1 also accumulate at tensionless kinetochores (Skoufias et al., 2007). However, in mammals, Mad2 accumulates to kinetochores more in the presence of the microtubule depolymerizing compound, nocodazole, than in the presence

of taxol (Waters et al., 1998). Hence, Mad2 seems to sense attachment over tension (Skoufias et al., 2001; Waters et al., 1998). In *Drosophila*, Mad2 and Bub1 seem to monitor microtubule occupancy while BubR1 and Bub3 monitor tension (Logarinho et al., 2004).

Tension can also regulate attachment. The results gained from reconstitution of kinetochore-microtubule attachments *in vitro* and purifying kinetochores from budding yeast suggest that tension in bi-oriented chromosomes increases the lifetime of the kinetochore-microtubule attachments (Akiyoshi et al., 2010). Therefore, tension can stabilize attachments (Foley and Kapoor, 2013), which makes it challenging to study them on their own.

2.4.4.3. Other SAC extinction pathways

Many substrates are phosphorylated when the SAC is active. It is thus logical to hypothesize that protein phosphatases can affect SAC inactivation. PP1 binds to kinetochores via a PP1-binding motif of Knl1 (Caldas and DeLuca, 2014; Rosenberg et al., 2011). In budding yeast, deletion of the PP1-interaction motif in Spc105 (the budding yeast homologue of Knl1) is lethal because of the constitutively active SAC (Rosenberg et al., 2011). In addition, a microtubule-binding domain in the N terminus of Knl1, next to the PP1-binding motif, (Caldas and DeLuca, 2014) is required for SAC silencing in *C. elegans* (Espeut et al., 2012). In human cells, the interaction of Knl1 and PP1 has been linked to kinetochore-microtubule attachments (Liu et al., 2010). The function of PP1 in SAC extinction can be masked by other mechanisms that silence the SAC, such as the removal of SAC proteins from the kinetochore by dynein (Gassmann et al., 2010; Howell et al., 2001).

Cytoplasmic dynein removes certain proteins from kinetochores along the microtubules towards their minus ends when the kinetochore-microtubule attachments are formed. These proteins include the RZZ complex, Spindly, Mps1, Mad1, and Mad2 (Gassmann et al., 2010; Howell et al., 2001; Musacchio and Salmon, 2007). Removal of Mad1 and Mad2 can be executed also by dynein-independent mechanisms, possibly dependent on the KMN complex, whereas the removal of Spindly is reliant on dynein (Gassmann et al., 2010). The presence of Spindly at the kinetochores interferes with SAC extinction, but the details of this process are not known. (Foley and Kapoor, 2013).

Extinction of SAC signaling requires the detachment of Cdc20 from the other partners of the MCC, which activates the APC/C. The APC/C-dependent

autoubiquitylation of Cdc20 promotes the detachment of Cdc20 and SAC inactivation (Foster and Morgan, 2012). In addition, $p31^{comet}$ binds to C-Mad2 (Mapelli et al., 2007) and promotes both the disassembly of the MCC and the autoubiquitylation of Cdc20 (Westhorpe et al., 2011). The formation of kinetochore-microtubule attachments might activate $p31^{comet}$ to compete with O-Mad2 for binding to C-Mad2 and to inhibit the interaction between C-Mad2 and O-Mad2, which is a prerequisite for amplification of the SAC signal according to "the Mad2-template model" (Musacchio and Salmon, 2007).

2.4.4.4. Mitotic kinases and phosphatases

Several kinases control chromosome segregation and the formation of correct kinetochore-microtubule attachments. Typically phosphatases PP1 and PP2A counteract the function of the kinases. The most crucial mitotic kinases are Aurora B, Plk1, Cdk1 and the checkpoint kinases Mps1, Bub1 and BubR1.

Plk1 is an important regulator of mitotic progression and it is needed for spindle assembly, chromosome congression, SAC signaling, and cytokinesis (Lenart et al., 2007; Petronczki et al., 2007; Sumara et al., 2004; van de Weerdt and Medema, 2006; Watanabe et al., 2009). The substrates of Plk1 include Cdc25C, Wee1, Myt1, Cyclin B, Shugoshin (Sgo), the APC/C and Cohesin (van de Weerdt and Medema, 2006). It creates a tension-sensing phosphoepitope 3F3/2 (Ahonen et al., 2005) and associates with kinetochore proteins such as Cenp-U (also called PBIP1) (Kang et al., 2006) and PICH via its polo-box domain (PBD) (Baumann et al., 2007).

Cdk1 is active when bound to Cyclin B. In mammalian cells, the dephosphorylation of Cdk1 by the phosphatase Cdc25C activates the kinase. Cdk1 promotes NEBD, chromatin condensation and spindle assembly (Nigg, 2001). Moreover, exit from mitosis depends on the inactivation of Cdk1–Cyclin B complexes when Cyclin B is degraded by the APC/C. Chemical inhibition of the kinase can also induce exit from mitosis. However, the cells stay in mitosis in the absence of Cdk1 activity if the proteolysis of Cyclin B or protein phosphatase activity is suppressed (Skoufias et al., 2007).

Kinetochore-localized Mps1 is a dual-specificity kinase that was originally identified in yeast to regulate centrosome duplication (Winey et al., 1991). Later, the kinase was recognized as an essential component of the SAC (Hewitt et al., 2010; Maciejowski et al., 2010), which is needed for the kinetochore localization of Mad1, Mad2, Plk1 and Cenp-E (Abrieu et al., 2001; Martin-Lluesma et al., 2002; Wong and Fang, 2005). In human cells, antibody

microinjections and RNAi were used to show that Mps1 regulates SAC signaling (Stucke et al., 2002). These experiments revealed that Mps1 is required for SAC-dependent arrest in response to microtubule depolymerization. The role of Mps1 in chromosome segregation was shown by mutating one of the autophosphorylation sites of the kinase (Jelluma et al., 2008). Furthermore, it has been suggested that the release of Mps1 from kinetochores is essential for SAC silencing and anaphase onset (Jelluma et al., 2010).

Bub1 kinase is localized to unattached kinetochores and its levels are decreased upon the formation of kinetochore-microtubule attachments and interkinetochore tension (Taylor et al., 2001). Bub1 contributes to recruiting Mad1, Mad2, BubR1, Bub3, Mps1, Cdc20, Cdc23, Cenp-F, PP2A, Sgo, MCAK and Cenp-E to kinetochores and centromeres (Boyarchuk et al., 2007; Johnson et al., 2004; Sharp-Baker and Chen, 2001; Tang et al., 2004; Tang et al., 2006; Vigneron et al., 2004). Bub1 also affects the formation of the MCC-APC/C complex (Morrow et al., 2005) and phosphorylates Cdc20 to promote SAC inhibition of the APC/C (Tang et al., 2004). Moreover, Bub1 contributes to the localization of the complex of Sgo and PP2A to centromeres (Tang et al., 2006). A loss of the complex induces a subsequent loss of sister chromatid cohesion (Kitajima et al., 2006).

The role of BubR1 kinase activity in SAC signaling appears to be somewhat contradictory. Human BubR1 is required for mitotic arrest when cells are treated with a microtubule inhibitor nocodazole (Chan et al., 1999). However, BubR1 ortholog in *Saccharomyces cerevisiae,* Mad3, lacks a kinase domain, which is present in the vertebrate BubR1 (Chan et al., 1998). Thus, the catalytic activity of BubR1 might not be necessary for the function of the SAC. However, the kinase might be needed for other aspects of kinetochore signaling or chromosome congression (Ditchfield et al., 2003; Lampson and Kapoor, 2005). BubR1 interacts with Cenp-E, a microtubule plus end directed motor protein that contributes to chromosome alignment (Chan et al., 1999; Yao et al., 2000). Cenp-E activates the BubR1 kinase when not bound to microtubules (Mao et al., 2005). Therefore, BubR1 activity is high before the formation of the kinetochore-microtubule attachments when Cenp-E binds to microtubules. In addition, the kinetochore levels of BubR1 are reduced in Cenp-E depleted cells (Weaver et al., 2003). In *Xenopus* egg extracts, the loss of Cenp-E by immunodepletion overrides the SAC when the spindle has been disrupted by nocodazole (Abrieu et al., 2000) suggesting that Cenp-E is needed for correct SAC signaling. In human cells, the depletion of Cenp-E by RNAi (Weaver et al., 2003; Yao et al., 2000) or antibodies (McEwen et al., 2001) leads to mitotic arrest with a few chromosomes misaligned while the majority are at the equator.

However, Cenp-E null mutations in mouse embryo fibroblasts induce even fewer unattached kinetochores, which are not able to maintain active SAC (Weaver et al., 2003). This suggests that, at least in mammals, Cenp-E mediates amplification of the SAC signal and becomes essential for correct SAC function when there are only a few unattached kinetochores (Weaver et al., 2003).

Protein phosphatases counteracting mitotic kinases localize to the kinetochores or the inner centromeres. PP1 and PP2A at kinetochores oppose Aurora B phosphorylation (Foley et al., 2011; Pinsky et al., 2006; Trinkle-Mulcahy et al., 2003). Furthermore, it has been suggested that PP1 can inhibit Mps1-dependent phosphorylation of Knl1 and thus dissociate Bub1 and Bub3 from Knl1 (London et al., 2012; Shepperd et al., 2012). PP1 can also reverse Zwint-1 phosphorylation by Aurora B and promote dynein-mediated stripping of the RZZ complex and the SAC proteins (Kasuboski et al., 2011). Finally, PP1 induced dephosphorylation of Cenp-E is able to stabilize kinetochore-microtubule attachments (Kim et al., 2010). PP2A is required to prevent proteolytic cleavage of centromeric Cohesin complexes and forms a complex with Sgo1. Sgo1 is required to recruit PPA2 to centromeres (Kitajima et al., 2006; Tang et al., 2006).

2.5. Chromosomal passenger complex (CPC)

2.5.1. Introduction to the CPC

The chromosomal passenger complex (CPC) consists of four core proteins: Aurora B kinase, INCENP (inner centromere protein), Survivin and Borealin (also known as Dasra). Aurora B is the enzymatic element of the complex whereas the other components have regulatory and targeting functions. The CPC participates in the regulation of chromosome condensation, correction of erroneous kinetochore-microtubule attachments, SAC signaling and cytokinesis (reviewed in Carmena et al., 2012b). The subcellular localization of the CPC changes according to its functions during mitosis (Fig. 9). Furthermore, if the localization or function of Aurora B, INCENP, Survivin or Borealin is perturbed, the other CPC subunits do not localize correctly and Aurora B activity is diminished (Adams et al., 2001; Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003; Vader et al., 2006). In early prophase, the CPC localizes to chromosome arms and translocates to centromeres later in prophase (van der Waal et al., 2012). The centromeric localization of the CPC is dependent on the phosphorylation of histone H3 Thr3 by Haspin (Dai and Higgins, 2005) and histone H2A Thr120 by Bub1 (Kawashima et al., 2010). In

anaphase, the CPC translocates towards the central spindle where it regulates chromosome compaction and later cytokinesis (van der Waal et al., 2012).

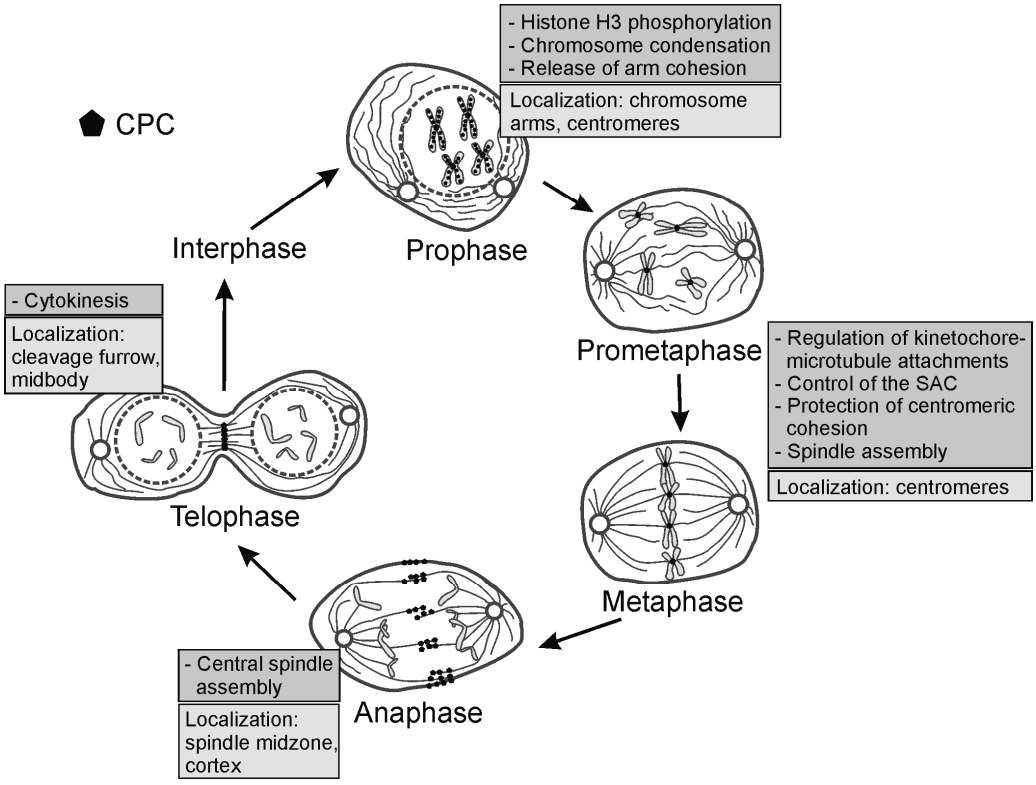

Figure 9. The localization and functions of the CPC. Adapted from Ruchaud et al. (2007).

2.5.2. Members of the CPC

2.5.2.1. Aurora B kinase - the catalytic subunit of the CPC

There are three members in the mammalian Aurora kinase family: Aurora A, B, and C. Aurora A is localized at the poles of the mitotic spindle where it regulates centrosome and spindle functions. Aurora B controls several mitotic processes at the centromeres, anaphase spindle and midzone. In addition, Aurora C controls meiosis and mitosis during early development (Carmena et al., 2012b). Aurora B kinase is the catalytic subunit of the CPC. It is an essential mitotic Ser/Thr kinase implicated in SAC signaling, stability of kinetochore-microtubule interactions and cytokinesis. Aurora B binds the IN-

BOX of INCENP that initially activates the kinase. Subsequently, Aurora B is able to phosphorylate INCENP (Bishop and Schumacher, 2002; Honda et al., 2003) and also autophosphorylate Thr232 in its own kinase domain (Yasui et al., 2004). This autophosphorylation results in full activation of Aurora B. Additionally, many mitotic proteins such as BubR1 and Bub1 regulate the activity of Aurora B (Ditchfield et al., 2003; Lampson and Kapoor, 2005; Ricke et al., 2011). The centromeres of misaligned chromosomes exhibit elevated levels of Aurora B in some cell lines (Salimian et al., 2011).

2.5.2.2. Regulatory subunits of the CPC

INCENP is the platform for CPC assembly, and its N-terminus is required for CPC centromere localization (Ainsztein et al., 1998). INCENP forms a triple-helix structure with Survivin and Borealin that targets the CPC to centromeres, anaphase spindle midzone and telophase midbody (Ainsztein et al., 1998; Jeyaprakash et al., 2007; Klein et al., 2006; Vader et al., 2006). The kinases Cdk1 and Aurora B regulate INCENP. In budding yeast, phosphorylation of the INCENP homolog Sli15 by Cdc28 (yeast Cdk1) and Ipl1 (yeast Aurora B) prevents the association of Sli15 with the midzone before anaphase (Nakajima et al., 2011; Pereira and Schiebel, 2003). The phosphorylation of Sli15 is required to activate the SAC, and dephosphorylation by Cdc14 inactivates the SAC in anaphase (Mirchenko and Uhlmann, 2010). In mice, phosphorylation of INCENP by Cdk1 is a prerequisite for the inner centromere targeting of Plk1 (Goto et al., 2006). Furthermore, both INCENP and Aurora B are required for the activation of Plk1 at the inner centromeric region in *Drosophila* and humans (Carmena et al., 2012a).

Survivin contains an N-terminal BIR (baculovirus IAP repeat) domain and a C-terminal helical extension. The BIR domain mediates the association between the CPC and the centromere but it does not affect the localization from anaphase onward (Lens et al., 2006; Yue et al., 2008). Survivin can be phosphorylated by kinases Aurora B (Wheatley et al., 2004; Wheatley et al., 2007), Cdk1 (O'Connor et al., 2000), Plk1 (Chu et al., 2011; Colnaghi and Wheatley, 2010) and Casein Kinase II (CK2) (Barrett et al., 2011). The loss of Survivin induces chromosome misalignment and failed cytokinesis (Lens et al., 2003; Yue et al., 2008). Originally Survivin was designated as an inhibitor of apoptosis that is highly expressed in cancer (Ambrosini et al., 1997). The phosphorylation of Survivin Thr34 has been suggested to be required for the anti-apoptotic function of the protein (O'Connor et al., 2000). However, the loss of Survivin failed to sensitize chicken DT40 cells to two drugs, staurosporine and etoposide, that normally induce apoptosis (Yue et al., 2008).

Borealin contributes to localization of the CPC together with Survivin and INCENP (Jeyaprakash et al., 2007). The phosphorylation of Borealin by Cdk1 is needed to allow its interactions with Sgo1 and Sgo2, which are important for centromeric localization of the CPC (Tsukahara et al., 2010). Borealin interacts with other CPC subunits and is in contact with almost all of the Survivin in mitotic cells (Gassmann et al., 2004). Depletion of Borealin by RNAi delays mitotic progression and induces chromosome misalignment, spindle defects, and failure of cytokinesis (Gassmann et al., 2004).

2.5.3. Functions of the CPC

Aurora B mediates its activity through a phosphorylation gradient (van der Waal et al., 2012; Wang et al., 2011; Welburn et al., 2010). Bi-oriented kinetochore-microtubule attachments pull the sister kinetochores apart, evoking a separation of the active Aurora B from its substrates and therefore differential substrate phosphorylation under distinct kinetochore attachment states (Liu et al., 2009; Welburn et al., 2010). On the other hand, erroneous kinetochore-microtubule attachments or the lack of attachments decrease interkinetochore tension (Sandall et al., 2006), which allows Aurora B to phosphorylate its substrates. This phosphorylation reduces microtubule-binding affinity (DeLuca et al., 2006; Welburn et al., 2010) and destabilizes erroneous kinetochore attachments (Tanaka et al., 2002). The destabilized connections are sensed by the SAC. Repositioning Aurora B closer to the kinetochore prevents stabilization of attachments and activates the SAC (Liu et al., 2009). In anaphase, the phosphorylation gradient formed by Aurora B determines the position of the spindle midzone and the cleavage furrow (Fuller et al., 2008).

Both PP1 and PP2A phosphatases can counteract the activity of Aurora B. Knl1/Spc105 recruits PP1 to the outer kinetochore where it dephosphorylates the substrates of Aurora B and stabilizes the kinetochore-microtubule attachments (Liu et al., 2010). Moreover, phosphorylation of Knl1 by Aurora B disrupts the interaction between Knl1 and PP1 creating a positive feedback mechanism (Liu et al., 2010). It is also suggested that PP2A containing a B56 regulatory subunit (B56-PP2A) can counteract the KMN phosphorylation status (Foley et al., 2011). The recruitment of B56-PP2A to kinetochores depends on BubR1 (Kruse et al., 2013; Suijkerbuijk et al., 2012). In addition, B56-PP2A regulates the phosphorylation status of BubR1 and the kinetochore localization of Plk1. B56-PP2A is concentrated in the inner centromere in the absence of microtubule attachments and dissociates upon bipolar attachment (Foley et al., 2011). Therefore, PP2A may contribute to the formation of stable connections

in prometaphase when interkinetochore tension is low and Aurora B can access its substrates (Foley and Kapoor, 2013).

The substrates of Aurora B at the kinetochores include the Dam1 ring complex (Cheeseman et al., 2002; Tien et al., 2010), the Ska complex (Chan et al., 2012b), the microtubule-depolymerizing kinesin MCAK (Andrews et al., 2004; Knowlton et al., 2006; Lan et al., 2004) and the Ndc80 complex (Cheeseman et al., 2006; DeLuca et al., 2006). Phosphorylation by Aurora B kinase localizes MCAK to the centromere suppressing microtubule depolymerizing activity and MCAK accumulation at the microtubule plus ends (Lan et al., 2004; Tanenbaum et al., 2011). Aurora B phosphorylation negatively regulates the association of the Dam1, Ska, and Ndc80 complexes with the microtubules (Chan et al., 2012b; Cheeseman et al., 2002; Tien et al., 2010).

