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NEW ROADS TO GENOMIC IMBALANCE: MicroRNA- AND PROTEIN PHOSPHATASE-MEDIATED REGULATION OF MITOSIS

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To my beloved family

“It always seems impossible until its done”
- Nelson Mandela

ABSTRACT

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NEW ROADS TO GENOMIC IMBALANCE: MicroRNA- AND PROTEIN PHOSPHATASE-MEDIATED REGULATION OF MITOSIS

University of Turku, Faculty of Medicine, Institute of Biomedicine, Department of Physiology; Drug Research Doctoral Programme (DRDP); FinPharma Doctoral Program (FPDP); VTT Health, VTT Technical Research Centre of Finland; Turku Centre for Biotechnology.

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The Spindle Assembly Checkpoint (SAC) works to maintain the genomic balance by monitoring the correctness of attachments between the chromosomes and microtubules during early cell division (mitosis). Importantly, chromosome missegregation and genomic instability is caused by defects in SAC function, which can lead to cell transformation and cancer. Moreover, malfunction of SAC can confer resistance to microtubule-targeting drugs (MTAs) such as paclitaxel. The identification and functional characterization of novel SAC regulating biomolecules and analysis of their expression profiles in tumor cells may in the future facilitate improved cancer diagnosis and predict patient's response to MTA therapy. The microRNA- (miRNA) and protein phosphatase-mediated regulation of mitotic signaling were investigated in my thesis. First, to identify new miRNAs that regulate SAC signaling, a cell-based high-throughput screen (HTS) was performed to test the ability of 810 different pre-miRNAs to override a drug imposed M phase arrest. The HTS led to the discovery of miR-378a-5p and miR-493-3p as suppressors of MTA action *in vitro*. Further molecular biology experiments revealed that miR-378a-5p and miR-493-3p negatively regulated the expression of *AURKB* and *MAD2L1,* respectively. Retrospective analysis of ovarian and breast cancer samples *in vivo* indicated deregulated expression of both miRNAs in different tumor grades and subtypes compared to healthy tissues. Moreover, high expression of miR-493-3p was found to associate with reduced survival of ovarian and breast cancer patients with aggressive tumors, especially if the patients were treated with paclitaxel chemotherapy in comparison to epirubicin. Secondly, the functions of the Dual specificity protein phosphatase 3 (Dusp3) in dividing cells were explored. The maintenance of bipolar spindle architecture during mitosis was found to be dependent on Dusp3. In summary, the previously unknown mitotic functions for three biomolecules, namely miR-378a-5p, miR-493-3p and Dusp3 were identified. The determination of miR-378a-5p and miR-493-3p expression profiles, in tumor samples can in the future assist in cancer subtyping and selecting the most effective chemotherapy for ovarian and breast cancer patients with advanced disease.

Keywords: mitosis, microRNA, spindle assembly checkpoint, Dusp3

TIIVISTELMÄ

Mahesh Tambe

MikroRNA- JA PROTEIINI FOSFATAASI – VÄLITTEINEN MITOOSIN SÄÄTELY

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia; Lääketutkimuksen tohtoriohjelma; Suomen lääketutkimuksen tohtoriohjelma; VTT Health, Teknologian tutkimuskeskus VTT; Turun Biotekniikan Keskus.

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Solujaon (mitoosin) tarkastuspiste on solunsisäinen viestinvälitysjärjestelmä, joka pyrkii ylläpitämään perimän tasapainoa tarkkailemalla solun kromosomien ja sukkularihmojen välisten kytkentöjen oikeaoppisuutta. Tarkastuspiste estää kromosomien jakautumisen, kunnes kaikki solun kromosomit ovat saavuttaneet kaksinapaisen kytkennän tumasukkulan säikeiden kanssa. Virheet tarkastuspisteen toiminnassa aiheuttavat kromosomien vääränlaisen jakautumisen ja perimän epätasapainotilan, joka puolestaan voi edistää solujen pahanlaatuista muuntumista ja syövän syntymistä. Lisäksi mitoottisen tarkastuspisteen toimintahäiriöt voivat aiheuttaa solujen resistenssiä sukkularihmaston toimintaa salpaaville lääkeaineille, kuten paklitakselille. Tarkastuspisteen toimintaa säätelevien uusien biomolekyylien tunnistaminen sekä niiden tehtävien ja ilmentymisen tutkiminen voi tulevaisuudessa edistää syövän diagnosointia ja taudin etenemisen ennustettavuutta. Tässä väitöskirjatyössä on tarkasteltu mikro-RNA (miRNA) ja proteiini fosfataasi–välitteistä solujaon säätelyä. Ensimmäisessä tutkimuslinjassa suoritettiin solupohjainen tehoseulonta uusien mitoottista tarkastuspistettä säätelevien miRNA-molekyylien tunnistamiseksi. 810:n pre-miRNA:n kyky kumota lääkeaineen aiheuttama mitoosin pysähtyminen testattiin ja miR-378a-5p:n ja miR-493-3p:n havaittiin estävän mikrotubuluslääkeaineiden normaalin toiminnan viljellyissä syöpäsoluissa. Molekyylibiologiset lisäkokeet paljastivat, että miR-378a-5 säätelee negatiivisesti *AURKB*-geeniä ja miR-493-3p *MAD2L1*-geeniä. Retrospektiivinen munasarja- ja rintasyöpäpotilasnäytteiden tutkiminen osoitti molempien miRNA-molekyylien ilmentymisen muuttuneen eriasteisissa tuumoreissa ja syövän eri alatyypeissä terveeseen kudokseen verrattuna. Lisäksi havaittiin yhteys kohonneen miR-493-3p:n ilmentymisen ja aggressiivista munasarja- tai rintasyöpää sairastavien potilaiden lyhentyneen elinajanodotteen välillä. Tämä havainto oli erityisen merkittävä paklitakseli -lääkeainetta saaneen potilasryhmän sisällä verrattuna ebirubisiini- terapiaryhmään. Toisessa tutkimuslinjassa tarkasteltiin Dusp3–fosfataasin toimintaa jakautuvissa soluissa. Havaittiin, että fosfataasia tarvitaan ylläpitämään tumasukkulan normaali rakenne solujaon aikana. Yhteenvetona voidaan todeta, että työssä löydettiin aikaisemmin tuntemattomia solujaon aikaisia tehtäviä kolmelle biomolekyylille, miR-378a-5p:lle, miR-493-3p:lle ja Dusp3 -fosfataasille. miR-378a-5p:n ja miR-493-3p:n ilmentymisen määrittäminen kasvaimista voi tulevaisuudessa auttaa syöpätyyppien luokittelussa ja tehokkaimman lääkehoidon valinnassa edennyttä munasarja- ja rintasyöpää sairastavilla potilailla.

Avainsanat: mitoosi, mikroRNA, mitoottinen tarkastuspiste, Dusp3

Table of Contents

ABBREVIATIONS

ABC	ATP binding cassette
A-Dusp	Atypical Dusp
AGO	Argonaute
APC/C	Anaphase Promoting Complex / Cyclosome
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5'-triphosphate
ATR	Ataxia Telangiectasia And Rad3-Related Protein
AURKA	Aurora Kinase A
AURKB	Aurora Kinase B
AURKC	Aurora Kinase C
BAK-1	BCL2-Antagonist/Killer 1
BBC	Bub3:BubR1:Cdc20
Bcl-2	B-Cell CLL/Lymphoma 2
BRCA1	Breast-Cancer-Associated-gene 1
Bub1	Budding uninhibited by benzimidazoles 1
Bub3	Budding uninhibited by benzimidazoles 3
BubR1	Budding uninhibited by benzimidazole-related 1
CCC	Clear cell carcinoma
Cdc14	Cell division cycle 14
Cdc20	Cell division cycle 20
Cdc25	Cell division cycle 25
Cdh1	Cdc20 homologue 1
Cdk1	Cyclin dependent kinase 1
Cdk2	Cyclin dependent kinase 2