Aurora B is required to maintain SAC-dependent mitotic arrest upon taxol treatment when kinetochores usually sustain their microtubule occupancy and the interkinetochore tension is low (Ditchfield et al., 2003; Hauf et al., 2003). Furthermore, when cells are incubated with nocodazole, the interkinetochore tension becomes reduced and Aurora B activity is needed to maintain the active SAC, particularly when Bub1 is silenced (Morrow et al., 2005). Nocodazole induced mitotic arrest is less affected by Aurora B inhibition compared to taxol induced arrest (Hauf et al., 2003). For example, Aurora B inhibitor Hesperadin rapidly overrides the SAC in taxol treated cells but not in nocodazole treated cells as they stay arrested for 3-5 hours (Hauf et al., 2003). This indicates that microtubules that are stabilized by taxol, and thus cannot establish interkinetochore tension, will be continuously destabilized by the CPC. When the CPC is silenced or chemically inhibited, the erroneous attachments are not corrected and the SAC will be inactive. On the other hand, in nocodazole treated cells, the lack of microtubules leads to unattached kinetochores even though the Aurora B is inhibited. However, the duration of the nocodazole induced mitotic arrest in the presence of an Aurora B inhibitor may depend on the concentration of both compounds (Santaguida et al., 2011). The role of Aurora B in correcting faulty kinetochore-microtubule attachments links the CPC with SAC signaling but the complex is thought to regulate the SAC also in a more direct manner. Antibody injections to *Xenopus* cells have indicated that Aurora B is needed for SAC-dependent mitotic arrest even when kinetochores are unattached (Kallio et al., 2002). Furthermore, the mutated INCENP lacking its coiled-coil domain has induced defects in taxol induced mitotic arrest (Vader et al., 2007). However, mutant INCENP could restore chromosome alignment and cytokinesis in unperturbed cells, and Aurora B exhibited normal localization and activity. This indicates that the coiled coil domain of INCENP is necessary for SAC activity and that the CPC regulates the SAC also

independently of its error correction functions. Aurora B also promotes kinetochore recruitment of Mad1, Mad2, Bub1, BubR1, Mps1, Cenp-E, ZW10 and Rod (Ditchfield et al., 2003; Famulski and Chan, 2007; Santaguida et al., 2011; Saurin et al., 2011), all implicated in SAC signaling.

In addition to error correction and SAC signaling, Aurora B regulates condensation of mitotic chromosomes by controlling the binding of condensin I, which is responsible for the maintenance of mitotic chromosome structure (Collette et al., 2011; Lipp et al., 2007). In prophase, Plk1 and Aurora B have been postulated to regulate the release of arm cohesion between the sister chromatids (Losada et al., 2002). However, the detailed mechanism is still not completely understood. In addition, Aurora B phosphorylates Sgo2, which recruits PP2A and MCAK to centromeres (Tanno et al., 2010). Sgo proteins prevent the premature release of sister chromatid cohesion through the interaction with PP2A. In *Drosophila* meiosis, phosphorylation of Sgo1 by Aurora B is essential for the stable kinetochore localization of Sgo1 and sister chromatid cohesion (Resnick et al., 2006). Furthermore, Aurora B can affect chromatin organization and timely regulation of cytokinesis by phosphorylating histone H3 (Hirota et al., 2005) and Cenp-A (Zeitlin et al., 2001), respectively. Therefore, the phosphorylation of histone H3 Ser10 (Hsu et al., 2000; Murnion et al., 2001) and Cenp-A Ser7 (Zeitlin et al., 2001) are well-established markers of Aurora B activity.

At the metaphase-anaphase transition, the CPC complexes transfer to central spindle microtubules and later to the equatorial cortex where cytokinesis occurs (Earnshaw and Cooke, 1991). The active phosphatase Cdc14 and Aurora B as well as diminished Cdk1 activity are associated with this relocalization process (Hummer and Mayer, 2009; Pereira and Schiebel, 2003; Xu et al., 2009). Furthermore, MKLP2 mediates the recruitment of the CPC to the central spindle (Gruneberg et al., 2004) and phosphorylation by Cdk1 opposes midzone localization of MKLP2 and the CPC (Hummer and Mayer, 2009). The antiparallel microtubule plus ends form the central spindle, which is the site of the CPC in late mitosis. Before cytokinesis, the contractile ring forms composed of actin, myosin and other cytoskeletal elements. The CPC that is localized at the midbody has an essential role in the regulation of cleavage furrow ingression and abscission (van der Waal et al., 2012). The CPC regulates the contractile ring via indirect control of RhoA, a small GTPase that promotes actin polymerization and myosin II activation. The CPC targets centralspindlin to the midzone (Kaitna et al., 2000), which promotes recruitment of the RhoGEF (guanine nucleotide exchange factor) ECT2 (Epithelial cell-transforming 2) to the equatorial cortex (Nishimura and Yonemura, 2006; Su et al., 2011; Yuce et al., 2005). In addition, Aurora B

phosphorylates a component of the centralspindlin complex called the Rho GTPase activating protein (MgcRacGAP) (Mishima et al., 2002). The phosphorylation induces the RhoGAP activity of MgcRacGAP (Minoshima et al., 2003; Toure et al., 2008). The other component of the centralspindlin complex, the kinesin MKLP1, is also phosphorylated by Aurora B, which induces centralspindlin clustering and stabilizes the central spindle (Douglas et al., 2010). Cytokinesis is completed by abscission when the membranes fuse to separate daughter cells. Aurora B has been proposed to delay abscission in the case when there are lagging chromosomes, thereby protecting the cell from polyploidization (Steigemann et al., 2009).

2.6. MicroRNAs (MiRNAs)

2.6.1. Discovery of miRNAs

MiRNAs are small 18-25 nucleotide regulatory RNA molecules regulating gene expression post-transcriptionally (reviewed in Ameres and Zamore, 2013; He and Hannon, 2004; Krol et al., 2010). The first miRNA discovered was lin-4, which was identified in *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993) in 1993. Four different larval stages (L1-L4) can be distinguished in *Caenorhabditis elegans*, and mutations of *lin-4* evoke defects in the temporal regulation of larval development (Chalfie et al., 1981). *Lin-4* encodes a 22-nucleotide non-coding RNA that negatively regulates the protein LIN-14, which initiates the development of the second larval stage (Lee et al., 1993). The negative regulation requires the presence of intact 3'UTR (untranslated region) of LIN-14 mRNA and a functional *lin-4* gene (Lee et al., 1993; Wightman et al., 1991), and thus lin-4 RNA was found to target LIN-14 mRNA (Wightman et al., 1993). In addition, lin-4 was found to negatively regulate the translation of LIN-28, a protein that initiates the development from the L2 to the L3 stage (Moss et al., 1997). The second miRNA identified was let-7, which was discovered almost seven years after the identification of lin-4. Let-7 was also discovered in worms (Pasquinelli et al., 2000; Reinhart et al., 2000). Its gene encodes a 21-nucleotide RNA that regulates the development from the L4 stage to the adult stage (Reinhart et al., 2000).

Today, the repository for miRNA sequences and annotation, miRBase (http://www.mirbase.org/, Kozomara and Griffiths-Jones, 2014) lists a total of 28645 mature miRNAs found in several organisms. In humans, 1881 precursor miRNAs and 2588 mature miRNAs are listed (29.1.2015). Furthermore, miRNAs have been reported to target several proteins and affect important signaling pathways in normal and pathologically relevant conditions. MiRNAs

have emerged as important regulatory units in different organisms (reviewed in Ameres and Zamore, 2013). However, miRNAs have not been found in fungi. Furthermore, the plant and animal miRNA production differs from each other suggesting that there have been at least two development lines for miRNAs during evolution (Jones-Rhoades et al., 2006). In plants, the development happened before multicellularity occurred as also unicellular algae *Chlamydomonas reinhardtii* produces miRNAs (Molnar et al., 2007). On the contrary, in animals the emergence of miRNAs seems to correlate with the development of multicellularity as small silencing RNAs are present in the earliest diverging extant lineage of animal life, the sponge *Amphimedon queenslandica* (Grimson et al., 2008), and have not been found in single cell organisms. Interestingly, viruses also seem to possess miRNAs.

2.6.2. MiRNA genomics

Animal miRNAs are evolutionarily conserved and mammalian miRNA genes also have multiple isoforms, paralogs, thought to be generated by duplication (Di Leva et al., 2014). The sequences of paralogs are similar with minor nucleotide differences and these paralogs form a family of miRNAs. The family members share the "seed sequence", which is composed of nucleotides 2-8 at the 5' end of the miRNAs. The family members are often implicated in the regulation of similar physiological functions. MiRNAs can be transcribed as a single transcript or as a polycistronic precursor that releases many mature miRNAs after the processing steps (Di Leva et al., 2014). Furthermore, miRNAs can be intergenic or intragenic depending on their position in the genome. Intergenic miRNAs are located between genes whereas intragenic miRNAs lie either within introns or exons of genes (Di Leva et al., 2014). The miRNA and its host gene can share the same promoter and therefore can be transcribed at the same time (Baskerville and Bartel, 2005; Rodriguez et al., 2004).

The nomenclature of miRNAs can be rather complex (Ambros et al., 2003). Family members with closely related mature sequences are separated by letters, for example let-7a, let-7b and let-7c. Identical mature miRNAs can be generated from distinct genomic loci and precursor sequences. In this case, the mature miRNAs are written for example let-7a-1 and let-7a-2. Moreover, pre-miRNA loops can release two different mature miRNAs, one from each side of the loop. The predominant product is usually called miRNA (for example miR-56) and the less dominant product is marked with an asterisk, miRNA* (for example miR-56*). However, miRNA* does not always have to be a by-product and can also be selected as an active strand (Ghildiyal et al., 2010;

Okamura et al., 2009). If the predominance is not known the miRNAs can be named after the arm of the loop from which they are generated, for example miR-378a-3p and miR-378a-5p.

2.6.3. MiRNA biogenesis

MiRNAs are processed to become mature miRNAs in several phases (Fig. 10; reviewed in Ameres and Zamore, 2013; Inui et al., 2010; Krol et al., 2010). MiRNA biosynthesis starts when miRNAs are transcribed as primary transcripts (pri-miRNAs) by RNA polymerase II. However, some miRNAs that are associated with Alu repeats are transcribed by RNA polymerase III (Borchert et al., 2006). The hairpin structure of a pri-miRNA is recognized by the microprocessor complex, which contains the RNase III type endonuclease Drosha and DGCR8 in vertebrates (known as Pasha in *Drosophila melanogaster*). They create a 70-nucleotide stem loop called the precursor miRNA (pre-miRNA) that is exported to the cytoplasm by Exportin-5. Some miRNAs are produced from introns as a result of splicing and debranching and are therefore called mirtrons. They bypass editing by Drosha and DGCR8. The processing continues in the cytoplasm where the pre-miRNA is recognized by Dicer, an RNase III type endonuclease, bound to Transactivation-response (TAR) RNA-binding protein (TRBP, also called TARBP2) and Protein kinase R-activating protein (PACT) (Chendrimada et al., 2005; Lee et al., 2006) in mammals. In *Drosophila melanogaster*, Dicer-1 binds two isoforms of the double stranded RNA (dsRNA) -binding protein Loquacious (Loqs-PA and Loqs-PB). They are needed for efficient miRNA processing because they increase the affinity of Dicer-1 for pre-miRNAs (Saito et al., 2005). Dicer-2 pairs with R2D2, which prevents Dicer-2 from processing pre-miRNAs. Instead, Dicer-2 processes long dsRNA substrates (Cenik et al., 2011). The pre-miRNA is cleaved by Dicer resulting in the formation of a mature miRNA duplex of about 20 nucleotides. In mammals, some miRNAs can have their 3' arm cleaved by Argonaute 2 (Ago2), which has RNaseH-like endonuclease activity. This adds an additional processing step prior to Dicer cleavage (Diederichs and Haber, 2007). Dicer processing of miRNAs can also be by-passed for example in the case with miR-451 as the pre-miR is too short to be recognized by Dicer. Instead, pre-miR-451 is directly processed by Ago2 in the RNA-induced silencing complex (RISC) (Cheloufi et al., 2010). Usually one of the strands of the duplex is selected as the biologically active miRNA (the guide strand or miRNA) and incorporated into the RISC, and the opposite strand (the passenger strand or miRNA*) is degraded. The active strand is usually the one with the less stably base-paired 5' end in the miRNA duplex.

2.6.4. Function of miRNAs

A single miRNA can target an average of more than 100 mRNAs (Di Leva et al., 2014). In turn, an mRNA can be targeted by several miRNAs. It has been predicted that more than 60 % of human protein-coding genes are targeted by miRNAs (Friedman et al., 2009). The mature miRNA associates with the RISC, which consists of Ago proteins, Glycine-tryptophan proteins of 182 kDa (GW182), and the miRNA. The amino-terminal part of GW182 interacts with Ago while the carboxy-terminal part interacts with Poly(A) binding protein (PABP) and recruits two deadenylases, CCR4 and CAF1 (Eulalio et al., 2009; Krol et al., 2010). The RISC recognizes target mRNAs via partial sequence complementarity between the miRNA and the target mRNA (reviewed in Ameres and Zamore, 2013; Krol et al., 2010). Generally, the seed sequence of the miRNA (nucleotides 2-8 at the 5' end), pairs with the 3'UTR of the mRNA. In some cases, miRNAs have been reported to bind 5'UTR or the coding region of the target mRNA (Forman et al., 2008; Kloosterman et al., 2004; Lytle et al., 2007). The RISC suppresses the expression of the target by either removing the polyA tail (deadenylation) followed by mRNA degradation, or by blocking translation at the initiation or elongation steps (Inui et al., 2010). Deadenylation occurs via modulation of the activity of deadenylases, such as CCR4 and CAF1. Translation blockage can be achieved for example by inhibiting Eukaryotic initiation factor 4E (EIF4E) or by inducing ribosome stalling followed by localization of RISC-bound mRNA to P-bodies, where it is stored or degraded. Sometimes RISC containing Ago2 binds to target mRNA with nearly perfect complementarity to miRNA, which leads to endonucleolytic cleavage of the mRNA and its degradation (Krol et al., 2010). This mechanism is rare in animals but common in plants (Voinnet, 2009).

The software used to predict miRNA targets typically consider conservation, the seed sequence, the number of sites in the target, and the context of the surrounding mRNA sequence in their predictions The software that base predictions on miRNA conservation are for example miRanda, PicTar, TargetScan, and Diana-microT. The PITA and rna22 algorithms consider the free energy of binding and secondary structures of 3'UTRs (Di Leva et al., 2014). The following two sections describe the two miRNAs that have been in the focus of the present studies.

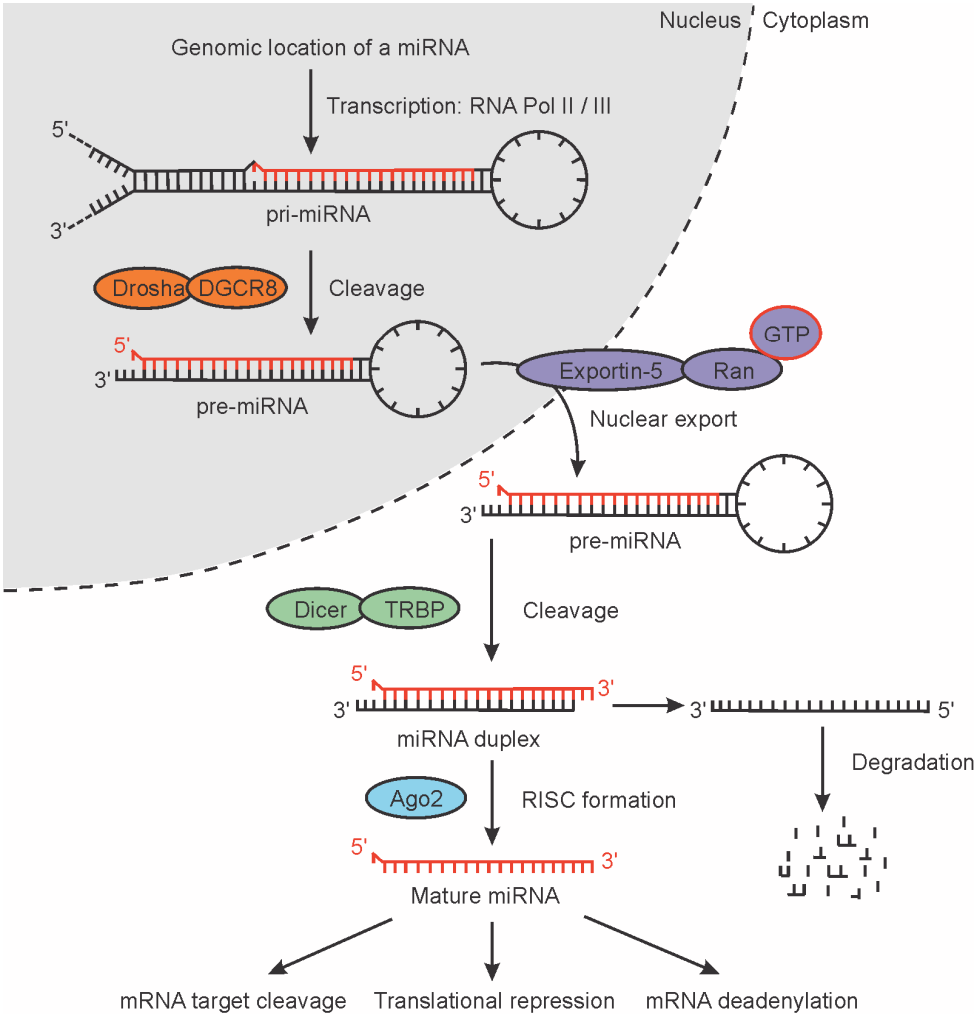

Figure 10. MiRNA biogenesis and function. Adapted from Winter et al. (2009).

2.6.5. MiR-378a-5p

MiR-378a-5p is located on chromosome 5 in an intron of *PPARGC1B* (*PGC-1β, PERC*), which is a co-activator of several transcription factors such as the Estrogen receptor-α (ER; Kressler et al., 2002) and the Estrogen receptor-related receptor (ERR; Kamei et al., 2003). MiR-378a-5p is co-transcribed with its host gene and has been associated with cell survival, tumor growth and angiogenesis due to direct targeting of SuFu and Fus-1, two proteins that repress the transcription factor Gli-1 (Lee et al., 2007). Gli-1 stimulates the transcription of angiogenesis related Vascular endothelial growth factor A (VEGF-A) when not repressed. Moreover, miR-378a-5p also directly targets

VEGF-A and thus competes with miR-125a for binding to VEGFA, which results in an increase in the expression of VEGF-A (Hua et al. 2006). In breast cancer cells, miR-378a-5p promotes the metabolic shift from oxidative to glycolytic metabolism by targeting ERRγ and α-subunit of GA binding protein transcription factor (GABPA), which are both binding partners of PGC-1β coded by the host gene of the miRNA (Eichner et al., 2010). This metabolic shift is a characteristic of cancer cells, and in line with this, miR-378a-5p expression increases while breast cancer progresses (Eichner et al., 2010). It has been suggested that in the future miR-378a-5p levels in a patient's serum could be used as a biomarker for gastric cancer (Liu et al., 2012b). For other cancer types, the possible use of miR-378a-5p as a biomarker has yet to be tested or is controversial as in case of renal carcinoma (Hauser et al., 2012; Redova et al., 2012).