Cenp-A	Centromere protein-A
Cenp-C	Centromere protein-C
Cenp-E	Centrosome-associated protein E
Cenp-T	Centromere protein-T
CEP57	Centrosomal Protein 57kDa
Chk1	Checkpoint Kinase 1
CIN	Chromosomal instability
Cks	Cyclin-dependent kinase regulatory subunit
C-Mad2	closed-Mad2
c-miRNA	Circulating miRNAs
CPC	Chromosomal Passenger Complex
D-box	Destruction box
DGCR8	DiGeorge syndrome critical region 8
DKK1	Dickkopf-1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
sno-RNA	Small nucleolar RNA
DSB	Double strand break repair
DSS	Disease specific survival
Dusp	Dual specificity phosphatases
E2F1	E2F transcription factor 1
Eg5	Kinesin Family Member 11
Egf	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence *in situ* hybridization
Emi1	Early Mitotic Inhibitor 1
ER	Estrogen Receptor
ErbB2	Erythroblastic leukemia viral oncogene homolog 2

Erk1/2	Extracellular signal-regulated kinase 1 and 2
ERRγ	Estrogen-Related Receptor Gamma
Esco1	Establishment Of Sister Chromatid Cohesion N-Acetyltransferase 1
Esco2	Establishment Of Sister Chromatid Cohesion N-Acetyltransferase 2
FOXM1	Forkhead box protein M1
FZD4	Frizzled class receptor 4
G1	Gap1
G2	Gap2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLEBS motif	Gle2-binding-sequence
GW	Greatwall kinase
GW182	Glycine tryptophan repeat-containing protein of 182 kDa
HCV	Hepatitis C-virus
Hec1	Highly expressed in cancer 1
Her2	Human epidermal growth factor receptor 2
HGSC	High-grade serous ovarian cancer tumors
HPV	Human Papiloma Virus
HTS	High-throughput screen
IGF1R	Insulin-like growth factor 1
INCENP	Inner centromeric protein
Jnk	c-Jun N-terminal kinase
KARD domain	Kinetochore attachment regulatory domain
KDM2A	Lysine (K)-Specific Demethylase 2A
Kgf	Keratinocyte Growth Factor
Kif2b	Kinesin Family Member 2B
KMN	Knl1 Mis12 Ndc80
Knl1	Kinetochore null 1

M18BP1	Mis18-binding protein 1
Mad1	Mitotic arrest deficient 1
Mad2	Mitotic arrest deficient 2
MAP	Microtubule associated proteins
Mapk	Mitogen-activated protein kinase
MCAK	Mitotic Centromere-Associated Kinesin
MCC	Mitotic Checkpoint Complex
MELT	methionine-glutamate-leucine-threonine
Mek1/2	Mitogen-Activated Protein Kinase Kinase 1
miRISC	miRNA induced silencing complex
miRNA	Micro-RNA
Mis12	Missegregation 12
MKB	Mapk binding
MKK7	Mitogen-Activated Protein Kinase Kinase 7
Mklp2	Mitosis kinesin-like protein 2
MKP	Mapk phosphatase
M phase	Cell division phase
Mps1	Monopolar spindle 1
mRNA	messenger RNA
MTA	Microtubule targeting agent
MTOC	Microtubule organizing center
MVA	Mosaic variegated aneuploidy
NSCLC	Non-small cell lung cancer
Ndc80	Nuclear division cycle 80
NEBD	Nuclear envelope breakdown
Nek2A	NIMA-related kinase 2A
Nuf2	Nuclear filamentous 2
O-Mad2	open-Mad2

P19ARF	Cyclin-Dependent Kinase Inhibitor 2A
$P21^{cip1}$	Cyclin-Dependent Kinase Inhibitor 1A
PCM	Pericentriolar material
PCR	Polymerase chain reaction
Pdgf	Platelet-Derived Growth Factor
piRNA	PIWI-interacting RNA
Pkc	Protein kinase C
Plk1	Polo like kinase 1
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1 beta
PR	Progesterone receptor
Pre-miRNA	Preliminary-miRNA
Pri-miRNA	Primary-miRNA
PTEN	Phosphatase and tensin homologs
PTP	Protein tyrosine phosphatase
qRT-PCR	Quantitative real-time polymerase chain reaction
Rb	Retinoblastoma
REST	Repressor-element-1-silencing transcription factor
Rhoc	Ras homolog family member C
RKIP	Raf kinase inhibitory protein
RNA	Ribonucleic acid
RNAi	RNA interference
RTK	Receptor tyrosine kinase
RZZ	Rod-ZW10-Zwilch
S phase	Synthesis phase
SA1	Stromal Antigen 1
SA2	Stromal Antigen 2

SAC	Spindle assembly checkpoint
Ser/Thr	serine/threonine
Sgo	Shugoshin
siRNA	Small interfering RNA
Ska	Spindle and kinetochore-associated protein
Smc1	Structural Maintenance Of Chromosomes 1
Spc	Spindle pole component
STAT5	Signal transducer and activator of transcription-5
TCGA	The Cancer Genome Atlas
TRBP	Transactivation-responsive RNA binding protein
Trip13	Thyroid Receptor Interacting Protein 13
TSB	Target-site blocker
UTR	Untranslated region
VEGF-A	Vascular endothelial growth factor-A
Vhr	Vaccinia Virus Phosphatase VH1-Related

LIST OF ORIGINAL PUBLICATIONS

The thesis consists of three publications as listed below. In the text the Roman letters I-III are used to denote these publications. The thesis consists of unpublished data as well. The original communications have been reproduced with the permission of the copyright holders

I. 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.

II. **Tambe M*,** Pruikkonen S*, Mäki-Jouppila J, Chen P, Vilming Elgaaen B, Straume A, Huhtinen K, Cárpen O, Lønning P, Davidson B, Hautaniemi S, & Kallio M. Novel Mad2-targeting miR-493-3p controls mitotic fidelity and cancer cells' sensitivity to paclitaxel. Oncotarget. 2016. 7(11): 12267-12285.

III. **Tambe M,** Narvi E**,** Kallio MJ. Reduced levels of Dusp3/Vhr phosphatase impair normal spindle bipolarity in an Erk1/2 activity-dependent manner. FEBS Letters. 2016. *doi: 10.1002/1873-3468.12310.* (Epub ahead of print).

* Equal contribution

1 INTRODUCTION

Cell division (mitosis) forms the basis for cellular proliferation and normal growth and development of an organism. Mitosis aims for equal distribution of the DNA of a mother cell between two forming daughter cells. Mechanistically and morphologically mitosis is a demanding task for a cell: many cellular organelles and elements of the cytoskeleton are reorganized, a bipolar spindle apparatus is built, chromatin condenses and folds into chromosomes that bind to microtubules and later segregate, and the cell is split in two. All this is achieved in a short time-frame of about 60 min in human cells. The complexity and temporal overlap of many of mitotic events require precise functional coordination between several signaling cascades and more than a hundred different proteins that work in concert to ensure fidelity of cell division. The most important mitotic signaling pathway is the Spindle Assembly Checkpoint (SAC), which blocks the cell division if errors are detected in chromosomal and/or spindle events (Musacchio, 2015). For this reason, defective function of the SAC puts the dividing cells at risk of delivering a wrong number of chromosomes and/or structurally deformed chromosomes to the daughter cells, an event also known as aneuploidy.

Aneuploidy is one of the hallmarks of cancer (Hanahan and Weinberg, 2011) and a common cause of birth defects (Hassold et al., 1980; reviewed in Hassold and Hunt, 2001). In the context of malignant cell growth abnormal chromosome number, especially presence of extra copies can provide cells with selection advantage for cancer initiation and progression (Rutledge et al., 2016). It should be noted that aneuploidy favors malignant cell transformation only to a certain threshold above which cells' tolerance for genomic imbalance is exceeded leading to cell cycle arrest and/or cell death (Thompson and Compton, 2008; Silk et al., 2013; Weaver et al., 2007). This fact is exploited by the microtubule targeting agents (MTAs) that are used for cancer treatment; one consequence of the MTA treatment is deregulation of mitosis and elevation of frequency of aneuploidy (Topham et al., 2015; Gascoigne and Taylor, 2008; Zasadil et al., 2014) which leads to suppression of cancer cell proliferation. However, due to the mechanism of action of MTAs (inhibition of microtubule dynamics via tubulin targeting), a number of issues arise that limit their best clinical utility. Firstly, the MTAs affect all microtubule-mediated processes in the human body and therefore cause serious side effects such as myelosuppression and neuropathy (Rowinsky et al.,

1993). Secondly, intrinsic and acquired drug resistance is frequently observed in the patients impeding the best therapy outcome. To overcome these and many other therapeutic issues, several scientists around the world are working to find novel cell cycle regulating biomolecules that can be the basis for the development of cancer-specific drugs and improved cancer diagnostics.

miRNAs are evolutionary conserved short non-coding RNA molecules that regulate gene expression post-transcriptionally. miRNAs inhibit the translation of the target mRNA and/or induce its decay thereby exerting a negative impact on gene expression. Importantly, besides being present intracellularly miRNAs are also found circulating in body fluids such as plasma, urine and saliva. This is an important feature of miRNAs that diversifies their use as molecular biomarkers. Specifically in cancer, miRNAs have been found to exhibit differential expression according to the grade and subtype of cancer, which may enable their use in early diagnosis (Pritchard et al., 2012; Nair et al., 2012). From the point of view of our studies, the mitosis and SAC regulating miRNAs have a potency to become prognostic markers to assist in the prediction of tumor cells response to MTAs before initiation of the chemotherapy (Furlong et al., 2012). The dual-specificity protein phosphatases (Dusps) compose a class of protein phosphatases that remove phosphate groups from Serine/Threonine or Tyrosine residues of their substrates (Pavic et al., 2015). Atypical Dusps, a sub-class of the Dusp family include small molecular weight phosphatases that are poorly studied in terms of their role in mitosis and tumorigenesis.

The present study describes our findings in regards of novel mitosis regulating biomolecules, two miRNAs and one Dusp protein. The data provides new insights to the mechanisms of acquired drug resistance and cancer development.

2 REVIEW OF THE LITERATURE

2.1 Cell Cycle

The fundamental task of the cell cycle is to generate daughter cells that resemble the parent cell and contain similar cellular makeup. In order to achieve this, the DNA and cellular organelles in the parent cells are duplicated and distributed equally between the progeny cells in a very precise and highly regulated manner.

During the cell cycle, a cell progresses through four major phases known as Gap1 (G1), Synthesis (S), Gap2 (G2) and cell division (M) phase, in a sequential and irreversible manner. The G1, S, and G2 phases together are termed *the interphase*, while *the M phase* constitutes the mitosis and cytokinesis. Interphase, which is significantly longer in time than the M phase is a metabolically active growth phase. In G1, based on the received extracellular stimulus, the cell either initiates a new cell cycle or enters quiescence, a reversible cell cycle arrest referred as G0 phase. It is only after passing the restriction point in late G1 when the cell commits to duplicate itself and also becomes less dependent on extracellular stimuli for the growth. In S phase, the cell synthesizes a copy of its DNA. In G2 phase, the cell prepares for chromosome segregation and cell cleavage that take place at M phase. The M phase involves two main stages; nuclear division called mitosis and cytokinesis that involves redistribution of the cytoplasm and cellular organelles to the two daughter cells. Mitosis is categorized into five phases known as prophase, prometaphase, metaphase, anaphase (A and B) and telophase (Fig. 1). During prophase, the DNA starts to condense and organize into compact structures called the chromosomes that each contain a pair of sister chromatids held together by cohesion complex. Also, the microtubule cytoskeleton starts to re-organize; two spindle poles form upon separation of the centrosomes and the microtubules become more dynamic. The beginning of prometaphase is marked by nuclear envelope breakdown (NEBD) and formation of the mitotic spindle apparatus that captures the chromosomes and starts to maneuver them towards the cell equator. At metaphase, all the chromosomes are in the middle of the spindle and form a structure called the metaphase plate. At the onset of anaphase, the release of cohesion enables separation of the sister chromatids. During anaphase A, the sister chromatids are pulled apart towards the opposite spindle poles by the spindle microtubules. During anaphase B, antipar-

allel microtubule arrays form a tight structure called the spindle midzone that contributes to sister chromatid separation by pushing the spindle poles further away from each other. At telophase, the sister chromatids start to decondense and nuclear envelope reforms around the segregated sets of chromosomes. Upon formation of the spindle midzone, starts also the cytokinesis that cleaves the cell in two parts. First, a contractile ring forms at the midzone to facilitate the cell cleavage. At the end of M phase, the daughter cells remain attached by a thin bridge called the midbody, which is cut during a process called abscission in early G1. This completes a full round of cell cycle and starts the journey of new progeny cells.

The fate of individual cells and their progression in the cell cycle is controlled by evolutionally conserved cell signaling cascades called the checkpoints. In general, there are three cell cycle checkpoints that individually work at G1/S boundary, G2/M boundary and metaphase-anaphase boundary. The G1/S checkpoint is also known as the restriction point that in the absence of extracellular stimuli, such as growth factors or nutrients, prevents the progression of a cell from G1 to S phase. The G2/M checkpoint is also called the DNA damage checkpoint, which detects and responses to damaged DNA by inducing a cell cycle arrest that provides time for the repair process. The metaphase-anaphase checkpoint is called the spindle assembly checkpoint (SAC), which works during mitosis to ensure fidelity of chromosome segregation. The SAC prevents the onset of anaphase until all the chromosomes have attained stable connections with the microtubules originating from opposite poles of the spindle apparatus.

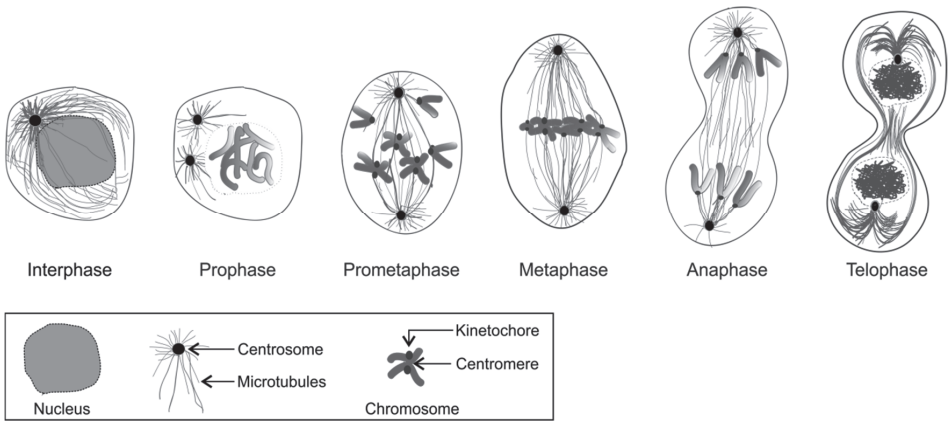

Figure 1: Simplified illustration of mitotic phases.

2.2 Key elements controlling mitosis signaling

Below I will list the main structures and organelles of mitosis that participate in chromosome segregation.

2.2.1 Chromosomes

The DNA molecules associated with histone proteins are called chromatin. At the beginning of M phase, the chromatin undergoes tight packaging to form typical highly compressed mitotic chromosomes. A basic unit of the chromatin packaging is called a nucleosome that consists a histone octamer and fragment of DNA (~145 to 147bp) wrapped around it (Luger et al., 1997). The histone octamer is formed by the association of two copies of each histone H2A, H2B, H3 and H4; while an additional histone molecule, H1 serves as a linker between two successive nucleosome core particles. In a diploid human cell, there are 23 pairs of chromosomes (22 pairs of autosomes and one pair of sex chromosomes) making 46 chromosomes in total. One full set of 23 chromosomes comes from both parents. Furthermore, each chromosome undergoes duplication during S phase, and the resulting two copies, called the sister chromatids, separate in mitosis.

2.2.2 Microtubules

In eukaryotes, the microtubules are an essential structural component of the cytoskeleton that in concert with several microtubule-associated proteins (MAPs) and motor proteins facilitate and control several cellular processes such as cell movement, cilia function, morphogenesis and signal transduction. Microtubules are hollow cylindrical structures made up of α- and β-tubulin heterodimers. The α:β heterodimer polymerizes with another α:β heterodimer in a linear way to form a protofilament. An association of 12-15 such protofilaments builds a microtubule measuring ~25nm in diameter (Wade et al., 1990; Chretien et al., 1992). The microtubules are subjected to continuous cycles of growth and shrinkage, which occurs due to addition and removal of tubulin dimers at the polymer ends. This behavior is called as dynamic instability (Mitchison and Kirschner, 1984). During mitosis, the dynamic characteristics of a microtubule are important in the search and capture of the free kinetochores and facilitation of chromosome movements.