2.6.6. Let-7b

Let-7 family members are some of the most widely studied miRNAs and were first discovered to control developmental timing in *Caenorhabditis elegans* (Pasquinelli et al., 2000; Reinhart et al., 2000). The miRNA family consists of 10 different gene isoforms (let-7a, b, c, d, e, f, g, i, miR-98, miR-202) coded from 13 genomic loci in humans and is well conserved across the species. Let-7 miRNAs are considered to be tumor suppressor miRNAs as they target and suppress many known oncogenes such as *RAS* (Johnson et al., 2005) and *MYC* (Sampson et al., 2007). Other targets of let-7 miRNAs implicated in tumorigenesis are *HMGA2*, *IMP-1*, *Dicer*, *CDC34*, *IL-6*, *E2F2*, *CCND2*, *Bcl-XL*, *ZEB1*, and *ZEB2* (Di Leva et al., 2014). In xenograft models and in cells, let-7 family members have been shown to suppress cancer development and proliferation (Esquela-Kerscher et al., 2008; Johnson et al., 2007; Johnson et al., 2005; Kumar et al., 2008). Altered expression of let-7 miRNAs can lead to tumorigenesis and cancer metastasis (Johnson et al., 2007; Yu et al., 2007). In patients, low expression of let-7 miRNAs is linked to poor prognosis in lung and ovarian cancer (Nam et al., 2008; Takamizawa et al., 2004). Moreover, expression of let-7a, let-7b, and let-7g has been found to be reduced in breast cancer patients with lymph node metastasis when compared to patients without lymph node metastasis (Hu et al., 2013). However, in some lymphomas upregulated let-7 has been associated with cancer progression (Lawrie et al., 2009).

Mature let-7 miRNAs are produced abundantly in normal and differentiated cells whereas in cancer cells and non-differentiated cells their production is decreased due to diminished miRNA processing (Thomson et al., 2006). Protein

LIN28 is an important post transcriptional regulator of let-7 miRNAs. It interacts with pri-let-7 in the nucleus and blocks Drosha processing of the miRNA (Viswanathan et al., 2008). Furthermore, LIN28 can bind pre-let-7 in the cytoplasm and induce uridylation of the miRNA by recruiting the terminal uridyl (U) transferase (TUT) Zcchc11 (Hagan et al., 2009). The poly(U) tail at the 3' end of the pre-miRNA blocks Dicer processing and directs the miRNA for degradation. The expression of LIN28 is inversely correlated with mature let-7 expression during development (Wu and Belasco, 2005) and tumorigenesis (Thornton and Gregory, 2012). In addition, let-7 miRNAs also regulate LIN28 as let-7 binds to LIN28 mRNA repressing its expression (Viswanathan and Daley, 2010), which creates a positive feedback loop.

2.7. Cancer – defects in mitosis and miRNA expression

2.7.1. Aneuploidy and chromosomal instability (CIN) in cancer

Cancer is characterized by excessive and uncontrolled cell proliferation, which occurs due to defects in important signaling pathways, for example those involved in cell viability, apoptosis and cell cycle progression. Tumorigenesis is accompanied by genomic imbalance, which is an enabling factor of cancer (Hanahan and Weinberg, 2011), including numerical and structural chromosome abnormalities. Structural chromosomal rearrangements contain deletions, insertions, duplications, translocations and inversions. They can activate oncogenes or inactivate tumor suppressors. Numerical chromosome abnormalities refer to changes in the whole chromosome number of a cell. The state of an abnormal chromosome number is called aneuploidy (Gordon et al., 2012). An aneuploid cell has either more or fewer chromosomes than a diploid cell. It is important to distinguish this concept from chromosomal instability (CIN), which refers to the rate of karyotypic changes including mis-segregation of whole chromosomes or parts of chromosomes. CIN and aneuploidy are connected as CIN can lead to aneuploidy. However, not all aneuploid cells exhibit CIN. Aneuploidy seems to have a dual role in tumorigenesis as it can either drive cancer or suppress it, depending on the level of aneuploidy (Weaver et al., 2007; Weaver et al., 2008). It has been hypothesized that low levels of aneuploidy can promote tumorigenesis whereas high levels of aneuploidy induce cell death of cancer cells.

Defects in several signaling pathways have been implicated in increased CIN. The mechanisms promoting CIN can be divided into four main categories (Fig. 11): (I) defects in kinetochore-microtubule attachments, (II) multipolarity and abnormal centrosome number, (III) defects in SAC signaling and (IV) defects in

chromosome cohesion (Gordon et al., 2012; Kops et al., 2005). In cancer cells, merotelic attachments between kinetochores and microtubules occur frequently and can lead to chromosome missegregation (Cimini et al., 2001; Cimini, 2008; Compton, 2011). Merotelic attachments can be caused by chromatin alterations induced by the inhibition of histone deacetylases and loosely organized centromeric chromatin (Cimini et al., 2003), condensin defects (Samoshkin et al., 2009), cohesion defects (Loncarek et al., 2007), multipolarity and hyperstable kinetochore-microtubule attachments (Bakhoum et al., 2009a; Ganem et al., 2009; Gregan et al., 2011; Silkworth et al., 2009). The cell will strive to correct merotelic attachments and thus erroneous connections need to be released. If this process is prevented, the merotelic attachments may persist leading to chromosome missegregation (Bakhoum et al., 2009a). Sister chromatids might segregate towards the same pole or a chromosome can lag behind and be excluded from both daughter cells as the cell divides. Both cases will result in aneuploidy. Kinetochore-microtubule attachments seem to be more stable in cancer cell lines with CIN compared to non-cancerous diploid cell lines (Bakhoum et al., 2009a), which makes the error correction more difficult in cancer cells. Manipulating the stability of kinetochore-microtubule attachments can alter the rate of CIN (Bakhoum et al., 2009b). Importantly, the SAC does not detect merotelic attachments because there is still interkinetochore tension existing in this type of anomaly. Destabilizing erroneous attachments by Aurora B kinase has been proposed as a primary correction mechanism of merotelic attachments (Cimini et al., 2006). Attachment to opposite poles can bring Aurora B substrates into the region of high Aurora B activity regardless of interkinetochore tension and thereby enable destabilization of merotelic attachments (Gregan et al., 2011).

The extent of centrosome amplification in tumors strongly correlates with CIN (Nigg, 2006; Pihan et al., 2003). Already the classical experiments by Theodor Boveri showed that extra centrosomes induce multipolar cell division and chromosome segregation errors (Boveri, 1929; Holland and Cleveland, 2009). However, multipolar cell divisions are rare and usually the progeny cells are not viable. Cancer cells tend to cluster their multiple centrosomes to form a pseudo-bipolar spindle (Brinkley, 2001; Ganem et al., 2009; Silkworth et al., 2009). This increases the cell survival of the progeny cells.

A defective SAC may not be able to arrest cells in mitosis leading to CIN and aneuploidy (Rajagopalan et al., 2003). A cell with the compromised SAC can exit from mitosis prematurely with unaligned chromosomes producing aneuploid daughter cells or proceed without completion of cytokinesis resulting in the formation of a polyploid cell. Polyploidization might also result from cell-cell fusion, endoreduplication, or defects in duplication, maturation or

segregation of centrosomes (Nigg, 2002; Storchova and Pellman, 2004). The altered expression or activity of the SAC related proteins is known to induce CIN and aneuploidy (Hauf et al., 2003; Sotillo et al., 2007). In mice, the combined overexpression of proteins Mad2 and KRAS has resulted in a larger scale lung tumor formation compared to mice with only KRAS overexpression (Sotillo et al., 2007; Sotillo et al., 2010). In addition, aberrant expression or altered kinase activity of Aurora B can induce polyploidy and aneuploidy (Hauf et al., 2003; Honda et al., 2003; Kallio et al., 2002; Ota et al., 2002; Terada et al., 1998). Aurora B is commonly overexpressed in cancers (Carter et al., 2006) and its overexpression correlates with poor prognosis in patients (Kurai et al., 2005).

Cohesion defects are one of the causes of aneuploidy. Inactivation of the yeast homologues of Securin and Separase has resulted in chromosome loss (McGrew et al., 1992; Uzawa et al., 1990; Yamamoto et al., 1996), and in human cancer cells, CIN is increased when Securin is inactivated by homologous recombination (Jallepalli et al., 2001). Inactivation of Stromal antigen 2 (*STAG2*) results in defective sister chromatid cohesion and increased aneuploidy (Solomon et al., 2011). *STAG2* encodes one of the two human orthologs of the yeast SCC3 Cohesin subunit. However, it should be noted that cohesion defects have a variety of cellular effects in addition to induction of aneuploidy. For example, cohesion also regulates transcription (Dorsett, 2011).

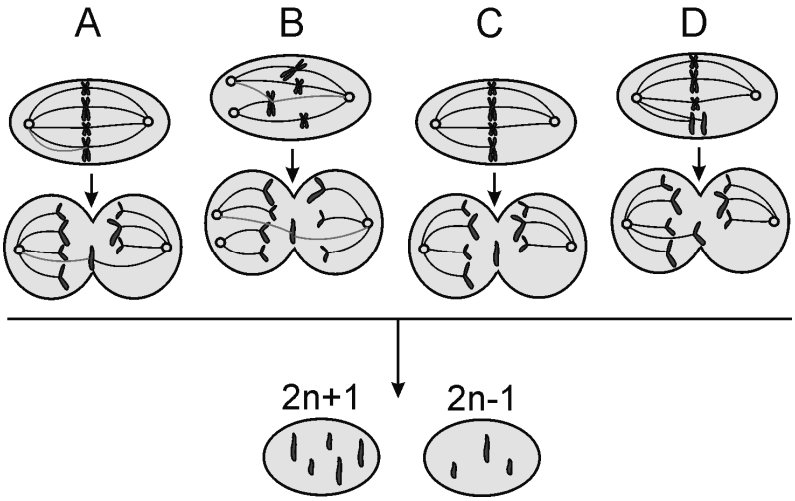

Figure 11. The mechanisms promoting CIN: defects in kinetochore-microtubule attachments (A), multipolarity (B), defects in SAC signaling (C) and defects in chromosome cohesion (D). Modified from Gordon et al. (2012).

2.7.2. Mitotic cancer therapies

Mitosis is one of the most successful targets in cancer therapy. The traditional microtubule drugs, such as the vinca-alkaloids and taxanes, interfere with microtubules and thus inhibit normal spindle formation in mitosis. High drug concentrations either stabilize (taxanes and epothilones) or depolymerize (vinca alkaloids) microtubules. Low drug concentrations interfere with microtubule dynamics (Panda et al., 1996; Yvon et al., 1999). The microtubule drugs induce SAC-dependent mitotic arrest leading to cell death in the M phase or slippage from mitosis with abnormal chromosome segregation and cytokinesis defects. Unfortunately, the development of resistance to microtubule poisons is encountered in the clinics due to mutations and altered expression of tubulin isotypes and drug efflux pumps (reviewed in Chan et al., 2012a; Kavallaris, 2010). Furthermore, microtubule drugs affect not only cancer cells but also normal non-tumorigenic cells and therefore cause harmful side effects, such as neurotoxicity (Jordan and Wilson, 2004). It is hoped that new anti-mitotic therapies will be able to overcome the problems associated with microtubule-targeting drugs. Mitotic proteins are putative targets for novel drugs as their targeting may increase the cancer cell specificity of the therapy based on high proliferation rate of cancer cells. Moreover, many mitotic proteins are found to be overexpressed in tumors, which may make the cancer cells more vulnerable for their suppression. In addition, aneuploid cancer cells might be more sensitive to the subsequent genetic imbalances induced by anti-mitotic drugs (Janssen and Medema, 2011).

Many mitotic proteins are being evaluated for their potential as cancer drug targets (reviewed in Janssen and Medema, 2011; Salmela and Kallio, 2013). These proteins include mitotic kinases Plk1 and Aurora kinases A and B as well as motor proteins such as Cenp-E and Eg5. Moreover, indirect inhibition of microtubules could be achieved by targeting Hec1, which mediates the connection between microtubules and kinetochores (McCleland et al., 2004; see also section 2.3.1.1.). The modes of action of drugs under development rely on either induction of cell death of mitotic cells or mitotic slippage that can increase the rate of CIN and aneuploidy beyond the tolerance of cancer cells (Fig. 12). An aberrant exit from the M phase, also called forced mitotic exit, can be caused by decreased SAC activity and/or loss of Cdk1 activity, both of which drive cells out of the M phase potentially leading to changes in the chromosome numbers of the daughter cells. Alternatively, in the case of cytokinesis failure, the progeny cell will be tetraploid. After forced exit from mitosis, cells can undergo cell death, senescence, or endoreduplication (Keen and Taylor, 2009). Unfortunately, despite intense efforts into developing novel mitosis-targeting cancer drugs, many of the LMW compounds that have

advanced to clinical trials have failed either due to poor efficacy or excessive cytotoxicity (Salmela and Kallio, 2013).

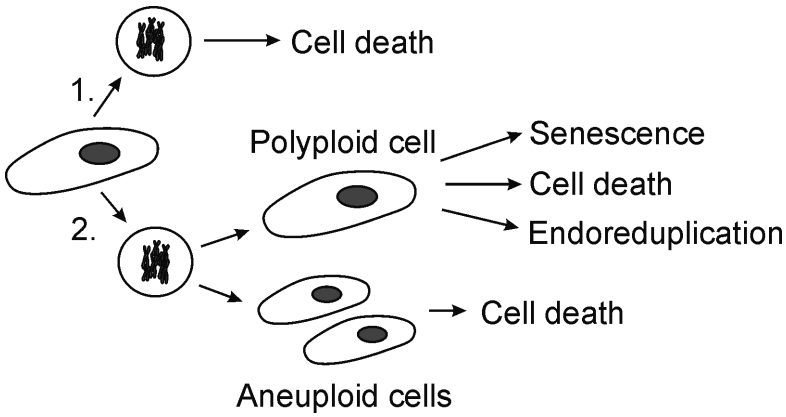

Figure 12. The mode of action of new anti-mitotic drugs under development. The drugs can induce cell death of mitotic cells (1) or mitotic exit (2) that can increase the rate of genomic instability followed by cell death or cell cycle arrest.

Inhibition of mitotic proteins can result in anomalies of the mitotic spindle, depending on the target protein. For example, suppression of Eg5, Plk1 and Aurora A induces the formation of monopolar spindles because the proteins are needed for normal centrosome maturation and separation (Barr et al., 2004; Blangy et al., 1995; Carmena and Earnshaw, 2003). This defect typically leads to mitotic arrest. On the other hand, inhibition of Aurora B and Mps1 overrides the SAC, causing forced mitotic exit (Colombo et al., 2010; Hauf et al., 2003). Moreover, Cenp-E inhibition induces defective chromosome congression resulting in the SAC-dependent mitotic arrest in human cells (Ding et al., 2010; Henderson et al., 2009; Wood et al., 2010).

Plk1 is often overexpressed in cancer, and its inhibition has been shown to decrease the viability of cancer cells (Liu and Erikson, 2003), making Plk1 an interesting drug target (Takai et al., 2005). Many Plk1 kinase inhibitors, such as BI2536 (Ellis et al., 2013), its analogue BI6727 (Rudolph et al., 2009) and GSK461364A (Gilmartin et al., 2009) block the ATP-binding site abrogating the catalytic function of the kinase. In addition, Plk1 has a PBD that binds phosphopeptides in substrates and affects the subcellular localization of the kinase (Lee et al., 1998). PBD-binding Plk1 inhibitors include natural products thymoquinone and purpurogallin as well as a synthetic thymoquinone derivative, poloxin (Reindl et al., 2008; Watanabe et al., 2009). Rigosertib (ON01910.Na) was first described as a Plk1 inhibitor by its developer,

Onconova, but is currently acknowledged to be a multikinase inhibitor affecting Phosphoinositide 3-kinase (PI3K), Plk1 and the MAPK pathways (Chapman et al., 2012; Gumireddy et al., 2005; Prasad et al., 2009). However, the mechanism of action of rigosertib has remained controversial (Lan et al., 2012; Prasad et al., 2009; Steegmaier et al., 2007; Strebhardt, 2010). The compound is currently undergoing phase II and III clinical trials for the treatment of solid tumors and blood malignancies (Jimeno et al., 2008; Ma et al., 2012; Seetharam et al., 2012). Rigosertib induces multipolarity and mitotic arrest, and decreases cell viability in a panel of cancer cell lines (Gumireddy et al., 2005). In addition, cancer cells appear to be more sensitive to the compound when compared to non-tumorigenic cells. Finally, tumor growth was suppressed by rigosertib in xenograft mouse models (Gumireddy et al., 2005).

Aurora A kinase regulates centrosome maturation, separation and spindle assembly (Vader and Lens, 2008). The inhibition of Aurora A induces defects in the spindle poles and chromosome congression (Manfredi et al., 2007). Many Aurora kinase inhibitors target both Aurora A and Aurora B (Salmela and Kallio, 2013). More specific towards Aurora A are compounds MLN8054 (Manfredi et al., 2007), MK-5108 (Shimomura et al., 2010), and ENMD-2076 (How and Yee, 2012). The inhibition of Aurora B induces defects in SAC signaling, chromosome misalignment and perturbation of cytokinesis (Hauf et al., 2003), which often result in a premature exit from mitosis without cytokinesis. Aurora B inhibitors include AZD1152 (Mortlock et al., 2007), ZM447439 (Ditchfield et al., 2003), hesperadin (Hauf et al., 2003), and barasertib (Dennis et al., 2012; Kantarjian et al., 2013; Kantarjian et al., 2013; Marxer et al., 2014; Schwartz et al., 2013). Furthermore, for example VX-680 (Harrington et al., 2004) and PHA-739358 are dual-inhibitors of Aurora A and B (Carpinelli et al., 2007).

Mps1 has a role in error correction of incorrect kinetochore-microtubule attachments (Jelluma et al., 2008) and SAC signaling (Stucke et al., 2002). Mps1 localizes to kinetochores and exhibits its highest activity during mitosis (Stucke et al., 2002). Mps1 inhibitors include cincreasin (Dorer et al., 2005), SP600125 (Schmidt et al., 2005), AZ3146 (Hewitt et al., 2010), MPI-0479605 (Tardif et al., 2011), NMS-P715 (Colombo et al., 2010) and reversine (Santaguida et al., 2010). Inhibition of Mps1 kinase overrides the SAC and thus increases aneuploidy (Colombo et al., 2010; Tardif et al., 2011).

Mitotic kinesins, such as Eg5 and Cenp-E, function in spindle assembly, chromosome congression, and SAC signaling (Abrieu et al., 2000; Blangy et al., 1995; Kapoor et al., 2000; Khodjakov et al., 2003; Lombillo et al., 1995; Sawin et al., 1992; Yao et al., 2000). Kinesin inhibitors either affect the motor

activity of kinesins or stabilize the binding of kinesins to microtubules (Salmela and Kallio, 2013). Eg5 regulates centrosome separation, and thus its inhibition leads to monopolarity and mitotic arrest (Blangy et al., 1995). There are several Eg5 inhibitors, for example SB-715992 (Purcell et al., 2010) and its analogue SB-743921 (Holen et al., 2011). Cenp-E inhibition induces defects in chromosome segregation and SAC signaling (Wood et al., 2008). Syntelin (Ding et al., 2010), UA62784 (Henderson et al., 2009) and GSK923295 (Wood et al., 2010) have been reported to inhibit Cenp-E.

2.7.3. MiRNAs and cancer

The expression of miRNAs is altered in cancer and the expression profiles can be associated with clinicopathological features of tumors such as grade, stage and vascular invasion, and also with features of the patient such as sex and age (Calin and Croce, 2006). Most miRNAs are more downregulated in cancer cells than in normal tissue, and this phenomenon is associated with the loss of differentiation in tumors (Lu et al., 2005). In addition, monoallelic loss of *Dicer1* resulting in global deletion of miRNAs promotes tumorigenesis, as shown in a mouse model of retinoblastoma (Lambertz et al., 2010). However, there are also miRNAs termed oncomiRs that are upregulated in cancer and can promote the development of malignancies. On the other hand, tumor suppressor miRNAs, which are often downregulated in cancer, prevent cancer formation and progression. For example, transgenic expression of oncomiR miR-21 (Medina et al., 2010) can contribute to the development of lymphoma in mice and an excess of tumor suppressor miRNA let-7 can inhibit tumor progression (Trang et al., 2010). In addition, a number of miRNAs have been reported to induce resistance to anticancer therapies (Blower et al., 2008; Miller et al., 2008; Schetter et al., 2008; Zhou et al., 2010). A few of these miRNAs will be discussed in the next section.

Cancer is characterized by chromosomal rearrangements and mutations such as deletions and amplifications. More than half of the miRNA genes are located in those genomic regions frequently altered in cancer such as amplified regions, fragile sites, as well as in common sites for sister chromatid exchange, translocation, deletion and tumor-associated viral integration (Calin et al., 2004). For example, the region coding for miR-15a and miR-16-1 is the most frequently deleted region in human chronic lymphocytic leukemia (CLL; Calin et al., 2002). In addition to genomic loss, transcription of a tumor suppressor miRNA can be repressed by the loss of transcription factors. On the other hand, overexpression of oncomiRs can occur by activation of their transcription factors (Di Leva et al., 2014). Epigenetic alterations such as DNA

hypermethylation of tumor suppressors, DNA hypomethylation, and alterations in histone modifications are common in cancer cells and can contribute to miRNA dysregulation (Fabbri et al., 2013; Lopez-Serra and Esteller, 2012).