The microtubules are nucleated and anchored at the microtubule organizing centers (MTOCs) typically consisting of centrosomes (see chapter 2.2.3). During interphase the MTOC's are near the nuclear surface in the cytosol whereas during

mitosis they are at the spindle poles. A microtubule filament has polar nature; the presence of α-tubulin marks the minus end that faces a centrosome while the β-tubulin marks the plus end that extends from a centrosome. Basically, three types of microtubules emanate from a mitotic spindle pole; kinetochore microtubules, interpolar microtubules and astral microtubules. Each type is needed for faithful chromosome segregation. Kinetochore microtubules are dynamic polymers that search for free kinetochores and become stabilized after a proper attachment. In vertebrate cells, a kinetochore at metaphase is attached to ~15-20 individual microtubules (Wendell et al., 1993; Walczak et al., 2010). On the other hand, interpolar and astral microtubules do not associate with the kinetochores but still contribute to chromosome segregation. The interpolar microtubules emanating from the opposite spindle poles overlap with each other at the center of the cell and provides support to the spindle structure and push the poles apart in late anaphase. The astral microtubules reach outwards to the cell periphery and are needed for correct positioning and orientation of the mitotic spindle (Palmer et al., 1992).

Furthermore, the function of microtubules is regulated by post-translational modifications of tubulin which include acetylation, detyrosination, polyglutamylation, polyglycylation, phosphorylation, polyamination (reviewed in Janke, 2014). In addition, the cofactors such as MAPs and motor proteins assist in microtubule function as well. As the name suggests, MAPs associate with microtubules and are important for microtubule integrity. In addition, the two classes of motor proteins namely dynein and kinesin assist microtubules in the movement of chromosomes, bipolar spindle formation and transport of biomolecules. Dyneins are minus-end directed while kinesins are plus-end directed motors (Banks and Heald, 2001; Wordeman, 2010).

2.2.3 Centrosomes

Centrosomes are the microtubule organizing centers (MTOCs) in a cell. They nucleate microtubules to facilitate several events such as cell cycle progression, cell adhesion, cell locomotion, cell polarity and chromosome segregation (reviewed in Conduit et al., 2015). A centrosome is composed of two centrioles, which are short cylindrical tubulin structures arranged perpendicularly to each other, and a protein rich pericentriolar material (PCM) surrounding the centriole pair (Pihan, 2013). After a normal cell division, the two newly formed daughter cells have each one centrosome containing a pair of centrioles (one “mother” and one “daughter” centriole). The centriole pair duplicates during S phase and attains PCM around it to form a new centrosome. The matured centrosomes separate during early M phase to represent the two spindle poles at mitosis (reviewed

in Conduit et al., 2015). Centrosome cycle is occurring parallel to the DNA replication cycle; one cycle of duplication is allowed per a cell cycle resulting in the formation of one centriole adjacent to the preexisting one (Nigg, 2007).

2.2.4 Centromere

The centromere is a specific part of the chromosome that serves as an attachment point for the spindle microtubules during mitosis and is the region where the sister chromatids stay connected by cohesion complex until the onset of anaphase. In human, the centromere is formed by multiple 171 basepair repeats of α-satellite DNA sequences (Masumoto et al., 1989). It should be noted that in vertebrate cells centromeres can also be formed without repeated α-satellite DNA sequences and that the α-satellite DNA sequence regions cannot always function as centromeres (Cheeseman, 2014). However, an epigenetic mark, replacement of the histone H3 with Centromere protein-A (Cenp-A), is critical for the identity of the centromere (Palmer et al., 1987; Blower, 2002). The replacement is facilitated by Mis18 and Mis18-binding protein 1 (M18BP1, also known as Knl2) (Fujita et al., 2007; Maddox et al., 2007).

2.2.5 Kinetochore

The Kinetochore is a multiprotein complex assembled at the centromere. Its main function is to facilitate the connection between a chromosome and the spindle apparatus and to control the stability of the attachment. There are more than 100 different proteins at a kinetochore; some are permanent components present throughout the cell cycle while many exhibit dynamic properties and show cell cycle-dependent expression profile (reviewed in Cheeseman and Desai, 2008). Through a complicated mechano-biochemical mechanism a cell responds to the presence of kinetochores that are not properly connected with the microtubules. The cell is unable to segregate its chromosomes and exit from M phase, until all the kinetochores have achieved stable microtubule attachments. These molecular events constitute the basis of the SAC signaling and are discussed in more details below.

2.2.6 Kinetochore-Microtubule attachments

The attachment of microtubules at the kinetochores is dependent on the KMN network formed by the association of Kinetochore null protein 1 (Knl1), mis-

segregation 12 (Mis12) complex and nuclear division cycle 80 (Ndc80) complex (Cheeseman and Desai, 2008) (Fig. 2B). The KMN network is also an important regulator of SAC signaling (reviewed in Foley and Kapoor, 2013). The Ndc80 complex and Knl1 both can bind to the microtubules and synergize to increase the binding affinity (Cheeseman et al., 2006). The Ndc80 complex is a tetramer formed by the association of Hec1, Nuf2, Spc24 and Spc25 proteins (Ciferri et al., 2005; Wei et al., 2005; Ciferri et al., 2008). The Hec1 and Nuf2 subunits have calponin-homology domains that facilitate the binding of Ndc80 complex to the tubulin interfaces of microtubules (Wei et al., 2007) whereas the Spc24-Spc25 heterodimer bind to Mis12 protein (Cheeseman et al., 2006), which is connected to the centromeric protein Cenp-C, to establish the link between microtubules and the kinetochore (Screpanti et al., 2011; Przewloka et al., 2011). Cenp-C binds to Cenp-A, a histone H3 variant nucleosome at the centromere (Carroll et al., 2010; Kato et al., 2013). Additionally, Cenp-T, an inner kinetochore protein, also contributes to the formation of kinetochore-microtubule attachment by directly interacting with Spc25 of the Ndc80 complex (Nishino et al., 2013; Gascoigne et al., 2011). Ndc80 complex in association with the spindle and kinetochore-associated (Ska) complex maintains the kinetochore attachments with the depolymerizing plus end of the microtubule (Welburn et al., 2009). The KMN network is hyperphosphorylated by Aurora kinase B (AURKB) that results in the destabilization of the microtubule attachments (Cheeseman et al., 2006; Alushin et al., 2010), which is an important event in the correction process of erroneous kinetochore-microtubule attachments. In early mitosis, after the NEBD, the microtubules originating from the centrosomes search for free kinetochores. Upon encounter, the microtubule and a kinetochore first interact laterally that later leads to the generation of a more stable end-on attachment (Magidson et al., 2011).

Four different kinetochore-microtubule attachment types exist (Fig. 2C). *Amphitelic attachment*: this is normal attachment type in which the two sister kinetochores of a chromosome are attached to microtubules emanating from opposite spindle poles. *Monotelic attachment*: this is an erroneous attachment type in which one sister kinetochore is connected to microtubules from one spindle pole and the other sister kinetochore has no microtubule attachment. *Syntelic attachment*: this is an erroneous attachment type in which both sister kinetochores are attached to microtubules originating from the same pole. *Merotelic attachment*: this is an erroneous attachment in which one sister kinetochore is connected to microtubules emanating from both spindle poles while the other kinetochore has a normal association with microtubules originating from a single pole.

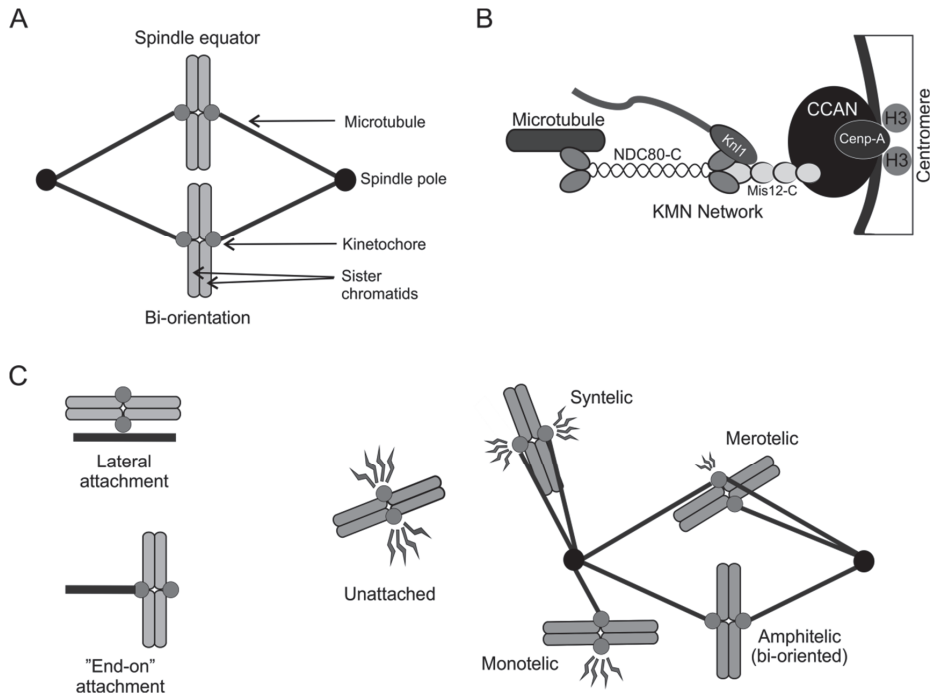

Figure 2: Kinetochore-microtubule attachments. Modified from Krenn and Musacchio, 2015. A) Two chromosomes attached to the microtubules originating from oppositely oriented spindle poles. B) Schematic illustration of the KMN network facilitating the connection between a microtubule and centromere. C) Different types of kinetochore-microtubule attachments (see text for details). NDC80-C: Nuclear division cycle 80 complex, Knl1: Kinetochore null protein 1, Mis12-C: Missegregation 12 complex, CCAN: Constitutive Centromere Associated Network, Cenp-A: Centromere protein A, H3: Histone 3.

2.2.7 Sister-chromatid cohesion

The cohesin ring complex is loaded onto the chromatin in G1 phase and is required to hold the two sister chromatids of a chromosome (formed in S-phase) together until their physical separation in mitosis. The cohesin ring is a heterodimer of Smc1 and either SA1 or SA2, linked by Scc1 protein. The other regulatory proteins such as Scc2, Scc4, Esco 1, Esco2, Pds5 (5A and 5B), Wap1 and sororin facilitate the timely loading of the cohesin ring on DNA and later its dissociation from DNA. During early mitosis the Polo-like kinase 1 (Plk1) phosphorylates the SA2 subunit in a process that is critical for the removal of cohesin complex from the chromosome arms (Hauf et al., 2005). The centromeric cohesion remains intact by the recruitment of shugoshin 1 (sgo1) and protein phosphatase 2A (PP2A) that dephosphorylates the SA2 subunit (Kitajima et al., 2006). At the

anaphase onset, the centromeric cohesion is resolved by the action of separase enzyme that cleaves the Scc1, which allows separation of the sister chromatids (Waizenegger et al., 2000).

2.2.8 Kinases and phosphatases

The progression through mitosis is regulated by counteracting activities of kinases and phosphatases that form a complex signaling network with feedback and feedforward loops. The key kinases functional during mitosis are Cyclin-dependent kinase 1 (Cdk1), Greatwall (GW), Aurora Kinase A (AURKA), Plk1, AURKB, Budding uninhibited by benzimidazoles 1 (Bub1) and Monopolar spindle 1 (Mps1) whereas the main mitotic phosphatases are Cell division cycle 25 (Cdc25), Cell division cycle 14 (Cdc14), Protein phosphatase 1 (PP1) and PP2A. Generally speaking, kinases are most active during early to mid-phases of mitosis when maximum phosphorylation in the mitotic proteins is observed, while towards the end of mitosis the phosphatases become active and reverse the phosphorylation status of many mitotic proteins. It should also be noted that the kinases and phosphatases constantly communicate with each other to regulate all phases of mitosis (Domingo-Sananes et al., 2011; Wu et al., 2009). More details about the mitotic tasks of these kinases and phosphatases are given in the forthcoming chapters.

2.3 Spindle Assembly Checkpoint

In eukaryotes, the SAC (also known as the mitotic checkpoint) aims to maintain genomic balance by facilitating equal segregation of chromosomes between the two daughter cells (reviewed in Musacchio, 2015). Errors in function of SAC can cause numerical chromosome changes in the progeny cells, which is an event linked with stimulation of malignant cell growth (reviewed in Kops et al., 2005).

2.3.1 SAC activation

SAC is activated in early mitosis to monitor the attachments between microtubules and kinetochores of chromosomes (Musacchio, 2015). In the case of erroneous attachments, SAC triggers a pre-anaphase delay allowing time for correcting the mistakes (Fig. 3A). The improper or absent attachment of even a single chromosome with the spindle microtubules generates a STOP signal at the

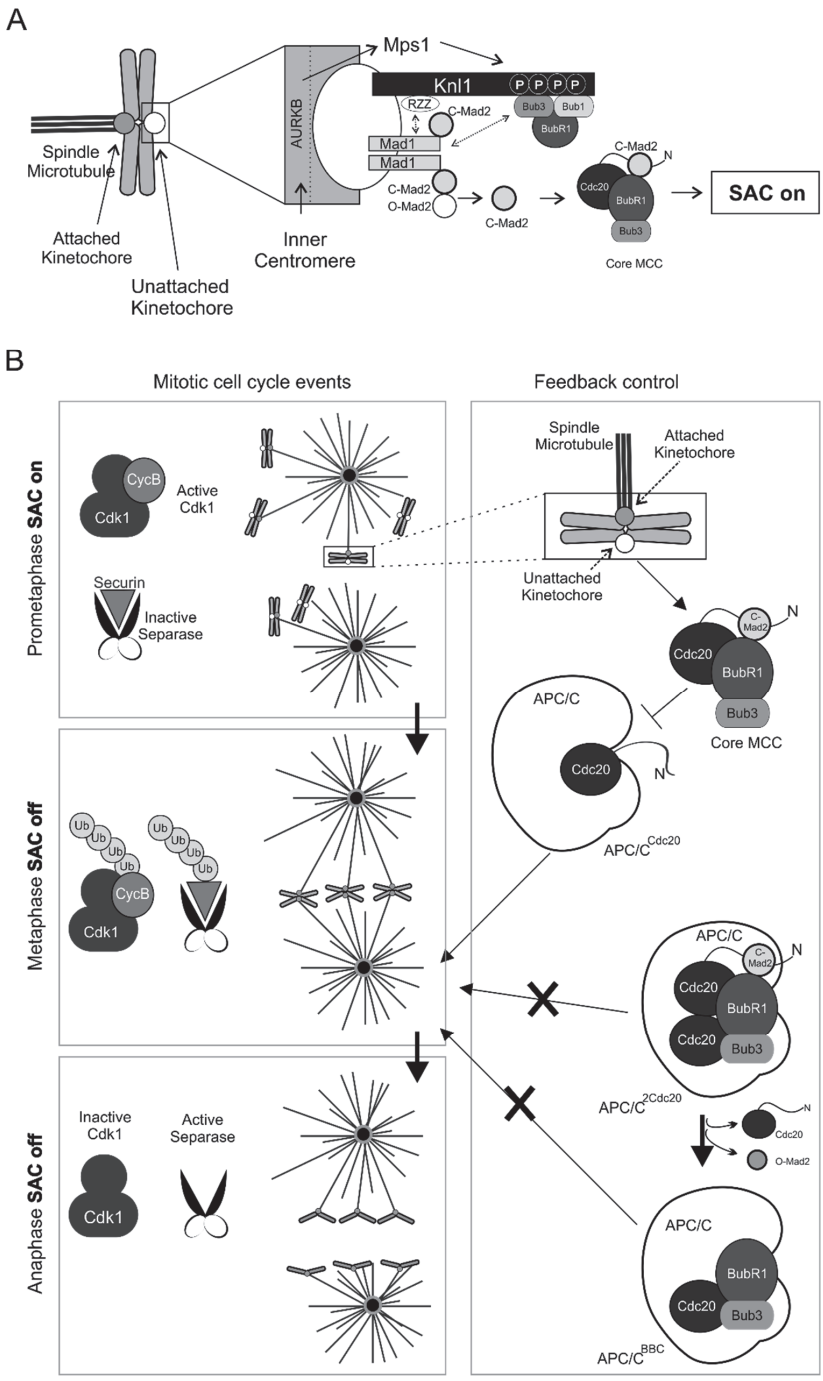

Figure 3: The Spindle Assembly Checkpoint (see text for details). (A) A simplified illustration of the assembly of SAC proteins at the unattached kinetochore during SAC activation. Modified from Foley and Kapoor, 2013, (B) The Spindle Assembly Checkpoint. Modified from Musacchio, 2015.