In cancer, 3'UTRs of mRNAs are often shortened by alternative polyadenylation site choice (Mayr and Bartel, 2009). This can contribute to loss of gene regulation by a miRNA and thereby have an impact on gene expression. Accordingly, the miRNA binding site in the mRNA can be mutated or become inaccessible, preventing miRNA-mediated regulation (Di Leva et al., 2014). Mutations in the enzymes responsible for biogenesis of miRNAs can also contribute to miRNA dysregulation in cancer. For example, in 39 % of patients with ovarian cancer, Dicer and Drosha are reported to be decreased by 60 % and 51 %, respectively (Merritt et al., 2008). Moreover, one copy of *Dicer1* has been found to be frequently deleted in retinoblastoma as stated earlier (Lambertz et al., 2010).

The role of miRNAs in mitosis is starting to emerge. Several miRNAs have been reported to participate in the control of cell cycle progression by targeting Cyclins, Cdks, phosphatases, and transcription factors (Bueno and Malumbres, 2011). For example, MiR-210 has been reported to target cell cycle regulators Plk1, Cdc25B, Cyclin F, Bub1, and Fam83D (He et al., 2013). An excess of miR-210 induces a delay of mitotic exit, chromosome misalignment and aberrant chromosome segregation. The variety of phenotypes induced by excess miR-210 is probably due to the multiple targets of the miRNA. Moreover, miR-210 has reduced tumor formation in a mouse metastatic tumor model (He et al., 2013). MiR-125b overexpression delays mitotic exit and induces chromosomal abnormalities and cell death by targeting Mad1 (Bhattacharjya et al., 2013). The cells undergo forced mitotic exit from nocodazole block. Finally, Mad1 expression is inversely correlated with miR-125b expression in head and neck tumors (Bhattacharjya et al., 2013).

2.7.4. MiRNA-based future cancer diagnostics and therapies

The altered expression of miRNAs in cancer suggests that miRNAs possess both diagnostic and prognostic value as biomarkers (Iorio and Croce, 2012). For example, in ductal adenocarcinoma, overexpression of miR-205 and miR-21 predicts phenotype changes characteristic of malignancies, which supports the use of miRNA expression profiling in early diagnostics (du Rieu et al., 2010). Moreover, different subtypes of cancer can be distinguished by their miRNA expression profiles. MiRNAs are differentially expressed for example in basal and luminal breast cancer subtypes (Blenkiron et al., 2007). Distinctive

miRNA expression can also indicate the ER, progesterone receptor (PR) and Human epidermal growth factor receptor 2 (HER2) status of tumors (Lowery et al., 2009). Poorly differentiated tumors can be classified by their miRNA expression signatures with better accuracy than can be obtained by their mRNA profiles and their tissue of origin can be identified (Lu et al., 2005; Rosenfeld et al., 2008). Furthermore, miRNA expression profiles can predict survival. Low expression of let-7 and high expression of miR-155 have been reported to correlate with poor prognosis in lung cancer patients (Takamizawa et al., 2004; Yanaihara et al., 2006). MiRNA expression profiling could also be utilized to plan personalized treatment strategies for patients. For example, high expression of miR-125b has been reported to predict a poor response to taxol-based treatments in breast cancer cells (Zhou et al., 2010), and high miR-21 expression is associated with a poor response to chemotherapy in adenocarcinomas (Schetter et al., 2008). In addition, overexpression of miR-221 and miR-222 is associated with resistance to anti-estrogenic therapies such as tamoxifen (Miller et al., 2008).

MiRNAs are abundant in body fluids and could therefore serve as non-invasive biomarkers. For example, they can be extracted and detected from blood (Mitchell et al., 2008), urine (Hanke et al., 2010), and saliva (Michael et al., 2010). MiRNAs can circulate in the body packed into exosomes (Taylor and Gercel-Taylor, 2008) or microvesicles, and can be taken up by the recipient cells via endocytosis. Exosomes are small (30-100 nm) and microvesicles larger (100-1000 nm) cell-derived vesicles (Etheridge et al., 2011) that are important for intercellular communication. Additionally, miRNAs can be released from a cell by passive leakage in the case of injury, inflammation, apoptosis, or necrosis (Ling et al., 2013).

Regardless of the encouraging results supporting the use of miRNAs as biomarkers, there still remain technical challenges to be tackled. Accurate diagnoses require more accurate methods to assess miRNA expression patterns in patients. The results from different studies are not always consistent, which is probably due to differences in sample preparation, experimental design, and analysis of the data (Xu and Wong, 2010).

In addition to the diagnostic and prognostic use of miRNAs, therapeutic approaches based on miRNAs are already being developed. The miRNA-based therapeutic approaches exploit two main strategies (Fig. 13). The first strategy is to inhibit the endogenous onco-miRNAs using miRNA antagonists such as antimiRs, locked nucleic acids (LNAs) or antagomiRs (Di Leva et al., 2014; Krutzfeldt et al., 2007). They are single-stranded RNA molecules about 21-23 nucleotides long and inhibit their target miRNA by complementary base-

pairing. The miRNA antagonists have been chemically modified to improve their binding affinities, nuclease resistance and cellular uptake (Krutzfeldt et al., 2007). In order to increase their nuclease resistance, antimiRs have phosphorothioate backbone linkages, which promote plasma protein binding to reduce clearance of the antimiRs in glomerular filtration and by urinary excretion. This eases the delivery of the antimiRs to their target sites *in vivo* (Krutzfeldt et al., 2005; Levin, 1999). AntimiRs that contain cholesterol are called antagomiRs. They are fully complementary to the miRNA sequence and contain several phosphorothioate parts to increase their stability. Also, additional high-affinity 2' sugar modifications such as 2'-O-methoxyethyl (2'-MOE), 2'-O-methyl, or LNAs improve nuclease resistance and increase affinity and melting temperature (van Rooij and Kauppinen, 2014). For example, the LNA-modified antimiR-122, named miravirsen by Santaris Pharma, has been tested successfully in nonhuman primates (Lanford et al., 2010; Lindow and Kauppinen, 2012) and is now in phase II clinical trials for the treatment of Hepatitis C virus (HCV) infection. MiR-122 is necessary for HCV RNA accumulation in liver cells. Chronically infected chimpanzees were treated with the LNA-modified antimiR, which led to suppression of hepatitis C viremia. Moreover, the chimpanzees did not have evident side effects. MiRNAs can be inhibited also by using miRNA sponges, which are RNA molecules expressed from strong promoters containing many binding sites to the miRNA of interest. Therefore, a miRNA sponge competes with the target mRNA for miRNA binding and inhibits miRNA function in cells (Ebert and Sharp, 2010).

In the second miRNA-based therapeutic strategy, it is hoped that the reintroduction of a synthetic double-stranded tumor suppressor miRNA mimic or viral vector-based overexpression of the miRNA will be able to enhance the function of the endogenous miRNA, expression of which is reduced or lost (Bader et al., 2010; van Rooij and Kauppinen, 2014). Chemical modifications of synthetic miRNAs are used to achieve better stability and improved cellular uptake (van Rooij and Kauppinen, 2014). The guide (antisense) strand of the mimic is identical to the miRNA of interest while the passenger (sense) strand is less stable and can be linked to cholesterol to enhance its cellular uptake (van Rooij and Kauppinen, 2014). It might also contain further chemical modifications to prevent it from RISC loading. The 2'-fluoro modification of the guide strand protects it from exonucleases, increasing the stability of the mimic (Chiu and Rana, 2003). The putative risks of the miRNA mimics include RISC saturation (Grimm et al., 2006) and possible unwanted side effects resulting from the miRNA expression in tissues where it is not normally expressed. However, studies conducted in mice investigating the delivery of tumor suppressor miRNAs have not revealed induction of adverse effects (Kota et al., 2009; Wiggins et al., 2010). The expression of a miRNA can be

introduced also by lenti-, adeno- and adeno-associated viruses, which has been reported by several studies (Kota et al., 2009; Miyazaki et al., 2012; Trang et al., 2010). Tissue-specific promoters increase the tissue-specificity of miRNAs (van Rooij and Kauppinen, 2014). In addition to oligo- and vector-based approaches, small molecules have been used to both inhibit specific miRNAs and to restore miRNA levels by enhancing miRNA biogenesis (Ling et al., 2013).

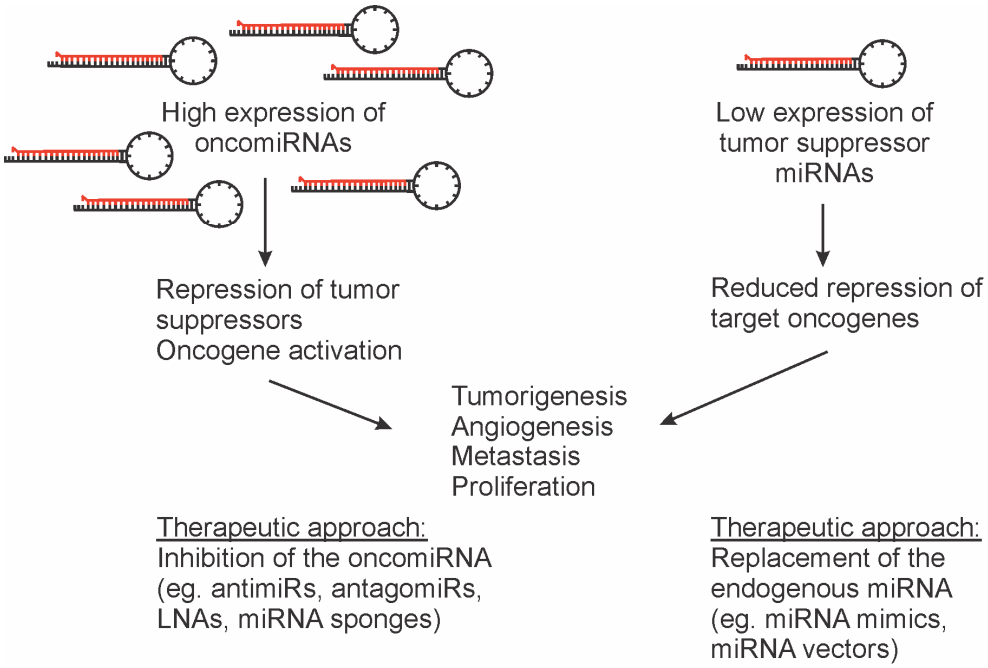

Figure 13. MiRNA expression in cancer and therapeutic approaches.

The first miRNA-based therapy forms are in preclinical tests and in clinical trials. The antimiR against miR-122, being developed by Santaris Pharma, is in phase II studies for the treatment of HCV infection, and the miRNA mimic of miR-34 by Mirna Therapeutics is in phase I for the treatment of liver cancer (van Rooij and Kauppinen, 2014). However, the delivery of miRNA-based therapy still remains a major challenge. Intravenously and intratumorally injected miRNA mimics can be delivered with liposome nanoparticles (Pramanik et al., 2011), polyethyleneimine (Ibrahim et al., 2011), or atelocollagen (Takeshita et al., 2010), which have been used to reintroduce tumor suppressor miRNAs in mouse models. Liposome delivery facilitates cellular uptake and protects the construct (Wiggins et al., 2010). Two tumor suppressor miRNAs, let-7 and miR-34, both of which are low expressed in lung cancer, were reintroduced as mimics using neutral lipid emulsion in a mouse

model of non-small cell lung cancer. The systematic treatment led to a significant reduction in tumor size (Trang et al., 2011). It has also been suggested that exosomes could be used to deliver miRNAs to their target sites (Momen-Heravi et al., 2014).

Extensive preclinical studies will be needed to evaluate the appropriate levels of inhibition by antimiRs. Similarly, with miRNA replacement therapies, the safe and well tolerated expression of miRNA mimics will have to be assessed in order to prevent side effects. Furthermore, the long term effects of miRNA therapies still need to be thoroughly investigated to minimize the off-target and adverse effects. Achieving cell- and tissue-specificity of future miRNA-based therapies will be one major challenge to be tackled. One option to increase the specificity could be to link the nucleic acids to targeting molecules, such as antibodies and peptides. In the meantime, local injections could be administered to achieve targeted delivery. Indeed, it does seem likely that the disease-associated miRNAs and their modulation will have therapeutic potential in the future (van Rooij and Kauppinen, 2014). However, the identification of miRNA targets and understanding the mechanism of action of individual miRNAs are essential aims to fully assess the future therapeutic potency of miRNAs.

3. AIMS OF THE STUDY

The research conducted in this thesis aims to identify novel anti-mitotic LMW compounds and miRNAs that could benefit cancer drug development and discovery of predictive biomarkers of tumorigenesis, respectively. The results are expected to elucidate the mechanisms of action of the identified compounds and miRNAs.

Objective 1
The aim is to identify LMW compounds with a similar chemical interaction field as but a different molecular structure than the compound rigosertib. The hit compounds are expected to induce an anti-mitotic phenotype analogous to rigosertib.

Objective 2
The aim is to characterize the cellular effects and anti-cancer potency of a LMW compound Centmitor-1 and to compare it with the compound rigosertib (ON01910.Na).

Objective 3
The aim is to identify novel Hec1 inhibitors and to validate their mechanism of action.

Objective 4
The aim is to identify novel anti-mitotic miRNAs, to characterize their cellular effects and to validate their target genes whose perturbation can explain the observed mitotic defects.

Objective 5
The aim is to generate knowledge on miRNA-mediated signaling in cancer cell proliferation that can be used in basic research and later in diagnostics.

4. MATERIALS AND METHODS

Detailed description of materials and methods is available in the original publications (I-IV).

Methods

Method	*Used in*
3D image acquisition and analysis	II
3D organotypic cell culture	II
Anisotropy	II
Cell based high-throughput screen (HTS)	I, II, III, IV
Cell culture	I, II, III, IV
Cell cycle synchronization	III, IV
Clinical data analysis	III, IV
Cloning	III, IV
Determining of microtubule dynamicity	I
Enzyme-linked immunosorbent assay (ELISA)	III
Flow cytometry	I, III, IV
Fluorescence in situ hybridization (FISH)	III, IV
FRET for analysis of Plk1 kinase activity	I
Image acquisition and analysis	I, II, III, IV
Immunofluorescence	I, II, III, IV
In vitro tubulin polymerization assay	I
Live cell imaging	I, II, III, IV
Luciferase assays	III, IV
Measurement of EC_{50} values	II
Microscopy	I, II, III, IV
Recombinant protein production	II
RNA isolation and qRT-PCR	III, IV
RTK phosphorylation and kinase phosphorylation array	III
Site-directed mutagenesis	IV
Statistical analysis	I, II, III, IV
Total internal reflection fluorescence (TIRF) microscopy	II
Transfections	II, III, IV
Virtual HTS	I, II
Western blotting	I, II, III, IV

Cell lines

Cell line	*Description*	*Used in*
A549	lung adenocarcinoma	II
A549 GFP-α-tubulin	A549 stably expressing EGFP-α-tubulin	I
Ep156T	prostate epithelial cells	II
HCT116	colorectal carcinoma	II, III, IV
HeLa	cervical adenocarcinoma	I, II, III, IV
HeLa GFP-Spc24	HeLa cells stably expressing GFP-Spc24	II
HeLa H2B-GFP mCherry-tubulin	HeLa cells stably expressing H2B-GFP and mCherry-tubulin	I
LNCap	prostatic adenocarcinoma	II
MCF10A	mammary epithelial cells	II
MCF7	breast adenocarcinoma	II
MDA-MB-231	breast adenocarcinoma	II, IV
MDA-MB-231 SA	breast adenocarcinoma	II, IV
Ovcar-3	ovarian adenocarcinoma	II
RWPE-1	prostate epithelial cells	II
RWPE-2-W99	tumorigenic derivative of RWPE-1	II
U2OS stably expressing a Förster resonance energy transfer (FRET)-based Plk1 reporter	osteosarcoma, used for FRET	I

Chemicals

Chemical/Reagent	*Supplier, catalog number*	*Concentration*	*Used in*
Barasertib	Selleckchem, S1147	3.125-50 nM	IV
BI2536	Selleck Chemicals, S1109	200 nM	I
Centmitor-1	ChemBridge Corporation, 5676127	5 μM	I

Dimethylenastron	Alexis Biochemicals, ALX-270-438	5 μM	I
FR180204	Tocris Bioscience, 3706	25 μM	III
Insulin	Sigma, 19278	100 ng/ml	I
MG132	Sigma, C2211	20 μM	II, IV
Monastrol	Sigma, M8515	100 μM	I, II
Nocodazole	Sigma, M1404	150 nM, 0.5-3 μM	I, II, IV
Rigosertib	Selleck Chemicals, S1362	250 nM unless stated otherwise	I
Staurosporine	Sigma, S5921	1 μM	I, II
Taxol	Sigma, T7191	1-600 nM	I, II, III, IV
Thymidine	Sigma, T9250	2 mM	III, IV
TPA (12-O-Tetradecanoylphorbol-13-acetate)	Sigma, P1585	200 nM	III
Vinblastine	Sigma, V1377	3 μM	I
VTT-006	ChemBridge Corporation, 11866250	1-20 μM	II
Wortmannin	Tocris Bioscience, 1232	0.2-1 μM	I
ZM447439	Tocris Bioscience, 2458	5 μM, 20 μM	I, II, IV

MiRNAs

miRNA	***Supplier***	***Used in***
Pre-miR™ miRNA Precursor hsa-miR-378a-5p	Ambion	III
Pre-miR™ miRNA Precursor hsa-let-7b-5p	Ambion	IV
Pre-miR™ miRNA Precursor negative control #1	Ambion	III, IV
Pre-miR™ miRNA Precursor negative control #2	Ambion	III, IV

Primary antibodies used in immunoblot experiments

Antigen	*Species*	*Supplier, catalog number*	*Dilution*	*Used in*
AKT	rabbit	Cell Signaling, #9272	1:1000	I
Aurora B	rabbit	Abcam, ab2254	1:800	III, IV
Aurora B (AIM1)	mouse	BD Biosciences, 611083	1:200, 1:250, 1:1000	III, IV
cleaved PARP	mouse	Cell Signaling, #9546	1:1000	I, II
Cyclin B	mouse	BD Bioscience-Pharmingen, 554178	1:500	I
GAPDH	mouse	Advanced ImmunoChemical Inc., mAb 6C5, #2-RGM2	1:30 000-1:50 000	I, II, III, IV
p44/42 MAPK (ERK ½) (137F5)	rabbit	Cell Signaling, #4695	1:1000	III
PDGFR-β	rabbit	Cell Signaling, #3169	1:800	III
phospho-AKT (Ser473)	rabbit	Cell Signaling, #4060	1:2000	I
phospho-histone H3 (Ser10)	rabbit	Upstate, 06-570	1:4000	I
phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	rabbit	Cell Signaling, #9101	1:800	III
phospho-PDGFR-β (Tyr857)	goat	Santa Cruz, sc-12907	1:1000	III

Primary antibodies used in immunofluorescence labeling

Antigen	*Species*	*Supplier, catalog number*	*Dilution*	*Used in*
Aurora B (AIM1)	mouse	BD Biosciences, 611083	1:1000	II, III, IV

Bub1	mouse	Upstate, 05-899	1:500	I, II
BubR1	mouse	Abcam, ab4637	1:200	I, II
BubR1	rabbit	Proteinatlas	1:200	I
Cenp-A	mouse	Abcam, ab13939	1:1000	I
Centrin	mouse	Abnova, H00001070-M01	1:300	I
CREST (human autoimmune serum)	human	Antibodies Incorporated	1:200	I, II, III, IV
EB1	mouse	gift from G. Gorbsky	1:200	I
Hec1	mouse	Abcam, ab3613	1:1000	II
INCENP	rabbit	gift from E. Nigg	1:1000	III
NuMA	mouse	gift from M. Kallajoki	1:5	I
Pericentrin	rabbit	Abcam, ab4448	1:200, 1:500	I, II, III, IV
phospho-Aurora B (Thr232)	rabbit	Rockland 600-401-677	1:500	II
phospho-Cenp-A (Ser7)	rabbit	Upstate, 05-792	1:1000	III, IV
Plk1	mouse	Abcam, ab17057	1:200	I
Survivin	rabbit	Abcam, ab469	1:300	III
α-tubulin	rat	Abcam, ab6160	1:200, 1:500	I, III
α-tubulin	mouse	Abcam, ab7291	1:200	I, II, IV
γ-tubulin	mouse	Abcam, ab11316	1:200	I

Primers used in qRT-PCR

Gene	*Forward*	*Reverse*	*Probe*	*Used in*
AURKB	ATTGCTGACTTCGG CTGGT	GTCCAGGGTGCCAC ACAT	#69	III, IV
VEGFA	CTACCTCCACCATG CCAAGT	CCACTTCGTGATGA TTCTGC	#29	III
GAPDH	ACGACCAAATCCGT TGACTC	CTCTGCTCCTCCTG TTCGAC	#60	III, IV
GAPDH	AGCCACATCGCTCA GACAC	GCCCAATACGACCA AATCC	#60	IV

Probes were from Roche Universal ProbeLibrary.

Primers used for making luciferase reporter constructs

Gene	*Forward*	*Reverse*	*Used in*
AURKB	ATCGACTAGTGGAGAG TAGCAGTGCCTTGGA	ATCGACGCGTTGAGTA CAAAAAGCTTCAGCC	III, IV
AURKB 3'UTR	ATCGACTAGTTGATGGT CCCTGTCATTCACT	ATCGACGCGTTGAGTA CAAAAAGCTTCAGCC	III, IV

Primers used in site-directed mutagenesis

Gene	*Forward*	*Reverse*	*Used in*
AURKB	GGATCCCTAACTGTTCC CTTATCTGTTTTCGCAT TCCTCCTTTGTTTAATA AAGGCTGAAG	CTTCAGCCTTTATTAAA CAAAGGAGGAATGCGA AAACAGATAAGGGAAC AGTTAGGGATCC	IV

5. RESULTS

5.1. Centmitor-1 is a novel anti-mitotic LMW compound (I)

5.1.1. Discovery of Centmitor-1

Rigosertib is an effective anti-mitotic small compound, which is undergoing clinical trials for the treatment of cancer (Gumireddy et al., 2005; Jimeno et al., 2008; Ma et al., 2012; Seetharam et al., 2012). It has been suggested to inhibit Plk1 and PI3K (Chapman et al., 2012; Gumireddy et al., 2005; Prasad et al., 2009) although its mechanism of action is not known in detail. To further investigate its anti-mitotic mechanism, a HTS screen was devised to discover compounds that would possess freedom to operate rights. A ligand-based HTS was performed to identify LMW compounds possessing a similar chemical interaction field as rigosertib but with a different molecular structure (I, Fig. 1A). A total of 65 000 LMW compounds were screened virtually using molecular alignment and virtual screening tools Almond and Brutus (Pastor et al., 2000; Ronkko et al., 2006; Tervo et al., 2005). The 200 best compounds from the hit lists were purchased and cell-based screens were performed in HeLa cells to assess the mitotic phenotype induced by the compounds. One of the hit compounds was an acridinyl-acetohydrazide ($C_{22}H_{16}BrN_3O_3$) named Centmitor-1 (I, Fig. 1B) because it induced centrosome fragmentation. Centmitor-1 scored high in the field-based similarity test when compared to rigosertib (1.62 while the maximum is 2.0). This indicates that the compounds closely resemble each other in their charge distributions and 3D structures (I, Fig. 1C).

5.1.2. Centmitor-1 induces mitotic abnormalities and cell death

The mitotic phenotype induced by Centmitor-1 was further studied by incubating HeLa cells with the drug and filming the cell cycle progression using an IncuCyte live-cell imager (I, Fig. 2A). The cell fate of 60 individual cells was recorded (I, Fig. 2B). Centmitor-1 (5 μM) treated cells were compared to cells treated with DMSO (negative control), taxol (microtubule stabilizing drug) and nocodazole (microtubule depolymerizer). In the DMSO treated cell population, the average duration of mitosis was 1.5 ± 1.8 hours. Treatment with nocodazole and taxol induced M phase arrest lasting on average for 14.3 ± 5.4 and 16.7 ± 5.9 hours, respectively, and the mitotic arrest was followed by cell death. Half of the Centmitor-1 treated cells (48.3 %) were arrested in mitosis on average for 14.3 ± 5.9 hours before undergoing cell death in mitosis. The rest of the cells in the population exited mitosis with (38.3 %) or without (13.3 %)

cytokinesis after a mitotic delay. In comparison, rigosertib (250 nM) induced cell death in mitosis in the majority of the cells (76.7 %) whereas the rest of the cells exited with (16.7 %) or without (6.7 %) cytokinesis (I, Supplementary Fig. S1A). Mitotic and cell death indices were recorded from cell populations treated with 5 µM Centmitor-1 0, 12, 24 and 36 hours after introduction of the drug to the cells (I, Fig. 2C). The mitotic index peaked at 24 hours and cell death was most prominent 36 hours after the initiation of the treatment. Cells treated with rigosertib exhibited similar mitotic and cell death index profiles as seen with Centmitor-1 (I, Supplementary Fig. S1B).

Rigosertib has been reported to induce apoptosis (Gumireddy et al., 2005), and therefore the possible cell death effects caused by Centmitor-1 were investigated. HeLa cells treated with Centmitor-1 were stained with propidium iodide and Annexin V-FITC 12, 24 and 48 hours post-treatment followed by flow cytometric analysis of the cell cycle and cell death. Moreover, HeLa cells treated with Centmitor-1 were lysed at the same time points and subjected to Western blotting with an antibody recognizing cleaved PARP, a well-established marker for apoptosis. Centmitor-1 induced an increase in the G2/M population and an accumulation of the sub-G1 cells compared to DMSO control (I, Supplementary Fig. S2A). After 48 hours in the presence of Centmitor-1, 32.8 % of cells were in early apoptosis and 14.3 % in late apoptosis or necrosis as determined by Annexin V-FITC and propidium iodide staining. At the same time point in the DMSO treated cell population, 10.4 % of cells were early apoptotic and 8.6 % were late apoptotic or necrotic (I, Supplementary Fig. S2B). Moreover, Centmitor-1 induced an increase in the amount of cleaved PARP, which also shows that Centmitor-1 causes apoptosis (I, Supplementary Fig. S2C).

HeLa cells stably expressing H2B-GFP and mCherry-tubulin were filmed upon the addition of Centmitor-1 or DMSO to the culture medium (I, Supplementary Movies S1 and S2). Control cells divided normally with a bipolar spindle (I, Supplementary Movie S2), whereas Centmitor-1 caused a long prometaphase delay followed by cell death or an abnormal exit from mitosis. Centmitor-1 treated cells exhibited a multipolar spindle structure immediately after NEBD and they underwent multipolar anaphase after mitotic delay (I, Supplementary Movie S1). Alternatively, the cells either fused the multiple poles to create a bipolar spindle or established bipolarity upon entry into mitosis (I, Supplementary Movie S1). In the bipolar phenotype, many chromosomes congressed to the cell equator but a number of chromosomes remained unaligned near to the spindle poles (I, Supplementary Movie S1). The phenotype was further validated in fixed and immunostained cells (I, Fig. 3A-B). Rigosertib induced a similar phenotype with multipolar and bipolar spindles (I, Supplementary Fig. S3).

Rigosertib has been reported to cause centrosome fragmentation and reduction of γ-tubulin signal at centrosomes (Gumireddy et al., 2005). In order to determine the effects of Centmitor-1 on centrosomes, HeLa cells were fixed and immunostained for γ-tubulin, pericentrin and centrin after 6-hour Centmitor-1 treatment. The signal of γ-tubulin was found to be decreased at the spindle poles (I, Fig. 3C). Moreover, the centrosomes appeared to be fragmented as based on the pericentrin signal (I, Fig. 3D). Morphometric analysis revealed significant ($p<0.01$-$p<0.001$) differences in centrosome area, roundness and aspect ratio (AR; major axis/minor axis) between Centmitor-1 and DMSO treated cells (I, Fig. 3E). Similar differences were observed between rigosertib and DMSO treatments. Centmitor-1 also induced multipolarity with extra α-tubulin foci, which were often negative for pericentrin and centrin indicating abnormal centrosome structure (I, Fig. 3F). Furthermore, rigosertib and Centmitor-1 treated cells exhibited a shorter spindle length, 8.0 ± 0.9 μm ($p<0.001$) and 8.9 ± 1.4 μm ($p<0.001$) respectively, compared to control cells (10.4 ± 1.0 μm) (I, Fig. 3G). Interestingly, rigosertib and Centmitor-1 treatments induced fragmentation and redistribution of the NuMA signal from the spindle poles to multiple cytosolic foci detected by immunofluorescence stainings (I, Fig. 3H). NuMA contributes to spindle pole assembly and the maintenance of the spindle structure (Silk et al., 2009). Depletion of NuMA induces defects in chromosome alignment (Haren et al., 2009), similarly to the treatments with rigosertib and Centmitor-1.

To investigate the molecular mechanisms influencing the mitotic arrest induced by Centmitor-1, the signal intensity of the SAC proteins BubR1 and Bub1 were determined at the unaligned kinetochores of control and Centmitor-1 treated cells. Kinetochore localization of BubR1 and Bub1 is considered to be a marker of active SAC (Logarinho et al., 2004; Skoufias et al., 2001). After culturing HeLa cells in the presence of Centmitor-1, taxol, or DMSO for 6 hours, the cells were fixed, immunostained and subjected to immunofluorescence microscopy. Similarly to the control cells treated with DMSO, BubR1 and Bub1 signals were almost undetectable at the kinetochores of aligned chromosomes in Centmitor-1 treated cells (I, Fig. 4A-B). In contrast, the signals remained elevated at the kinetochores of unaligned chromosomes, both in Centmitor-1 treated cells and in controls (I, Fig. 4C-D). Accordingly, when cells were treated with rigosertib, both BubR1 and Bub1 signals were present at the kinetochores of the unaligned chromosomes (I, Supplementary Fig. S4). Furthermore, introducing an Aurora B inhibitor ZM447439 induced exit from mitosis in the presence of Centmitor-1 and taxol. This was detected by measuring the protein levels of Cyclin B and phospho-histone H3 by Western blotting. The levels of both proteins were greatly reduced by ZM447439

treatment (I, Fig. 4E). The results indicate that the mitotic arrest induced by Centmitor-1 and rigosertib is dependent on normal SAC signaling and Aurora B activity.

5.1.3. Centmitor-1 modulates microtubule dynamics

Interference with microtubules induces spindle abnormalities and subsequent mitotic arrest (Jordan and Kamath, 2007). In order to test the effects of Centmitor-1 on microtubules, tubulin polymerization was assessed *in vitro* in the presence of the compound. Taxol, a compound that stabilizes microtubules, and vinblastine, which induces microtubule depolymerization, were used as controls. Centmitor-1 at 1 and 5 μM concentrations had no effect on microtubule polymerization whereas 10 and 20 μM drug induced a slight increase in the tubulin polymerization rate (I, Fig. 5A). Rigosertib has been reported not to have an effect on tubulin polymerization *in vitro* at 5 μM concentration (Gumireddy et al., 2005). In interphase cells, Centmitor-1 caused only slight curving of microtubules as compared to DMSO treated control cells detected by immunofluorescence stainings (I, Fig. 5B). As expected, the two control compounds, taxol and nocodazole, induced clear microtubule bundling and a fragmented microtubule network, respectively. Rigosertib (250 nM) induced only slight changes in interphase microtubules compared to DMSO control. Previously, it has been reported that rigosertib at a high concentration (2.5 μM) depolymerizes microtubules (Steegmaier et al., 2007). In our hands, most microtubules were also lost after a 6-hour incubation with 2.5 μM rigosertib (I, Supplementary Fig. S5).

Although Centmitor-1 and rigosertib did not have a major effect on microtubule filaments, the microtubule plus ends were affected by the compounds. This was detected by immunofluorescence staining of EB1, a protein that is needed for normal microtubule dynamics (Rogers et al., 2002). The compounds induced clear changes in the localization of EB1 in interphase as compared to DMSO treated cells (I, Fig. 5C). The effect of Centmitor-1 on microtubule dynamics was further investigated using a human A549 lung carcinoma cell line stably expressing EGFP-α-tubulin that had earlier been validated for the measurement of microtubule dynamics (Narvi et al., 2013). In these cells, 5 μM Centmitor-1 induced a similar phenotype as seen in the HeLa cells: chromosome misalignment, multipolarity and mitotic delay (I, Supplementary Fig. S6A-B). Furthermore, Centmitor-1 had only minor effects on interphase microtubules (I, Supplementary Fig. S6C). These cells were however more sensitive to rigosertib compared to HeLa cells as 250 nM rigosertib eliminated microtubules. For measuring microtubule dynamics, the cells were treated with

DMSO, 5 μM Centmitor-1 or 50 nM rigosertib for 1 to 2 hours prior to live cell imaging. 50 nM rigosertib did not abolish microtubules but was sufficient to induce mitotic arrest. The microtubule plus ends were tracked to measure average microtubule dynamics. In DMSO treated cells (n = 21), the average microtubule dynamicity was 0.096 ± 0.036 μm/s whereas in Centmitor-1 treated cells (n = 20) it was reduced to 0.051 ± 0.019 μm/s ($p<0.001$) (I, Fig. 5D, Supplementary Movies S3 and S5). Rigosertib treatment (n = 18) notably reduced the dynamicity (0.039 ± 0.014 μm/s, $p<0.001$) as compared to controls (I, Fig. 5D, Supplementary Movie S4). These results suggest that Centmitor-1 and rigosertib affect microtubule dynamics and microtubule plus ends in interphase cells.

5.1.4. Centmitor-1 has a mitotic target other than Plk1

To assess the effects of Centmitor-1 and rigosertib on mitotic cells and their microtubule functions, interkinetochore distances were measured in HeLa cells after treatments with the compound. Cenp-A was used as an inner centromere marker for the measurements. Tension across the sister kinetochores is necessary for stable kinetochore-microtubule interactions and SAC inactivation (Musacchio and Salmon, 2007). The interkinetochore distances of unaligned chromosomes were in a similar range in control prometaphase cells as in Centmitor-1 and rigosertib treated cells. However, Centmitor-1 and rigosertib treatment reduced the interkinetochore distance in aligned chromosomes to 1.07 ± 0.09 μm ($p<0.001$) and 1.06 ±0.04 μm ($p<0.001$), respectively, in comparison to control cells (1.51 ± 0.09 μm) (I, Fig. 6A-B). This indicates that Centmitor-1 and rigosertib both interfere with the mitotic spindle forces. In order to verify further that Centmitor-1 executes its actions by targeting a mitotic protein, the compounds were introduced into mitotic post-NEBD HeLa cells, which were time-lapse filmed for 24 hours. Both compounds caused an immediate effect on mitotic cells: mitotic arrest followed by cell death (I, Fig. 6C). In comparison, the Eg5 inhibitors monastrol and dimethylenastron did not affect post-NEBD mitotic cells as Eg5 functions earlier in centrosome separation (Blangy et al., 1995; Kapoor et al., 2000). These results indicate that the target protein of Centmitor-1 and rigosertib is expressed in mitotic cells.

Rigosertib has been suggested to target Plk1 (Gumireddy et al., 2005). Previously, it has been reported that failure to remove active Plk1 from metaphase aligned chromosomes leads to decreased interkinetochore tension (Liu et al., 2012a). This could explain the smaller interkinetochore distance in cells treated with Centmitor-1 or rigosertib. However, immunofluorescence stainings revealed that there were no changes in Plk1 localization after

treatments with Centmitor-1 or rigosertib (I, Supplementary Fig. S7A). Next, the effects of Centmitor-1 and rigosertib on Plk1 activity were evaluated in U2OS cells stably expressing a Förster resonance energy transfer (FRET)-based probe (Macurek et al., 2008). Both compounds induced a mitotic delay when introduced to U2OS cells (I, Fig. 6D). Plk1 activity was decreased by a Plk1 inhibitor BI2536 whereas cells treated with Centmitor-1 or rigosertib exhibited Plk1 activity similar to the DMSO treated mitotic cells (I, Fig. 6E). These results suggest that Centmitor-1 and rigosertib do not target Plk1 activity. Rigosertib has been also reported to inhibit phosphoinositide 3-kinase (PI3K) (Prasad et al., 2009). For this reason, the effects of Centmitor-1 and rigosertib on PI3K were investigated by using AKT phosphorylation at Ser473 as a marker of PI3K activity (Alessi et al., 1996). Under serum-free conditions, insulin addition activated the PI3K/AKT pathway in the presence of both compounds as it did in the DMSO treated HeLa cells (I, Supplementary Fig. S7B-C). In comparison, the PI3K inhibitor wortmannin decreased the phosphorylation of AKT and thus inactivated PI3K (I, Supplementary Fig. S7B-C). Thus, it was concluded that Centmitor-1 and rigosertib do not directly target PI3K or Plk1 in the used cell models.

5.2. VTT-006 is a putative Hec1 inhibitor (II)

5.2.1. Discovery of VTT-006

Hec1 mediates the interaction between chromosomes and microtubules during mitosis and participates in SAC signaling. Inhibition of Hec1 could therefore indirectly perturb microtubule-mediated mitotic processes causing fewer side effects than traditional microtubule drugs. Moreover, the protein is highly expressed in cancer cells, which makes it an attractive target for future cancer therapies. A virtual HTS was designed to identify LMW compounds that could interfere with the normal binding of Hec1 to microtubules. The docking software FRED (McGann, 2011) was used to virtually fit approximately four million LMW compound structures against the CHD of Hec1. The crystallized structure of $Hec1_{81\text{-}196}$ was used as the docking template (Wei et al., 2007). The best 138 hit compounds were purchased and tested in cell-based assays for their ability to induce mitotic arrest and/or cell death, which are the main phenotypes of Hec1 depletion by RNAi (Gurzov and Izquierdo, 2006; Martin-Lluesma et al., 2002). HeLa H2B-GFP cells were used to evaluate the phenotypic outcome of the compound treatments based on DNA morphology. Three putative lead compounds were identified: VTT-006, VTT-102 and VTT-106, all of which induced strong mitotic arrest (II, Fig. 1). VTT-006 was selected for further

studies. *In silico*, VTT-006 docked into the CHD of Hec1 and was predicted to interact with the protein via hydrogen bonds.

5.2.2. VTT-006 binds to the recombinant Ndc80 complex

The binding of VTT-006 to its target protein Hec1 was validated *in vitro* using fluorescence anisotropy measurements and the recombinant Ndc80 complex, called Bonsai (Fig. 5), as the bait. The recombinant Ndc80 complex contains the globular domains at both ends of the complex and a shortened rod domain and has been shown to bind to microtubules *in vitro* (Ciferri et al., 2008). The results from the anisotropy measurements indicate that VTT-006 could bind to Bonsai but not to Aurora B, which served as a negative control (II, Fig. 2A). The dissociation constant (K_d) for VTT-006 was calculated to be 2.1 ± 2.8 μM.

TIRF microscopy was used to determine the effects of VTT-006 on the interaction between taxol-stabilized microtubules and the recombinant Ndc80 complex *in vitro* (II, Fig. 2B-D). The residence time of Ndc80-GFP complexes on microtubules was shorter in the presence of VTT-006 in comparison with DMSO (II, Fig. 2B, $p<0.001$). Accordingly, the off-rate constant increased by VTT-006 (0.79 ± 0.04 s^{-1}) as compared to the DMSO control (0.47 ± 0.02 s^{-1}). Moreover, the diffusion constant was higher in the presence of VTT-006 (0.023 ± 0.0006 $\mu m^2 s^{-1}$) than with DMSO treatment (0.016 ± 0.0005 $\mu m^2 s^{-1}$) (II, Fig. 2C). These results suggest that VTT-006 binds to the Ndc80 complex, which perturbs the normal association of microtubules with the complex.