kinetochore that is transduced across the cell. This prevents sister chromatid separation until all chromosomes have achieved proper bipolar orientation and moved to the spindle equator (Rieder et al., 1995). However, the SAC can be activated at different strengths depending on at least the following factors; the number of unattached kinetochores in a cell (Dick and Gerlich, 2013), the amount of mitotic arrest deficient 2 (Mad2) protein present at the unattached kinetochores, and the amount of Mitotic Checkpoint Complex (MCC) formed (Collin et al., 2013). Under normal growth conditions the SAC is active ~ 30 to 60 min, from the NEBD to the onset of anaphase. However, in response to notable spindle damage induced for example by microtubule-targeting drugs, the SAC activity and consequent mitotic arrest can last much longer, even up to 20 hours before the cell eventually dies or slips out of mitosis (Topham et al., 2015; Musacchio, 2015). The molecular details of SAC signaling have been under extensive investigation for the past two decades. Despite many breakthroughs several unresolved issues continue to puzzle the mitosis researchers.

The current model suggests that AURKB operates in the frontline of SAC (Santaguida et al., 2011). For example, the kinase mediates the recruitment of Mps1 kinase to the unattached kinetochores (Vigneron et al., 2004; Santaguida et al., 2010; Nijenhuis et al., 2013), which then phosphorylates the methionine-glutamate-leucine-threonine (MELT) motifs of Knl1 protein. This event is identified by the Budding uninhibited by benzimidazole-3 (Bub3) that triggers the recruitment of SAC proteins and MCC formation at the unattached kinetochores (Yamagishi et al., 2012; Primorac and Musacchio, 2013; Zhang et al, 2014). Importantly, the MCC production needs to be continuous to maintain the SAC activity (Fig. 3A-B). The main task of MCC is to inhibit the ubiquitin ligase activity of Anaphase Promoting Complex / Cyclosome (APC/C) and thereby protect “anaphase inhibitors” such as Cyclin B and securin proteins from proteasome-mediated degradation. It is only after all the kinetochores have attained stable microtubule attachments and the chromosomes have aligned to the middle of the cell when the SAC is switched off. This leads to activation of the APC/C and ubiquitination consequent degradation of Cyclin B and securin resulting in separation of sister chromatids and exit from M phase (reviewed in Musacchio, 2015).

2.3.1.1 Anaphase Promoting Complex / Cyclosome (APC/C)

In vertebrates, APC/C (1.22MDa) is a large protein complex formed by the assembly of 19 subunits (Chang et al, 2014; 2015). APC/C is an E3 ubiquitin ligase enzyme that adds ubiquitin molecules onto the substrate proteins, which are then recognized by the 26S proteasome complex and degraded (Hwang et al., 1998;

Sudakin et al., 1995). The enzymatic activity of the complex is dependent on E2-ubiquitin enzyme that functions upstream in the ubiquitin pathway (Sivakumar and Gorbsky, 2015). The recognition of the substrate proteins by APC/C is facilitated by its association with co-factor Cell division cycle 20 (Cdc20) or Cdc20 homologue 1 (Cdh1) (Fang et al., 1998; Dawson et al., 1995; Visintin et al., 1997). Specifically, during early to late mitosis, Cdc20 acts as a co-activator of APC/C while at late mitosis and in G1 phase Cdh1 performs this task (Kramer et al., 2000; Listovsky and Sale, 2013). To facilitate ordered mitotic progression APC/C-Cdc20 ubiquitinates Cyclin A and NIMA-related kinase 2A (Nek2A) during early mitosis and Cyclin B and securin during mid-mitosis. During late mitosis, APC/C-Cdh1 takes charge, which leads to the ubiquitination of Cdc20, Aurora kinases and Plk1, and consequent exit from mitosis (Sivakumar and Gorbsky, 2015).

The activity of APC/C is regulated throughout the cell cycle and especially during early mitosis when its activity is tightly controlled at the substrate level. For example, in prometaphase APC/C activity is not completely inhibited; the protein complex is able to add ubiquitin tags on Cyclin A and Nek2A to facilitate their degradation by the proteasome. However, at the same time Cyclin B and securin are protected from APC/C ubiquitin ligase activity (den Elzen and Pines, 2001; Hayes et al., 2006; reviewed in Van zon and Wolthuis, 2010). Interestingly, both these activities are Cdc20 dependent (den Elzen and Pines, 2001; Hayes et al., 2006). However, many molecular details of signaling along the APC/C-SAC pathway remain to be solved. For example, degradation of Cyclin A requires its association with Cyclin-dependent kinase regulatory subunit (Cks) (Wolthuis et al., 2008; Di Fiore and Pines, 2010) but whether this contributes to the SAC independency in Cyclin A elimination is not known.

2.3.1.2 Mitotic Checkpoint Complex (MCC)

The MCC is formed by the association of Mad2, budding uninhibited by benzimidazole-related 1 (BubR1, in yeast known as Mad3) and Bub3 with Cdc20 in response to the presence of unattached kinetochores in a mitotic cell (Fig. 3A). Therefore MCC serves as an effector for the SAC. When formed, the MCC physically interacts with APC/C to negatively affect its ubiquitin ligase activity (Hein and Nilsson, 2014; Izawa and Pines, 2015). The individual components of MCC are known to dynamically exchange between the cytosol and unattached kinetochores (Howell et al., 2004; Shah et al., 2004; Howell et al., 2000; Kallio et al., 1998). However, the precise molecular mechanisms controlling this protein trafficking are not known, and the exact composition of MCC in time and cellular space is also poorly understood. Below is a list of the MCC components.

BubR1 (120kDa) is the largest protein in the MCC and represents a pseudo-kinase (Suijkerbuijk et al., 2012b). BubR1 is able to interact with two Cdc20 molecules via its KEN1 and KEN2 motifs (King et al., 2007; Malureanu et al., 2009; Izawa and Pines, 2015). It can bind Bub3 via its GLEBS motif (Overlack et al., 2015), dimerize with budding uninhibited by benzimidazole-1 (Bub1) using extended loop helix (Larsen et al., 2007), recruit Cdc20 to kinetochores via an internal Cdc20 binding site (Lischetti et al., 2014), and interact with PP2A phosphatase through a kinetochore attachment regulatory (KARD) domain (Suijkerbuijk et al., 2012a).

Bub3 (37kDa) contains a WD40 β-propeller domain and interacts with Bub1 and BubR1 to recruit them to the kinetochores (Taylor et al., 1998).

Cdc20 (55kDa) has a wide range of binding partners like APC/C subunits, Mad2, BubR1, Bub1, and several mitotic substrates of APC/C as e.g. Cyclin B and securin (Musacchio, 2015). The WD-40 β-propeller domain harbors a destruction box (D-box) motif that facilitates Cdc20 binding to its substrates. The binding of MCC to Cdc20 masks this D-box recognition site and prevents Cdc20 binding to substrates proteins (Chao et al., 2012).

Mad2 (24kDa) is a SAC protein that exists in two structural conformations, namely open-Mad2 (O-Mad2) and closed-Mad2 (C-Mad2), to control APC/C activity. The details of Mad2 function are reviewed below in a separate Mad2 chapter.