5.2.3. VTT-006 induces mitotic anomalies

The mitotic phenotype induced by VTT-006 was studied in more detail with live cell imaging. HeLa cells were treated with 1, 5 or 10 μM VTT-006 and monitored for 48 hours using time-lapse microscopy. Subsequently, mitotic and cell death indices were determined. In the presence of the compound, the cells typically arrested in mitosis and finally underwent cell death (II, Fig. 3A). Cells treated with 5 and 10 μM VTT-006 showed accumulation to the M phase already at the 6-hour time point when 18.0 ± 3.0 % and 25.6 ± 1.8 % of the cells were in mitosis, respectively. The mitotic indices peaked at the 12-hour time point when the mitotic indices were 36.1 ± 4.7 % and 44.8 ± 7.1 %, respectively (II, Fig. 3B). The cell death indices peaked at the 48-hour time point in populations treated with 5 μM (56.2 ± 12.8 %) and 10 μM (96.0 ± 2.7 %) VTT-006 (II, Fig. 3C). The analysis of mitotic duration and cell fate of

individual cells revealed that 10 μM VTT-006 resulted in a long mitotic arrest of 11.5 ± 5.5 hours whereas in the DMSO treated control cells mitosis lasted 1.2 ± 0.3 hours (II, Fig. 3D, $p<0.001$). The majority (84.0 %) of the arrested VTT-006 treated cells died in the M phase. Furthermore, the type of cell death induced by VTT-006 was confirmed to be apoptosis by Western blotting, which showed the appearance of cleaved PARP 24 hours after the drug was added to cells (II, Fig. 3E).

In order to test whether the mitotic arrest induced by VTT-006 was due to the active SAC, HeLa cells were treated with DMSO, taxol, or VTT-006 and subjected to fixation and immunofluorescence stainings 6 hours later. Kinetochore localization of Bub1 and BubR1 is a marker of the active SAC (Logarinho et al., 2004; Skoufias et al., 2001). As expected, the Bub1 and BubR1 signals were diminished at metaphase kinetochores in DMSO treated cells (II, Fig. 3F, Supplementary Fig. S1). In VTT-006 treated cells, chromosome alignment was not completed and Bub1 and BubR1 signals remained high at the kinetochores of unaligned chromosomes (II, Fig. 3F, Supplementary Fig. S1). This result indicates that the VTT-006-induced mitotic arrest is due to active SAC.

The effects of VTT-006 were further analyzed with high resolution live-cell imaging of HeLa H2B-GFP cells. The cells were filmed for 15 hours in the presence of 5 μM VTT-006. The compound was found to induce defects in chromosome congression as several chromosomes remained near to the spindle poles while most chromosomes congressed at the cell equator (II, Fig. 4A, Movie S1). The control cells divided normally (II, Fig. 4A, Movie S2). In early mitosis, kinetochores form lateral attachments with microtubules followed by the formation of end-on attachments (Kapoor et al., 2006; Magidson et al., 2011). Previous studies have indicated that the Ndc80 complex is not essential for the formation of lateral attachments (Vorozhko et al., 2008). To investigate the effects of VTT-006 on lateral connections, the kinetochores were tracked in live HeLa cells expressing GFP-Spc24 as a kinetochore marker. Early mitotic cells were filmed and the average speed of kinetochores was determined for chromosomes that exhibited rapid movements around the time of NEBD. Movements slower than 10.0 μm/min were excluded from these measurements. In control cells and in VTT-006 treated cells, the average velocity of movement was 14.4 ± 3.9 μm/min and 13.9 ± 3.3 μm/min, respectively (II, Fig. 4B). The results indicate that VTT-006 does not apparently affect the establishment of lateral connections between kinetochores and microtubules, which is in line with previous studies (Vorozhko et al., 2008). Although the Ndc80 complex is not needed for lateral connections, it is essential for the establishment of correct end-on attachments and needed for oscillatory movements (DeLuca et al., 2011;

Maure et al., 2011). The effect of VTT-006 on metaphase plate oscillations was determined by tracking kinetochore pairs in HeLa cells stably expressing GFP-Spc24 and transiently transfected with GFP-NuMA to allow spindle pole visualization. The cells were time-lapse filmed after partial chromosome alignment and kinetochore pairs of aligned chromosomes were tracked in relation to one of the spindle poles. The kymographs (II, Fig. 4C) showed that cells treated with VTT-006 exhibited significantly reduced oscillations in comparison to control cells. Accordingly, the deviation from the average position of kinetochores measured relative to a pole was decreased in VTT-006 treated cells in comparison to control cells (II, Fig. 4D, $p<0.001$). Moreover, VTT-006 decreased the interkinetochore distance (0.96 ± 0.15 μm) as compared to control (1.19 ± 0.18 μm) indicating that VTT-006 reduces interkinetochore tension (II, Fig. 4E, $p<0.001$). The results indicate that VTT-006 does not have an impact on lateral attachments, which are not affected by the Ndc80 complex. However, end-on attachments, oscillations and interkinetochore tension can be disrupted by VTT-006, possibly due to Hec1 inhibition.

Since Hec1 localized to kinetochores in the presence of VTT-006, as determined by immunofluorescence stainings, the observed phenotype was not due to any drug effects on the protein's subcellular localization (II, Supplementary Fig. S2A-B). Also, the total protein levels of Hec1 were not changed by VTT-006 measured by Western blotting (II, Supplementary Fig. S2C). The kinetochores' ability to establish stable attachments with microtubules was determined by lysing the cells in cold calcium buffer to remove non-kinetochore microtubules. In VTT-006 treated cells, microtubules were attached to chromosomes at the metaphase plate and to chromosomes near the poles (II, Supplementary Fig. S3). This result indicates that the kinetochores were able to establish stable connections with microtubules and proposes that VTT-006 perturbs normal attachments but does not completely abolish the kinetochore-microtubule interaction.

Next, the effects of VTT-006 were tested on chromosomes that have aligned to the metaphase plate and have established kinetochore-microtubule attachments. MG132 prevents exit from mitosis by inhibiting the activity of the 26S proteasome but allows normal chromosome congression to take place. HeLa H2B-GFP cells were incubated in the presence of MG132 for 2 hours to induce the accumulation of metaphase cells. Then, either 10 μM VTT-006 or 20 nM taxol was added to the cells and live cell filming was started. An analysis of the time-lapse films showed that in MG132 treated cells, the chromosomes remained at the cell equator as expected (II, Movie S3) whereas several chromosomes moved away from the metaphase plate in the VTT-006 treated cells (II, Movie S4). A more robust disruption of the metaphase plate was

observed in taxol treated cells (II, Movie S5). The results indicate that VTT-006 not only affects chromosome congression in early mitosis but also disturbs the ability of the chromosomes to maintain their metaphase alignment.

The reversibility of the anti-mitotic phenotype induced by VTT-006 was tested by treating HeLa cells with DMSO, 10 μM VTT-006 or 100 μM monastrol for 5 hours. Next, the cells were subjected to immunofluorescence staining in order to detect microtubules, spindle poles and DNA. VTT-006 induced the formation of bipolar cells with a partial chromosome alignment (58.0 ± 4.1 %), multipolar cells (25.8 ± 7.2 %), bipolar cells with totally unaligned chromosomes (8.4 ± 3.0 %), bipolar cells with normal metaphase alignment (1.3 ± 0.6 %) or monopolar cells (6.5 ± 5.1 %) (II, Fig. 5A and B). In the DMSO control, 82.0 ± 1.2 % of cells exhibited a normal metaphase chromosome alignment with a bipolar spindle. When VTT-006 was washed off prior to fixation, the majority (75.3 ± 5.8 %) of cells exhibited normal metaphase alignment after a 1.5-hour recovery time. This was comparable to the cells after monastrol washout (73.7 ± 7.9 %). The mitotic arrest was maintained after VTT-006 washout by introducing MG132 for 1.5 hours prior to fixation. These results suggest that the phenotype induced by VTT-006 is reversible.

Aurora B kinase regulates the affinity of Hec1 to microtubules by phosphorylating the N-terminal tail of Hec1 (DeLuca et al., 2006; DeLuca et al., 2011). In order to study the changes in Aurora B localization and activity after Hec1 inhibition, HeLa cells were treated with DMSO, VTT-006, taxol, or nocodazole for 6 hours. Cells co-treated with ZM447439, which is an Aurora B inhibitor, and MG132 served as a control. After the incubations, the cells were fixed and immunostained to detect Aurora B, phospho-Aurora B (Thr232) and Crest. The Aurora B signal intensity was significantly higher in the centromeres of unaligned chromosomes after VTT-006 treatment as compared to control prometaphase cells (II, Fig. 6A-B, $p<0.05$). Furthermore, line-scan analysis of signal distribution indicated that Aurora B signal was spread out from the inner centromeres towards the kinetochores in unaligned chromosomes of VTT-006 treated cells (II, Supplementary Fig. S4). In control cells, Aurora B was more clearly localized at the inner centromere. The activity of Aurora B in unaligned chromosomes of VTT-006 treated cells was at the same level as in control prometaphase cells (II, Fig. 6A-B). The results suggest that VTT-006 induces the accumulation of Aurora B to centromeres of unaligned chromosomes, which could reflect amplification of the SAC signal or error correction.

5.2.4. VTT-006 sensitizes cells to taxol treatment

Hec1 depletion has previously been shown to induce sensitization to taxol (Mo et al., 2013). Possible synergistic effects of VTT-006 with taxol were studied in HeLa cells with both drugs being used either separately or together at different concentrations. The cell viability and mitotic indices were determined using IncuCyte live-cell imager. Combinations of sub-lethal concentrations of VTT-006 (1-2 μM) and taxol (1-5 nM) increased mitotic index more than single drug treatments at the same concentrations (II, Fig. 7A). The mitotic index was increased the most by the combination of 2 μM VTT-006 and 5 nM taxol as compared to treatments with each drug alone determined 12 hours after beginning the treatment ($p<0.05$). Furthermore, cells treated with a combination of 2 μM VTT-006 and 5 nM taxol exhibited a clear decrease in cell viability at 24-48 hours after the introduction of the drugs (II, Fig. 7B) in comparison to cells treated with either 2 μM VTT-006 or 5 nM taxol alone. The type of cell death was further verified to be apoptosis as detected by Western blotting with an anti-cleaved PARP antibody (II, Fig. 7C). According to these results, VTT-006 sensitizes cells to taxol treatment at certain sub-lethal concentrations.

5.2.5. Cell viability is suppressed by VTT-006

In addition to HeLa cells, the effects of VTT-006 were investigated in other cancer cell lines of prostate, breast, colon, lung and ovarian origin and also in three non-tumorigenic cell lines (II, Table 1). Cells treated with 1, 5, or 10 μM VTT-006 were imaged with the IncuCyte live-cell imager. VTT-006 induced accumulation of mitotic cells in all of the cell lines studied except for the non-tumorigenic MCF10A cells. Furthermore, EC_{50} values of VTT-006 were determined in different cell lines with CellTiter Glo assays (II, Table 1). The EC_{50} measurements also showed that MCF10A cells are more resistant to VTT-006. For other cell lines, the EC_{50} values ranged from 4.8 to 11.9 μM. Moreover, the effects of VTT-006 were studied in a 3D organotypic cell culture system (Harma et al., 2010), in which two breast cancer cell lines, MCF7 and MDA-MB-231 SA, were used as models. The area of cell spheroids was reduced by 5 μM and higher VTT-006 concentrations compared to controls (II, Supplemetary Fig. S5). These results show that VTT-006 suppresses the growth of several cancer cell lines and reduces the size of cell spheroids in 3D organotypic cell culture.

5.3. MiR-378a-5p regulates genomic balance and tumorigenesis (III)

5.3.1. Excess miR-378a-5p induces anti-mitotic phenotype

Many miRNAs have been implicated in tumorigenesis, both in the initiation and progression of cancer (Di Leva et al., 2014). This is the first time that a HTS of all commercially available human pre-miRNAs (810 pre-miRNAs) has been designed and conducted to identify miRNAs that affect mitotic regulation. In taxol treated cells, an excess of miR-378a-5p was found to decrease the mitotic index in comparison to control miRNA-transfected cells and to induce progeny cells with multilobed nuclei (III, Fig. 1A-C). In a drug-free cell population, the size of the nucleus was increased in miR-378a-5p-transfected cells compared to controls (III, Fig. 1A). The time-lapse filming of synchronized HeLa cells validated the mitotic phenotype and revealed that in drug-free culture conditions cells overexpressing miR-378a-5p entered mitosis about 4 hours later than the control cells (III, Fig. 1D). After entering mitosis, synchronized cell population overexpressing miR-378a-5p also exhibited M phase arrest for at least 9 hours, during which the average mitotic index was 18 %. As expected, control cells arrested in mitosis for at least 7 hours in the presence of taxol whereas excess miR-378a-5p forced the cells prematurely out of the M phase (III, Fig. 1D-E, Supplementary Movies 1 and 2). The phenotype was further confirmed by flow cytometry that showed the accumulation of the G2/M population due to excess miR-378a-5p (III, Supplementary Fig. S1A). Furthermore, the cell fate analysis validated the observed phenotype of miR-378a-5p overexpressing cells cultured in either the presence or absence of taxol (III, Supplementary Fig. S1B). An excess of miR-378a-5p induced also multipolarity since at 72 hours post-transfection, 73.7 ± 3.5 % of cells were multipolar whereas in the control population 23.0 ± 7.1 % of the cells exhibited multipolarity ($p<0.01$, III, Fig. 1F). These results indicate that excess miR-378a-5p perturbs normal mitosis and induces polyploidy, multipolarity, mitotic delay, and forced mitotic exit.

5.3.2. Overexpression of miR-378a-5p regulates ERK1/2 pathway

MiR-378a-5p has been implicated in angiogenesis via upregulation of VEGF-A (Hua et al., 2006). We confirmed that VEGF-A mRNA in cells and protein levels in the culture medium were upregulated in HeLa cells transfected with miR-378a-5p in comparison to controls (III, Fig. 2A). When measured by the phosphorylation array, the phosphorylation of receptor tyrosine kinases (RTKs) Platelet-derived growth factor receptor β (PDGFR-β), Erythroblastic leukemia

viral oncogene homolog 2 (ErbB2), Ephrin type-A receptor 7 (EphA7) and Vascular endothelial growth factor receptor 2 (VEGFR-2) was elevated by excess miR-378a-5p (III, Fig. 2B). VEGF-A has been reported to stimulate the activity of PDGFR-β, EphA7, and VEGFR-2 (Ball et al., 2007). The results were validated for the phosphorylation of PDGFR-β tyrosine (Tyr) 857 by Western blotting (III, Fig. 2B), whereas VEGFR-2 was not detectable in HeLa cells in sufficient amounts to allow Western blot quantification. Another phosphorylation array was used to investigate downstream signaling of VEGF-A, and increased phosphorylation of ERK1/2 (Thr202/Tyr204 and Thr185/Tyr187) by excess miR-378a-5p was detected (III, Fig. 2C). The result was validated by Western blotting showing that the phosphorylation level of ERK1/2 (Thr202/Tyr204) had been increased by excess miR-378a-5p whereas the total ERK1/2 protein levels remained at a level similar to that in the control cells (III, Fig. 2C). The ERK1/2 inhibitor, FR180204, was found to rescue the phenotype induced by excess miR-378a-5p (III, Fig. 2D). MiRNA-transfected cells were treated with DMSO or 25 μM FR180204 and time-lapse filmed prior to fixation and DNA staining. The duration of mitosis, frequency of exit from mitosis without cytokinesis and the number of cells with large nuclei were all significantly reduced by FR180204 in cells transfected with miR-378a-5p as compared to miR-378a-5p-transfected cells without FR180204 treatment. These results suggest that excess miR-378a-5p activates RTKs and the ERK1/2 pathway.

5.3.3. Excess miR-378a-5p indirectly affects Aurora B

Forced exit from microtubule drug-induced mitotic arrest is a phenotype characteristic of inhibiting mitotic regulators, including Aurora B (Hauf et al., 2003). Thus, it was decided to investigate the effects of excess miR-378a-5p on Aurora B kinase. MiR-378a-5p-transfected HeLa cells cultured in the absence or presence of taxol exhibited significantly ($p<0.01$) reduced Aurora B mRNA levels (III, Fig. 3A). Accordingly, protein levels were diminished as compared to control cells ($p<0.01$, III, Fig. 3B), and the centromeric localization of the kinase was found to be perturbed as detected by immunofluorescence stainings ($p<0.001$, III, Fig. 3C). Furthermore, the activity of Aurora B was decreased as the signal of phosphorylated Cenp-A (Ser7), a marker for Aurora B activity, was diminished at the kinetochores. The CPC components INCENP and Survivin were also mislocalized from kinetochores by excess miR-378a-5p reflecting the tight interdependency of the complex subunits (III, Supplementary Fig. S2). In order to test whether miR-378a-5p could bind to Aurora B mRNA, *AURKB* 3'UTR and the full gene sequence of *AURKB* were cloned to reporter vectors under the same promoter with a firefly luciferase

reporter gene. The reporter constructs were co-transfected with control miRNA or miR-378a-5p to HeLa cells. However, no significant decrease in the luciferase activity was induced by miR-378a-5p indicating that the effect of miR-378a-5p on Aurora B is indirect (III, Fig. 3D). Interestingly, the decreased protein levels of Aurora B were partially rescued by treatment with the ERK1/2 inhibitor, FR180204, indicating the presence of a link between ERK1/2 and Aurora B pathways (III, Fig. 3E).

Inhibition of Aurora B increases the frequency of syntelic attachments that can subsequently induce aneuploidy (Hauf et al., 2003). The effects of miR-378a-5p on aneuploidy induction were studied using a near-diploid human colorectal cancer cell line HCT-116. These cells exhibited polyploidy and reduced Aurora B protein levels upon overexpression of miR-378a-5p (III, Supplementary Fig. S3A-B). The copy numbers of chromosomes 12, 13 and 21 were analyzed 65 hours post-transfection using FISH probes. The chromosome number was increased by miR-378a-5p as trisomy and tetrasomy were more frequent in the miR-378a-5p-transfected cells as compared to controls (III, Fig. 4). According to these results, excess miR-378a-5p indirectly regulates Aurora B levels and induces aneuploidy characteristic of Aurora B inhibition.

5.3.4. Expression of miR-378a-5p correlates with tumorigenesis

The expression of miR-378a-5p in breast cancer patients was retrospectively analyzed to observe the possible correlations between miRNA expression and clinicopathological parameters. MiR-378a-5p expression was elevated with increasing tumor grade indicating that the miRNA is more expressed in later phases of tumorigenesis ($p<0.05$, III, Fig. 5A). Five molecular subtypes of breast cancer have been established based on gene expression profiling. The subtypes have prognostic value and predict patient survival. Luminal A/B and basal-like tumors have reciprocal gene expression profiles, with basal-like cancers being more aggressive (Naume et al., 2007; Perou et al., 2000; Sorlie et al., 2001). MiR-378a-5p was more expressed in basal-like tumors than in luminal A and B type tumors (III, Fig. 5B). ER is a widely used prognostic marker in breast cancer. Mir-378a-5p was more expressed in the aggressive ER negative cancers compared to ER positive cancers ($p<0.001$, III, Fig. 5C). Moreover, the tumors were divided into low and high proliferation groups based on the expression of the proliferation marker Ki67 and mitotic index. MiR-378a-5p was more expressed in the high proliferation group compared to low proliferation group ($p<0.001$, III, Fig. 5D). Intriguingly, the same expression pattern was observed with the miR-378a-5p host gene *PPARGC1B* when associated with the tumor grades, molecular subtypes, ER status and

proliferation (III, Supplementary Fig. S4A-D). These results suggest that the expression of miR-378a-5p is elevated in aggressive breast tumors.

5.4. Let-7b directly targets Aurora B 3'UTR (IV)

5.4.1. Excess let-7b perturbs mitosis

Let-7b was identified in a HTS as an anti-mitotic miRNA that could induce the formation of multilobed nuclei because the cells underwent a premature exit from chemically induced mitotic arrest. In drug-free culture conditions, excess let-7b caused polyploidy. In order to determine the fates of individual cells in more detail, the HeLa cells overexpressing let-7b or control miRNA (IV, Supplementary Fig. A.1A-B) were time-lapse filmed in the presence of nocodazole (IV, Fig. 1A and Supplementary Movies S1-2). Let-7b overexpressing cells exhibited an increased frequency of forced exit from the nocodazole block (47.5 %) compared to controls (18.8 %) (IV, Fig. 1A-B). In drug-free culture conditions, excess let-7b induced a slight mitotic delay as the average duration of mitosis was 3.7 ± 4.6 hours in comparison to 1.3 ± 1.3 hours in the control cells (IV, Fig. 1B). Overexpression of let-7b also decreased cell viability as in drug-free culture conditions 31.3 % of the cells transfected with let-7b underwent cell death after prolonged (9.1 ± 4.9 hours) mitosis. In comparison, only 5.0 % of control cells cultured under drug-free conditions died in mitosis after experiencing mitotic arrest (5.3 ± 4.8 hours) (IV, Fig. 1B).

The phenotype and effects of let-7b on the cell cycle were further studied by synchronizing let-7b- or control miRNA-transfected cells with a double thymidine block. After thymidine washout, the cells were time-lapse filmed and mitotic indices were recorded (IV, Fig. 1C). In drug-free culture conditions, there was no significant difference in the timing of mitotic entry between let-7b and control miRNA transfections. However, during the time-lapse filming the mitotic index was slightly higher in the cell population transfected with let-7b as compared to controls. In the synchronized and microtubule drug (nocodazole or taxol) treated cell populations, the mitotic index was higher in the control population in comparison to let-7b overexpressing cells (IV, Fig. 1C). When let-7b and control miRNA overexpressing HeLa cells were subjected to flow cytometric analysis, 13.3 ± 2.0 % of control cells were in G2/M (4n) whereas significantly more ($p<0.01$), 25.7 ± 4.0 %, of let-7b-transfected cells exhibited 4n DNA content (IV, Fig. 1D). Not only G2/M phase cells but also cells that had exited the M phase without cytokinesis can exhibit 4n DNA content shown as the G2/M peak in the flow cytometric analysis. In addition, the polyploid 8n cell population was increased by excess let-7b compared to control ($p<0.05$).

These results indicate that excess let-7b can induce the mitotic anomalies associated with forced exit from chemically induced mitotic block, increased levels of polyploidy and decreased cell viability.

5.4.2. Let-7b targets Aurora B mRNA

In order to explain the observed cellular phenotype, TargetScan target prediction software (TargetScanHuman 6.2; Garcia et al., 2011) was used to search for predicted mitotic targets of let-7b. Interestingly, Aurora B kinase was among the predicted targets. As noted above, Aurora B inhibition can be associated with the forced exit from mitosis (Kallio et al., 2002), which was one of the outcomes induced by let-7b overexpression. Thus, it was decided to study the effects of excess let-7b on Aurora B expression. In HeLa cells, an excess of let-7b reduced Aurora B mRNA and protein levels by 42.3 % and 34.6 %, respectively, as compared to controls (Fig. 2A-B, $p<0.05$). The immunofluorescence stainings also showed that an excess of let-7b reduced the signal of centromeric Aurora B and phosphorylated Cenp-A (Ser7), a substrate of Aurora B (Zeitlin et al., 2001) (Fig. 2C, $p<0.001$). The reduced Aurora B levels were also observed in let-7b-transfected breast cancer cell lines, MDA-MB-231 and highly bone metastatic MDA-MB-231 SA (IV, Supplementary Fig. A.2A-B). Moreover, in MDA-MB-231 SA cells, the activity of the kinase was diminished, as indicated by the reduced signal of phosphorylated Cenp-A (IV, Supplementary Fig. A.2C). In the same cell line, excess let-7b increased the amount of cells with multilobed nuclei measured after taxol treatment and fixation (IV, Supplementary Fig. A.2D). Direct binding of let-7b to Aurora B was determined with a luciferase reporter assay. *AURKB* 3'UTR or the full length *AURKB* gene was cloned into a luciferase reporter vector, which was co-transfected with let-7b or control miRNA. Excess let-7b decreased luciferase activity significantly by 24.0 % ($p<0.05$) and 21.1 % ($p<0.01$) compared to controls determined with *AURKB* 3'UTR plasmid and the full length *AURKB* plasmid, respectively (IV, Fig. 3A). The binding to the 3'UTR was further validated by mutating the predicted binding site of let-7b on Aurora B 3'UTR via site-directed mutagenesis. When either one of the two mutated constructs was co-transfected with let-7b, luciferase activity remained at the basal level indicating that the mutated residues were important for let-7b binding (IV, Fig. 3B). Furthermore, the suppression of Aurora B protein levels was rescued by a target site blocker (TSB), which was designed to bind the binding site of let-7b and thus compete with let-7b for binding to Aurora B (IV, Fig. 3C).

Alterations in Aurora B expression and activity are associated with polyploidy and aneuploidy (Hauf et al., 2003; Kallio et al., 2002; Ota et al., 2002).

Therefore, the effect of excess let-7b on aneuploidy induction was measured by FISH in a near-diploid colon cancer cell line HCT-116. Excess let-7b reduced Aurora B mRNA and protein levels also in this cell line (IV, Fig. 4A-B). A significant increase in the frequency of monosomy, trisomy, and tetrasomy was observed in let-7b-transfected cells in comparison to controls when the copy number changes of chromosomes 12, 13 and 21 were determined (Fig. 4C). The multipolarity of the cells transfected with let-7b was determined by immunofluorescence stainings with antibodies against pericentrin and α–tubulin. The frequency of cells with three or more poles was significantly increased in the let-7b-transfected cells (57.7 ± 12.7 %) compared to controls (8.9 ± 5.0 %) (IV, Fig. 5, $p<0.01$). This phenotype resembles that encountered with Aurora B inhibition by the compound ZM447439, which induced a high frequency (68.9 ± 8.6 %) of multipolarity (IV, Supplementary Fig. A.3). The results suggest that let-7b targets Aurora B and the excess of let-7b induces multipolarity and aneuploidy, both associated with Aurora B inhibition.

5.4.3. Excess let-7b has an additive effect on polyploidy induction in cells treated with an Aurora B inhibitor

Barasertib (AZD1152) is an Aurora B specific inhibitor, which is undergoing phase II clinical trials for the treatment of acute myeloid leukemia (Dennis et al., 2012; Kantarjian et al., 2013; Kantarjian et al., 2013; Schwartz et al., 2013). Barasertib induces polyploidy and mitotic slippage (Marxer et al., 2014), and therefore it was decided to examine its possible synergistic or additive effects on the phenotype of let-7b overexpressing cells. HeLa cells were transfected with let-7b or control miRNA and incubated in the presence of barasertib, with a concentration range from 3.125 nM to 50 nM, for 24 hours before cell harvest. The flow cytometric analysis showed that the 4n and 8n populations had become increased in control miRNA-transfected cells incubated in the presence of 12.5 nM barasertib as expected based on previous studies (Marxer et al., 2014). However, let-7b overexpression elevated the frequency of polyploidy in barasertib treated cell populations when compared to barasertib with excess control miRNA (IV, Fig. 6). The increase in polyploidy in let-7b-transfected cells compared to controls was restored throughout the barasertib concentration gradient but the difference was smaller at higher barasertib concentrations. Thus, let-7b has an additive effect on polyploidy induction in combination with barasertib.

5.4.4. Let-7b expression negatively correlates with tumor grade and Aurora B expression in breast cancer patients

In cancer patients, low let-7b expression has been linked to a poor prognosis (Nam et al., 2008; Takamizawa et al., 2004). Let-7b expression was retrospectively analyzed in a breast cancer patient cohort consisting of 101 patients. The tumors had been profiled for the expression of 799 miRNAs (Enerly et al., 2011). Let-7b expression negatively correlated with increasing tumor grade (IV, Fig. 7A). Additionally, the miRNA expression was analyzed in previously established molecular subtypes of breast cancer. The subtypes are based on gene expression (Perou et al., 2000; Sorlie et al., 2001) and associated with survival, where the basal-like tumors have the worst prognosis. Let-7b expression was lower in the HER2 and basal-like tumor subtypes than in the normal-like and luminal A and B subtypes (IV, Fig. 7B). Relapse-free survival records, available for 96 patients, showed that low let-7b expression was associated with poorer survival as compared to high let-7b expression (IV, Fig. 7C). Moreover, let-7b was lower expressed in HER2 positive tumors, ER negative tumors and *TP53* mutated tumors (IV, Fig. 7D-F). Interestingly, let-7b expression was found to negatively correlate with Aurora B expression in different tumor grades (Pearson correlation -0.41; $p<0.001$) supporting our results showing that let-7b targets Aurora B. For instance, let-7b expression was low in grade 3 tumors whereas Aurora B expression was elevated (IV, Fig. 7G). Moreover, these observations were reproduced by examining The Cancer Genome Atlas Network database (Cancer Genome Atlas Network, 2012). An analysis of let-7b expression, available for 395 breast cancer patients, revealed a high similarity to the Micma cohort. Let-7b expression was low in the HER2 and basal-like subtypes of breast cancer and there was also lower expression in HER2 positive and in ER negative tumors as compared to the opposite statuses (IV, Supplementary Figure A.4A-C). In addition, Aurora B expression was found to negatively correlate with let-7b expression (Pearson correlation -0.37; $p<0.001$; IV, Supplementary Figure A.4D).

Additionally, the expression of other let-7 family members was analyzed in different tumor grades and correlated with clinicopathological markers (IV, Supplementary Fig. A.5 and Supplementary Table A.1). The analysis revealed that let-7a, let-7b, let-7c, let-7e and let-7f were all downregulated in high grade tumors. This was more evident for the -5p strands as compared to the -3p strands processed from the same pre-miRNA. Interestingly, of all the analyzed let-7 miRNAs, let-7b-5p was the most significantly downregulated in HER2 positive, ER negative, and *TP53* mutated tumors. Let-7b-5p also displayed the clearest inverse correlation with increased tumor grade (IV, Supplementary Table A.1). The results suggest that low let-7b expression correlates with tumor

aggressiveness and high Aurora B expression. Furthermore, these results from patient sample analysis and cell experiments support previous studies indicating that overexpression and inhibition of Aurora B can both induce polyploidy and aneuploidy (Hauf et al., 2003; Honda et al., 2003; Kallio et al., 2002; Ota et al., 2002; Terada et al., 1998), which are characteristic of cancer cells.

6. DISCUSSION

6.1. The characterization of Centmitor-1 (I)

A sequential virtual and cell-based HTS was designed in order to identify LMW compounds possessing a similar chemical interaction field and inducing a comparable anti-mitotic phenotype as the template compound rigosertib, which is in clinical trials for the treatment of solid tumors and blood malignancies (Jimeno et al., 2009; Ma et al., 2012; Seetharam et al., 2012). Rigosertib perturbs the growth of several cancer cell lines and shows efficacy against tumors in xenograft models (Gumireddy et al., 2005). However, the mechanism of action of the compound remains to be clarified. To further investigate the mechanism, the HTS was set up to find compounds that would possess freedom to operate rights. Therefore, the aim was to identify compounds with different molecular structures but similar chemical interaction fields as rigosertib. The combined virtual and cell-based HTS led to the identification of Centmitor-1, a compound that induces multipolarity, centrosome fragmentation and errors in chromosome congression leading to SAC-mediated mitotic arrest and cell death or aberrant exit from mitosis. The anti-proliferative phenotype induced by Centmitor-1 very closely resembles that induced by rigosertib. The aim of this study was to characterize the anti-mitotic effects of both compounds in cells.

Centmitor-1 also shows a similarity with its template compound in terms of its impact on microtubules. Both compounds interfered with microtubule dynamics in interphase cells. Moreover, the subcellular localization of the plus end protein EB1 was changed by both compounds as compared to control cells. Depletion of EB1 has been shown to reduce microtubule dynamics and spindle length (Goshima et al., 2007; Rogers et al., 2002; Tirnauer et al., 1999), which suggests that the protein controls the mitotic spindle structure. Indeed, Centmitor-1 and rigosertib also shortened the spindle length in mitotic cells. Thus, it is possible that the compounds impair EB1-mediated processes but whether this is a direct or an indirect action remains to be clarified. Furthermore, mitotic cells treated with Centmitor-1 or rigosertib showed decreased interkinetochore distances indicative of low tension between the sister chromatids. The treated cells also exhibited active Aurora B and SAC. At the moment, it is not possible to state whether the effect of the compounds on microtubules is a direct effect on tubulin or caused by inhibition of a protein facilitating microtubule-mediated functions. Alternatively, the impact on microtubules might be mediated by a combination of both. However, low doses of microtubule drugs can induce acentrosomal spindle poles (Bian et al., 2010), chromosome misalignment (Kelling et al., 2003), mislocalization of NuMA and

EB1 (Morrison et al., 1998; Rousselet, 2009; Woodard et al., 2010), and reduced interkinetochore tension (Kelling et al., 2003), which are all in line with the phenotype caused by Centmitor-1 and rigosertib. Nevertheless, the compounds do not seem to perturb tubulin polymerization *in vitro* at least at low concentrations (Gumireddy et al., 2005; Maki-Jouppila et al., 2014), which would support the concept that the inhibition of microtubule dynamics is indirect. It should be noted that this was previously reported at only one rigosertib concentration (Gumireddy et al., 2005).

Rigosertib has been claimed to target the PI3K and Plk1 pathways (Chapman et al., 2012; Gumireddy et al., 2005; Prasad et al., 2009). However, this mechanism of action has been controversial (Lan et al., 2012; Steegmaier et al., 2007; Strebhardt, 2010). According to our results, the target(s) of Centmitor-1 and rigosertib do occur in mitotic cells, but the compounds do not target the Plk1 or PI3K, as determined by FRET and Western blotting. In general, Plk1 inhibition by small compounds induces the formation of a monopolar spindle (Lenart et al., 2007) whereas Centmitor-1 and rigosertib treatments lead to multipolarity according to the immunostainings. This further supports the hypothesis that the compounds are not targeting Plk1 activity. However, the compounds might still affect the substrates of Plk1 and PI3K and thus influence their pathways. Furthermore, the compounds can have cell line specific effects, for example due to alterations in the expression levels of the target protein.

In summary, the results suggest that the virtual HTS approach can identify novel anti-mitotic drug candidates by using a known compound as a template. A LMW compound termed Centmitor-1 was identified that induced similar phenotype in cells as rigosertib, a drug that is in clinical trials for the treatment of cancer. Both compounds reduce microtubule dynamics and cause mitotic defects by targeting a protein or proteins present in mitotic cells (Fig. 14). The detailed mechanism of action of Centmitor-1 and rigosertib and the identity of their target(s) remain to be investigated in future studies.

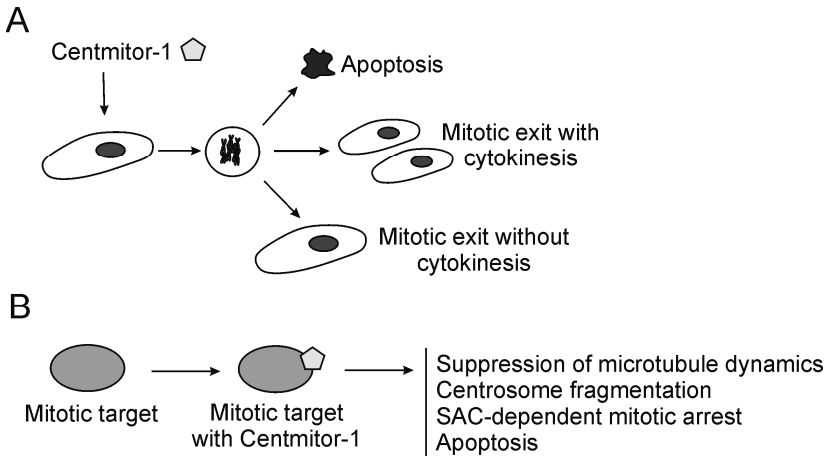

Figure 14. A summary of Centmitor-1 effects on mitosis on cellular (A) and molecular (B) levels.

6.2. Hec1 inhibition by LMW compounds (II)

Tubulin polymerization is a successful target of cancer therapies. In proliferating cells, spindle microtubules are highly dynamic and sensitive to microtubule drugs that bind directly either to soluble tubulin or to the microtubules. However, microtubule-targeting drugs also affect the microtubules of differentiated cells and show poor cancer cell selectivity. Microtubule-stabilizing drugs such as paclitaxel, docetaxel and epothilones as well as microtubule-depolymerizers like vinblastine and vincristine cause side effects such as neurotoxicity (Dumontet and Jordan, 2010) since microtubules have important functions in neuronal signaling. Inhibition of Hec1 could be a more cancer cell specific way to indirectly suppress microtubule functions because the protein is highly expressed in tumors and in proliferating cells and acts to facilitate proper microtubule-chromosome association. This approach could also reduce side effects in patients as compared to the drugs directly affecting microtubules. In breast cancer, high Hec1 expression is associated with the early and late stages of tumorigenesis (Bieche et al., 2011), which suggests the use of future Hec1 therapies in the treatment of breast cancer patients. Moreover, Hec1 overexpression in a transgenic mouse model was related to increased tumorigenesis (Diaz-Rodriguez et al., 2008). Depletion of Hec1 by siRNA or virus-mediated RNAi has been shown to suppress cancer cell growth in cell culture and in xenograft models (Gurzov and Izquierdo, 2006; Li et al., 2007), which further emphasizes the anti-cancer potency of Hec1 inhibition. Finally, modification of Hec1 N-terminus (EGFP-Hec1) was recently shown to suppress tumor growth in a mouse xenograft model by

disrupting mitosis and interfering with kinetochore-microtubule dynamics (Orticello et al., 2014). A sequential virtual and cell-based HTS was designed and executed to identify novel LMW compounds that bind to Hec1 and perturb its association with microtubules.

From the *in silico* HTS for compounds that could target the CHD of Hec1, the most promising compounds were purchased and tested in a cell-based phenotype screen. One compound, termed VTT-006, was selected for further validation based on the cellular anti-mitotic phenotype that it induced and its ability to bind the recombinant Ndc80 complex. Anisotropy measurements indicated binding of VTT-006 to the Ndc80 complex although the affinity was relatively low. VTT-006 may belong to a category of general-affinity ligands that typically exhibit K_d values in the range of 1 μM to 100 μM and have reduced target selectivity (Labrou and Clonis, 1994). Therefore, the possibility of VTT-006 having other targets in addition to Hec1 cannot be excluded. The intermediate binding affinity of VTT-006 can enable switching between bound and unbound states.

The results from cell-based and biochemical *in vitro* assays are in line with the intermediate affinity of VTT-006 for Hec1 possibly leading to continuous switching between the attached and detached states of the compound (Fig. 15). VTT-006 shortened the residence time of Ndc80-GFP on taxol stabilized microtubules, as detected by TIRF microscopy but did not completely abolish the binding of the complex. Previously, a similar effect has been reported when Aurora B phosphorylation sites in the N-terminal tail of Hec1 were mutated or when the whole tail region was removed (Umbreit et al., 2012). Live cell imaging and immunofluorescence stainings revealed that VTT-006 induces partial chromosome misalignment with stable kinetochore-microtubule attachments and reduced interkinetochore tension. These results are supported by an earlier study showing that mutating the Hec1 CHD at lysine (Lys) residues 89, 115 and 116 induces chromosome misalignment, reduces interkinetochore tension and causes mitotic arrest (Tooley et al., 2011). Furthermore, mutations in the CHD of Nuf2, Hec1 binding partner, also induce chromosome misalignment and cold stable kinetochore-microtubule attachments as well as reduced interkinetochore tension (Sundin et al., 2011). Thus, VTT-006 induces a similar phenotype as mutating microtubule binding sites in the Ndc80 complex, supporting the concept that VTT-006 can target the CHD of Hec1. Moreover, VTT-006 was observed to perturb normal metaphase chromosome oscillations, which can indicate errors in end-on attachments or changes in microtubule dynamics. The Ndc80 complex has been reported to regulate microtubule dynamics (Umbreit et al., 2012), and the phosphorylation status of the Hec1 tail region has been shown to correlate with the amplitude of

oscillations (Zaytsev et al., 2014). The present results, combined with the previous findings, suggest that the inhibition of Hec1 by VTT-006 interferes with microtubule dynamics and interkinetochore tension observed as the dampened oscillations. VTT-006 also perturbed the ability of cells to maintain correct kinetochore-microtubule attachments as the introduction of the compound into metaphase cells led to a disruption of the metaphase plate. However, the lateral attachments formed in early mitosis were not affected by VTT-006, which is in line with previous studies indicating that the Ndc80 complex is not required for lateral attachments (Vorozhko et al., 2008).

The intermittent binding of VTT-006 to Hec1 is also supported by the range of phenotypes observed in fixed immunostained cells as well as in time-lapse filmed cells. The amount of misaligned chromosomes varied between cells of the same population and cells with either bipolar or multipolar spindle structure were observed although a bipolar spindle was the more prominent phenotype. One may speculate that the chromosomes instantaneously establishing proper amphitelic attachments in early mitosis can congress to the metaphase plate in the presence of VTT-006 while the chromosomes with erroneous syntelic attachments stay near the poles due to perturbed error correction. In addition, the accessibility of the VTT-006 binding pocket may vary in different kinetochores due to conformational differences between individual Ndc80 complexes, which can contribute to the occurrence of different phenotypes. Furthermore, when VTT-006 is administered to metaphase cells, the amphitelic attachments become disrupted, leading to a loss of chromosome alignment. This could be due to the dynamic instability of microtubules, which allows binding of VTT-006 upon microtubule depolymerization.