2.3.1.3 MCC assembly

The assembly of MCC at unattached kinetochores is a step-wise process (Fig.3A). Based on current knowledge, Bub3 first interacts with Bub1 to localize at the kinetochores, guided by Mps1-mediated phosphorylation of Knl1 MELT motifs (Yamagishi et al., 2012; Primorac and Musacchio, 2013). Once at the kinetochores, Bub1 mediates the recruitment of BubR1 to the kinetochores (Overlack et al., 2015; Zhang et al., 2015). Moreover, Bub1 and BubR1 also serve as recruiters of Cdc20 to the kinetochore (Vleugel et al., 2015; Lischetti et al., 2014). In addition, kinetochore localization of Mad2 is Mad1 dependent, and facilitated, at least, by Bub1 (Moyle et al., 2014) and Rod:ZW10:Zwilch (RZZ) complex (Kops et al., 2005; Buffin et al., 2005). Furthermore, Bub3 and C-Mad2 independently promote the binding of BubR1 to Cdc20 (Han et al., 2013; 2014).

The MCC subunits C-Mad2 and BubR1 are indispensable for a robust SAC activity (Meraldi et al., 2004; Michel et al., 2004) and both synergize to mediate a strong mitotic checkpoint activity (Meraldi et al., 2004; Fang, 2002). Interestingly, for normal SAC signaling, Mad2 localization at the unattached kinetochore is absolutely needed (Meraldi et al., 2004) whereas BubR1's is not (Overlack et al., 2015).

2.3.1.4 Bub3:BubR1:Cdc20 (BBC): a sub-complex of MCC

Another checkpoint sub-complex consisting of Bub3:BubR1:Cdc20, called BBC, exists in association with APC/C (Westhorpe et al., 2011; Kulukian et al., 2009) (Fig. 3B). This sub-complex is able to bind and inhibit APC/C. However, BBC is less stable than MCC and therefore its inhibitory capacity towards APC/C is not as high as the MCC's. The explanation for the existence of BBC comes from the study by Izawa et al (Izawa and Pines, 2015). The authors showed that MCC can bind to two Cdc20 molecules. Specifically, the complex containing core MCC (Mad2, Bub3 and BubR1) and a Cdc20 molecule (referred as MCC^{1Cdc20}) later binds to another Cdc20 molecule, which is already bound to APC/C (referred as APC/C^{Cdc20}) to forms APC/C^{2Cdc20} (Fig. 3B). It was proposed that BubR1 would be a linking factor for MCC^{1Cdc20} and APC/C^{Cdc20} as it harbors two KEN box domains, both capable of binding one Cdc20 molecule and both needed for a functional SAC (King et al., 2007; Malureanu et al., 2009; Izawa and Pines, 2015; Musacchio, 2015). Importantly, the Cdc20 molecule bound to core MCC undergoes APC/C-mediated ubiquitination and is constantly degraded by the 26S proteasome during mitosis (Nilsson et al., 2008; Uzunova e al., 2012; Mansfeld et al., 2011). In addition, C-Mad2 bound to core MCC is reverted back to O-Mad2 by Thyroid Receptor Interacting Protein 13 (Trip13/Pch-2), an AAA ATPase enzyme, and p31comet proteins that leads to the release of Mad2 from the MCC along with the bound Cdc20 molecule (Westhorpe et al., 2011; Miniowitz-Shemtov et al., 2012; Miniowitz-Shemtov et al., 2015). So, taken together these studies propose that MCC^{1Cdc20} binds to APC/C^{Cdc20} via BubR1, and after the dissociation of Cdc20 and Mad2 from MCC^{1Cdc20}, the Bub3:BubR1 and a second Cdc20 molecule forms BBC that inhibits APC/C activity (Musacchio, 2015).

2.3.2 SAC inactivation

SAC is satisfied when all kinetochores of a cell have achieved stable amphitelic attachments and the chromosomes are aligned at the spindle equator. In vertebrate cells, the onset of anaphase occurs 10-14 min after the amount of Mad2

protein has dropped below the level of detection in all kinetochores of a cell (Howell et al., 2000; Musacchio, 2015). As mentioned earlier, SAC is a mechano-chemical system that responds to kinetochore-microtubule attachments and physical tension created by the pulling forces of attached microtubules.

Recent studies have shown that rather than the intra-kinetochore tension, the stable microtubule attachments at the kinetochores is a major driver for SAC inactivation (Tauchman et al., 2015; Etemad et al., 2015). This notion is supported by other studies in which metaphase cells were physically and biochemically manipulated; a metaphase chromosome that was under tension was able to reactivate the SAC when Mad1 was artificially put back to the metaphase kinetochore, which also led to the kinetochore recruitment of Mad2 (Ballister et al., 2014; Kuijt et al., 2014). This means that displacement of the SAC proteins such as Mad1 and Mad2 from the kinetochores after the proper kinetochore-microtubule attachments have formed is one of the factors needed for SAC inactivation. Below the details of this and other mechanisms are highlighted.

2.3.2.1 Stable binding of microtubules prevents rebinding of Mps1 to the kinetochores

Two recent independent studies provide evidence for the Mps1-mediated initiation of SAC extinction upon establishment of stable kinetochore-microtubule attachments (Hiruma et al., 2015; Ji et al., 2015). The Mps1 kinase is a dynamic SAC component at the kinetochores that constantly shuttles between cytosol and kinetochore. The authors show that microtubules bind to Ndc80 complex in close proximity to a domain where the Mps1 binds. The occupancy of the domain by microtubules prevents rebinding of Mps1 to the kinetochore and therefore results in loss of Mps1-mediated phosphorylation of Knl1, which is believed to limit the recruitment of other SAC proteins needed for the continuous MCC production (Hiruma et al., 2015; Ji et al., 2015). It is, however, important to note that SAC extinction would also need the delocalization of SAC proteins already present at the kinetochores as well as dephosphorylation of key kinetochore proteins and disassembly of the MCC complexes in the cell.

2.3.2.2 Dynein-mediated stripping of the SAC proteins from the kinetochores

The SAC proteins Mad2 and Mad1 are transferred to spindle poles via microtubules after the establishment of stable kinetochore-microtubule attachments (Howell et al., 2000; Howell et al., 2001; Gassmann et al., 2010). The stripping of Mad2 and Mad1 from kinetochores is a dynein-mediated process that requires

ATP (Howell et al., 2001). In addition, Spindly and RZZ complexes are implicated in the system as they direct dynein to the kinetochores (Gassmann et al., 2010; Chan et al., 2009; Griffis et al., 2007). However, the SAC silencing can also be independent of dynein and Spindly; it has been noted that abolishing dynein kinetochore localization and depletion of Spindly by RNAi did not result in any detectable defects in SAC extinction (Gassmann et al., 2010).

2.3.2.3 Reversal of protein phosphorylation to silence SAC

Control of SAC protein function by post-translational modifications, e.g. addition and removal of phosphate groups, is one of the mechanisms to silence the mitotic checkpoint. The studies demonstrate that SAC cannot be turned off in the presence of constitutively phosphorylated KMN complex (Kemmler et al., 2009) and SAC proteins (Huang et al., 2008). Importantly, the PP2A-B56 in humans (Espert et al., 2014) and PP1 in yeast have been identified as phosphatases that mediate dephosphorylation of many SAC proteins at the kinetochores (Vanoosthuyse and Hardwick, 2009). Moreover, PP2A-B56 and PP1 also oppose the AURKB mediated generation of unattached kinetochores that would otherwise induce SAC activation (Foley et al., 2011; Liu et al., 2010; Pinsky et al., 2009). In addition, in animal cells, the PP1-mediated dephosphorylation of dynein facilitates the stripping of SAC proteins to induce anaphase (Whyte et al., 2008).