VTT-006 is a novel anti-mitotic compound that induces mitotic arrest followed by cell death (Fig. 15). The compound causes a stronger mitotic arrest than INH1, a Hec1 inhibitor that perturbs the interaction between Hec1 and Nek2 and that was reported to induce cell death after mitotic slippage (Wu et al., 2008). The cell viability was decreased by VTT-006 in several cancer cell lines and also in the organotypic 3D culture of breast cancer cells. However, since cell viability became suppressed only at micromolar concentrations of VTT-006, it is clear that further chemical optimization of the compound will be needed to improve its efficacy. Moreover, the ADME properties of VTT-006 need to be improved before xenograft mouse assays can be performed. These *in vivo* experiments would reveal whether VTT-006 can retard tumor growth similar to INH1. In the meantime, VTT-006 can however be used as an experimental tool in the field of mitosis research.

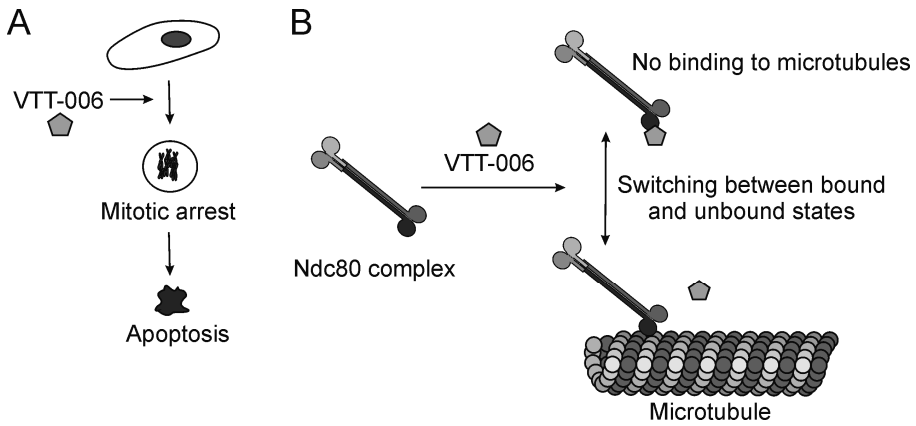

Figure 15. A summary of the cellular phenotype induced by VTT-006 (A) and the hypothesized mode of action on the level of a single microtubule (B).

6.3. Perturbation of mitotic fidelity by excess miR-378a-5p (III)

Previously, miR-378a-5p has been implicated in angiogenesis (Lee et al., 2007) and energy metabolism (Eichner et al., 2010). Our results show that miR-378a-5p also perturbs mitosis and genomic stability. An excess of miR-378a-5p upregulated VEGF-A in accordance with earlier studies (Hua et al., 2006), and increased the phosphorylation of PDGFR-β, VEGFR-2, ErbB2 and EphA7. Earlier, VEGF-A has been reported to phosphorylate PDGFR-β, VEGFR-2, and EphA7 (Ball et al., 2007). By activating PDGFR-β, VEGFR-2 and ErbB2 pathways, VEGF-A can subsequently activate ERK1/2 kinase (Jurek et al., 2011; Narasimhan et al., 2009; Pinkas-Kramarski et al., 1998), which is supported by our results showing increased phosphorylation of ERK1/2 by excess miR-378a-5p. Furthermore, the miRNA decreased levels of Aurora B kinase, which can explain the observed phenotype: forced exit from mitosis, multipolarity and aneuploidy. Accordingly, chemical inhibition and depletion of the kinase have been shown to cause an override of microtubule drug-induced mitotic arrest and to induce polyploidization due to interference with the functions of Aurora B in SAC signaling and cytokinesis (Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002). However, miR-378a-5p did not directly bind to the mRNA of Aurora B. Therefore, the detailed mechanism of action remains to be revealed. In addition, one cannot exclude the possibility of other miRNA targets contributing to the mitotic phenotype observed in cells that overexpress miR-378a-5p.

What could be the direct target of miR-378a-5p? The effects of miR-378a-5p on cells resemble the depletion of Raf kinase inhibitory protein (RKIP), which

has been reported to activate the Raf-1/MEK/ERK pathway, reduce Aurora B activity and mRNA levels, and induce insensitivity of cells towards taxol treatment (al-Mulla et al., 2011; Eves et al., 2006). Therefore, it is possible that the miRNA targets RKIP. However, the link between Aurora B and the RKIP/ERK pathway is unknown. On the other hand, the target could be a transcription factor or a co-factor that controls Aurora B mRNA expression. One possible candidate is Forkhead box protein M1 (FOXM1), which has been reported to regulate Aurora B expression (Wang et al., 2005). However, FOXM1 also controls transcription of other mitotic regulatory genes such as Cdc25B, Survivin, Cenp-A, and Cenp-B (Wang et al., 2005), which could contribute to the anti-mitotic phenotype in miR-378a-5p-transfected cells.

Altered Aurora B expression has been linked to the CIN and aneuploidy characteristic of tumorigenesis (Hauf et al., 2003; Honma et al., 2013; Ota et al., 2002). It was found that the expression of miR-378a-5p and its host gene *PPARGC1B* correlate with the clinicopathological markers of breast cancer. The miRNA expression was elevated in high grade tumors and in basal-like tumor subtype. The basal-like subtype is associated with poor survival and tumor aggressiveness, and the basal-like tumors lack the expression of ER and PR as well as the amplification of HER2 (Valentin et al., 2012). The expression of miR-378a-5p was accordingly higher in ER negative compared to ER positive tumors. Furthermore, the high miRNA expression was associated with a high proliferation status in a tumor. These results support the concept that miR-378a-5p expression increases during breast cancer progression (Eichner et al., 2010). The host gene *PPARGC1B* showed a similar expression correlation with the clinicopathological markers as did miR-378a-5p. This can be explained by the frequent coexpression of a host gene and a miRNA (Baskerville and Bartel, 2005).

In summary, the results show that excess miR-378a-5p induces elevated aneuploidy that is a typical characteristic of tumors (Fig. 16). Together with previous results, these findings suggest that excess miR-378a-5p has multiple ways to promote tumorigenesis. The miRNA can increase tumor angiogenesis (Lee et al., 2007), regulate the shift from oxidative to glycolytic metabolism (Eichner et al., 2010), and increase aneuploidy and thus impair genomic fidelity (Winsel et al., 2014). Furthermore, according to the results, excess miR-378a-5p may reduce the cancer cells' sensitivity to microtubule-targeting drugs such as taxanes, which may lead to the induction of drug resistance in the clinics. It will be interesting to see whether anti-miRs antagonizing miR-378a-5p can tackle the oncogenic properties of this miRNA in the future. Moreover, the results suggest that miR-378a-5p could be used as a biomarker in cancer diagnostics for example to assist in tumor grading and drug response prediction.

Earlier, expression analysis of miR-378a-5p has shown potential in the detection of gastric cancer (Liu et al., 2012b) supporting the present results. However, in renal cell carcinoma, the use of the miRNA as a biomarker remains controversial (Hauser et al., 2012; Redova et al., 2012) and needs to be further investigated.

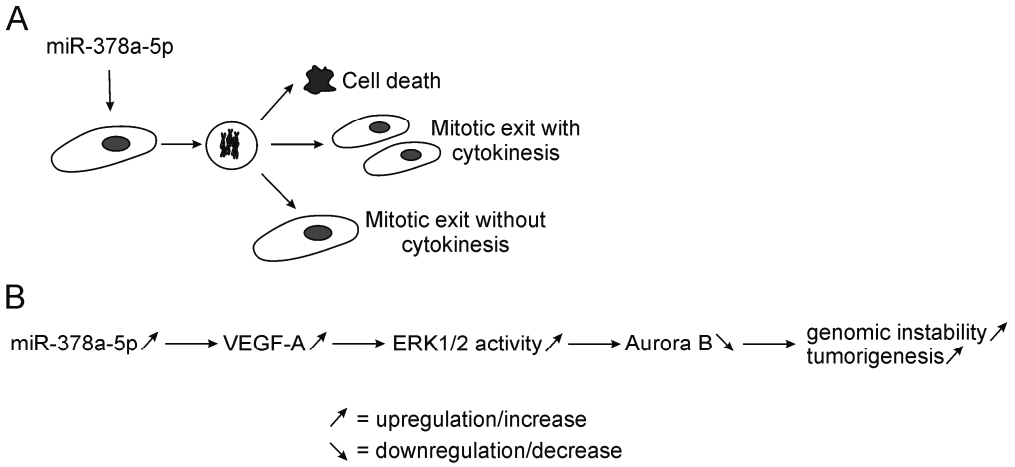

Figure 16. A summary of the mitotic phenotypes induced by excess miR-378a-5p (A) and a model of how the miRNA affects genomic stability and tumorigenesis (B).

6.4. Let-7b as a regulator of Aurora B kinase (IV)

The members of the let-7 miRNA family have been shown to act as tumor suppressor miRNAs that reduce cell proliferation and inhibit cancer progression (He et al., 2010; Johnson et al., 2005; Kumar et al., 2008). However, the influence of let-7 miRNAs on mitosis and cell cycle regulation has not been described earlier. Our results show that let-7b binds to Aurora B 3'UTR, subsequently reduces Aurora B mRNA and protein levels, and thereby participates in the control of SAC signalling and mitotic fidelity. Excess let-7b causes forced exit from chemically induced mitotic arrest, polyploidy, multipolarity, and aneuploidy, all associated with loss-of-function of Aurora B kinase (Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003; Tanaka et al., 2002). Multipolarity and polyploidy are likely consequences of Aurora B perturbation in late mitosis where the kinase controls cleavage furrow localization and proper cytokinesis by phosphorylating its substrates such as centralspindlin (Guse et al., 2005). Furthermore, aneuploidy can be caused by chromosome missegregation resulting from the persistent presence of erroneous syntelic attachments that are not corrected in the absence of Aurora B kinase

activity (Hauf et al., 2003). Although the phenotype induced by let-7b overexpression greatly resembles that caused by Aurora B inhibition, one cannot exclude the influence of other possible mitotic targets of let-7b. For example, let-7b targets two cell cycle regulators, Cyclin D1 and Cdc25A (Guo et al., 2013; Johnson et al., 2007; Schultz et al., 2008). Cyclin D1 forms a complex with Cdk4 or Cdk6 and drives the transition from the G1 to the S phase. The phosphatase Cdc25A activates Cdk kinases by dephosphorylation at G1/S and G2/M transitions. Therefore, the inhibition of both proteins can delay the progression of the cell cycle.

In cancer, Aurora B is commonly overexpressed (Carter et al., 2006) whereas the expression of let-7 miRNAs is low (Takamizawa et al., 2004; Yu et al., 2007). Both, high Aurora B expression and low let-7b expression have been separately reported to correlate with poor patient prognosis (Kurai et al., 2005; Nam et al., 2008). Furthermore, overexpression of let-7 miRNAs can suppress tumorigenesis and cancer metastasis (He et al., 2010; Johnson et al., 2007; Yu et al., 2007). The present results revealed a negative correlation between Aurora B and let-7b expression in tumors from breast cancer patients. Aurora B is higher expressed in grade 3 tumors whereas let-7b is low expressed in the same tumor type. The results from cell experiments and the analysis of breast cancer gene expression data suggest that let-7b can regulate Aurora B levels in cancer. It should be noted that both overexpression and inhibition of Aurora B can induce genomic instability (Hauf et al., 2003; Honda et al., 2003; Kallio et al., 2002; Ota et al., 2002; Terada et al., 1998). This indicates that the correct expression level of Aurora B is essential for accurate progression of mitosis. Low let-7b expression was found to correlate with poorer survival compared to patients with high let-7b expression. In addition, let-7b was less expressed in HER2 positive, ER negative and *TP53* mutated breast cancers, which are all associated with reduced patient survival. Therefore, let-7b could be used as a biomarker in cancer diagnostics in the future. Moreover, restoration of let-7b expression for example by miRNA mimics might reduce or halt tumorigenesis. However, it should be noted that since excess let-7b was observed to cause a forced exit from microtubule drug induced mitotic arrest, exogenous overexpression of the miRNA may lead to the development of resistance towards microtubule drugs via premature inactivation of the SAC. On the other hand, an analysis of let-7b expression could assist in prediction of clinical drug efficacy; patients with high let-7b expression in tumor tissue may not benefit from taxane therapies as much as those patients whose tumors show low let-7b levels. Finally, in cells, the overexpression of let-7b had an additive effect on polyploidy induction in the presence of barasertib, an Aurora B inhibitor. This observation suggests the use of combinatorial treatments of miRNA-based therapies and LMW compounds in future cancer therapies. Furthermore, the use

of let-7b expression profiles in the stratification of patients for future Aurora B inhibition therapies could be evaluated. If the additive effect on polyploidy induction is repeated in patients, high let-7b and low Aurora B expression could possibly predict a better response to Aurora B inhibition. On the other hand, if cancer cells are dependent on the overexpression of Aurora B, excess let-7b may lower the response to Aurora B drugs. However, one cannot exclude the impact of other let-7b targets on the additive effect observed in the presence of barasertib. Therefore, the effects of altered let-7b expression together with an Aurora B inhibition will need to be further studied.

The let-7 family members share the same seed sequence and often regulate the same processes (Farazi et al., 2011; Roush and Slack, 2008). Also in our gene expression analysis, let-7a, let-7b, let-7c, let-7e, and let-7f all showed a similar expression profile in different breast cancer grades. Interestingly, let-7c, let-7e, and let-7f emerged as hit miRNAs in addition to let-7b in the original HTS. However, let-7b expression had the clearest negative correlation with the increasing tumor grade in comparison to other let-7 family members. Let-7a and let-7b possessed very similar expression profiles in breast tumors, probably resulting from the close genomic location of these miRNAs on chromosome 22, which makes common promoter regulation possible. Furthermore, let-7a has been reported to target Aurora B in endometrial carcinoma (Liu et al., 2013) although the effects of let-7a on mitotic processes have not been investigated.

In summary, the results indicate that let-7b participates in the regulation of genomic balance by acting as a modulator of Aurora B expression (Fig. 17). In cancer, altered let-7b expression may contribute to changes in Aurora B expression, which can further promote tumorigenesis. In addition, altered let-7b expression can affect all of the oncogene targets of let-7b further stimulating cancer initiation and progression. Therefore, the potential diagnostic and therapeutic value of let-7b should be investigated in the future.

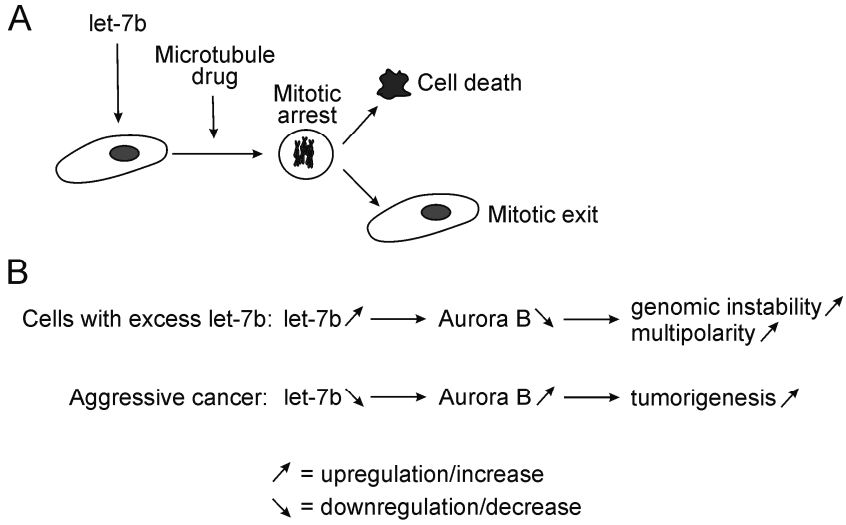

Figure 17. A summary of the main cellular phenotypes induced by excess let-7b (A) and a model of how the miRNA affects genomic stability in cells and tumorigenesis *in vivo* (B).

7. CONCLUSIONS

Mitosis is a potential target for cancer therapies due to the high proliferation rate of tumor cells. Microtubule-targeting drugs, such as taxanes, are extensively used in the clinics but they induce side effects as they target both cancer cells and normal non-tumorigenic cells. Therefore, novel anti-mitotic therapy forms are being developed. Furthermore, miRNA expression profiling could be used in cancer diagnostics to predict the stage of malignancy and the most suitable treatment strategies. The role of miRNAs in mitosis is emerging and it is anticipated that the new information will contribute to the future development of miRNA-based diagnostics and therapy forms.

The first part of the research presented here introduces a new approach for the identification of novel drug candidates that share similar chemical interaction fields with an already known drug or experimental compound. A HTS discovered a potential anti-mitotic compound termed Centmitor-1 that possesses similar steric and electrostatic features as the template compound rigosertib, which is in clinical trials for the treatment of cancer. Both compounds mediated their actions by interfering with microtubule dynamics. Another HTS identified a novel Hec1 inhibitor VTT-006 that can indirectly suppress the microtubule functions in dividing cells. Hec1 is highly expressed in cancer and therefore its inhibition is predicted to result in better cancer cell selectivity and cause fewer side effects compared to current microtubule drugs in clinical use. VTT-006 was shown to bind to the recombinant Ndc80 complex and perturb kinetochore-microtubule interactions *in vitro*. In cells, the compound induced SAC-dependent mitotic arrest with misaligned chromosomes followed by cell death. VTT-006 reduced the cell viability of several cancer cell lines and diminished the growth of cell spheroids in an organotypic 3D model. Both new LMW compounds investigated in this thesis could possibly be used in the development of novel cancer therapies and/or as research tools in the fields of cancer and cell biology.

Altered miRNA expression is associated with the initiation and progression of tumorigenesis. It was discovered that angiogenesis-related miR-378a-5p impaired genomic stability by inducing forced exit from mitosis, polyploidy, and aneuploidy. Excess miR-378a-5p activated the MAPK pathway and suppressed Aurora B kinase expression, which can explain the observed cellular phenotype. In breast cancer patients, high miR-378a-5p expression has correlated with tumor aggressiveness, indicating that the miRNA has oncogenic properties. Based on the observed phenotype, forced exit from microtubule drug imposed mitotic arrest, an excess of miR-378a-5p may induce resistance to the

microtubule drugs in clinical use. Moreover, the chromosomal imbalance induced by excess miR-378a-5p may further promote tumorigenesis. Forced exit from microtubule drug induced mitotic arrest was also a consequence of let-7b overexpression. In addition, excess let-7b caused polyploidy, multipolarity, and aneuploidy, all phenotypes typical of Aurora B loss-of-function. Aurora B 3'UTR was confirmed to be a direct target of the miRNA. Moreover, excess let-7b had an additive effect on the induction of polyploidy in combination with an Aurora B inhibitor basasertib, which suggests that let-7b expression could be used to assess patient response to future Aurora B drugs. Furthermore, combinatorial treatments could be evaluated when the delivery of miRNA-based therapies has improved. In breast cancer patients, let-7b expression negatively correlated with Aurora B expression. Low let-7b expression was characteristic of aggressive forms of cancer and may further allow overexpression of Aurora B and other targets of let-7b, many of which are oncogenes. Both of the identified miRNAs are hoped to possess diagnostic value in the evaluation of tumor progression.

In summary, the results of this thesis provide new information about the function of miRNAs in cancer-associated signaling circuits and introduce HTS approaches to cancer drug discovery. Furthermore, novel anti-mitotic compounds Centmitor-1 and VTT-006 could be used in the development of future cancer therapies and utilised as experimental tools in mitosis research. The miRNAs miR-378a-5p and let-7b both participate in the regulation of mitosis progression and their expression profiling could be utilised in the clinics for the prediction of cancer progression and for planning the treatment strategies. Hopefully, the challenges associated with miRNA delivery into target sites will be resolved in the future allowing the wider use and development of miRNAs as cancer therapeutics. In the meantime, the search for new mitosis targeting small compounds continues towards more cancer cell specific, better tolerable and more efficient therapies.

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