2.3.2.4 p31 comet and Trip-13 –mediated disassembly of MCC

p31 comet binds to C-Mad2 present in MCC, and functions as an endogenous Mad2 inhibitor (Yang et al., 2007; Xia et al., 2004). During early mitosis, the phosphorylation of p31comet, by a yet unknown kinase, diminishes its anti-Mad2 activity to keep the SAC active (Westhorpe et al., 2011; Hagan et al., 2011; Date et al., 2014). However, active p31comet facilitates the extraction of Mad2 from the MCC (Westhorpe et al., 2011), and also promotes the Cdk-dependent phosphorylation of Cdc20 to release Cdc20 from MCC (Miniowitz-Shemtov et al., 2012; Varetti et al., 2011; Teichner et al., 2011). It is, however, becoming clear that p31comet is assisted by Trip13 to inactivate the SAC. Specifically, Trip13 localizes to kinetochores where it binds to p31comet and provides the energy for reversal of C-Mad2 to O-Mad2 (Wang et al., 2014; Westhorpe et al., 2011). Trip13 associates with the p31comet:C-Mad2:Cdc20 complex and mediates the disassembly of MCC (Miniowitz-Shemtov et al., 2012; Miniowitz-Shemtov et al., 2015). Notably, the downregulation of either p31comet or Trip13 delays mitotic exit while the overexpression of the proteins inactivates SAC and disrupts

Mad1:Mad2 complex, respectively, resulting in mitotic exit (Westhorpe et al., 2011; Varetti et al., 2011; Wang et al., 2014; Eytan et al., 2014).

2.4 Chromosomal Passenger Complex (CPC)

The Chromosomal Passenger Complex (CPC) is formed by the association of four core proteins, namely the Inner centromeric protein (INCENP), AURKB, borealin and survivin (reviewed in Carmena et al., 2012). The enzymatic activity of the CPC is dependent on the AURKB, which is a serine/threonine (Ser/Thr) kinase. The other core members of CPC are required for the proper localization and function of the complex (reviewed in Van der Horst and Lens, 2014). CPC participates in the regulation of mitotic progression and fidelity of cell division. Importantly, the CPC core members are highly interdependent; absence of any subunit renders the complex as non-functional (Carvalho et al., 2003; Honda et al., 2003; Klein et al., 2006). The CPC is needed for normal chromosome congression, kinetochore-microtubule attachments, SAC signaling and cytokinesis (Adams et al., 2001; Kallio et al., 2002; Mackay et al., 1998; Vagnarelli and Earnshaw, 2004). As a result, the loss-of-function of CPC is implicated in erroneous chromosome segregation and induction of aneuploidy (Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003).

2.4.1 CPC subunits

INCENP was the first identified core member of CPC (Cooke et al., 1987). It is a large protein (140 kDa) that holds the CPC together. INCENP interacts with borealin (31kDa) and survivin (16kDa; splice variants with lower molecular weight are also expressed) via its N-terminal domain to form a three-helix bundle while the C-terminus binds to AURKB (Jeyaprakash et al., 2007). The ternary-complex formed between INCENP, survivin and borealin facilitate CPC localization at the centromeres (Jeyaprakash et al., 2007; Klein et al., 2006; Lens et al., 2006). AURKB belongs to the Aurora family of kinases that in humans contain two other kinases, namely AURKA and Aurora kinase C (AURKC). The *Saccharomyces cerevisiae* has only one aurora kinase (Ipl1) while *Xenopus laevis*, *Drosophila melanogaster* and *Caenorhabditis elegans* have each two (Ke et al., 2003). Characteristically, AURKA and AURKC kinases share a conserved phosphorylation motif with AURKB but function at different cellular locations during mitosis and target specific substrates (reviewed in Hochegger et al., 2013). For example, AURKA mainly localizes to spindle microtubules (dependent on co-factor TPX2) and spindle poles (dependent on co-factor Ajuba) (Hirota et al., 2003;

Kufer et al., 2002). On the other hand, the localization of AURKC in mitosis is not well-known; most of the data shows its association with meiosis (Quartuccio and Schindler, 2015).

2.4.2 Localization of CPC

The localization of the CPC changes at different phases of cell cycle (Fig. 4) (Earnshaw and Bernat, 1991). In interphase cells, CPC associates with pericentromeric heterochromatin (Monier et al., 2007) while in early mitosis it concentrates at the inner centromeres. At the onset of anaphase, CPC relocates to the spindle midzone microtubules and subsequently concentrates to the midbody at telophase (reviewed in Carmena et al., 2012; Van der Horst and Lens, 2014).The release of CPC from the pericentric heterochromatin is facilitated by AURKB-mediated phosphorylation of histone H3 (Ser10) (Hirota et al., 2005). Also, the centromere localization of CPC is guided by the histone 3 (Thr3) and histone H2A (Thr120) phosphorylation by the Haspin kinase (Kelly et al., 2010; Wang et al., 2010) and Bub1 kinase (Yamagishi et al., 2010), respectively. After the phosphorylation of Thr3 of H3, survivin identifies this signal and recruits CPC to the site (Kelly et al., 2010; Wang et al., 2010). In addition, the phosphorylation of Thr120 of H2A recruits shugoshin (Sgo) proteins that in turn recruit borealin to the site (Yamagishi et al., 2010).

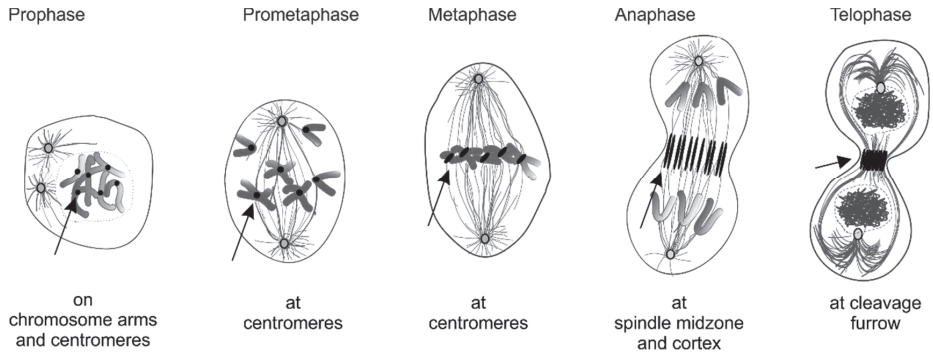

Figure 4: The localization of Chromosomal Passenger Complex (CPC). Modified from Ruchaud et al., 2007.

The mitotic accumulation of CPC to the centromeres is under a number of feedback mechanisms. First, CPC itself stimulates its own centromere recruitment;

AURKB mediated activation of haspin kinase enhances the phosphorylation of H3 to increase CPC localization at the centromere (Wang et al., 2011). Furthermore, AURKB enhances CPC centromere accumulation via phosphorylating the phosphatase scaffold protein Repo-Man and thereby preventing the PP1:Repo-Man complex from dephosphorylating the Thr3 of H3 (Qian et al., 2013). AURKB also influences the Bub1 activity via ATM kinase (Yang et al., 2011) and regulates Bub1 localization to the centromere via Mps1 kinase (van der Waal et al., 2012). Finally, Plk1 and AURKB itself regulate the enrichment of AURKB at the misaligned chromosomes (Salimian et al., 2011).

Upon onset of anaphase, CPC starts to move from the centromeres to the central spindle. The central spindle consists of anti-parallel microtubules with plus ends bundled together. At this site, the centralspindlin protein complex composed of Mitosis kinesin-like protein 1 (Mklp1/Kif23) and Rho GTPase activating protein (GAP) MgcRacGAP stabilizes the central spindle in an AURKB kinase activity-dependent manner to facilitate normal cell cleavage (Douglas et al., 2010). The central spindle localization of CPC is achieved by diminished phosphorylations of Thr3 of H3 and Thr120 of H2A due to a drop in the activity of Cdk1 and enhanced activity of PP1 gamma (Qian et al., 2011; Vagnarelli et al., 2011). In addition, the INCENP dephosphorylation at Thr 59 facilitates its binding to Mitosis kinesin-like protein 2 (Mklp2) to change the affinity of CPC from centromere to spindle microtubules (Gruneberg et al., 2004).

2.4.3 Activity of CPC

The normal function of CPC is needed for the maintenance of genomic stability (Ditchfield et al., 2003; Hauf et al., 2003; Honda et al., 2003). The full activity of CPC is achieved at mitosis when the complex works to correct erroneous kinetochore-microtubule associations and promotes normal SAC signaling. As mentioned above, the catalytic subunit of CPC is the AURKB, which is maximally active when the kinase is bound to the complex. Specifically, the binding of INCENP to AURKB followed by autophosphorylation of the kinase at Thr232 *in trans* (Sessa et al., 2005; Yasui et al., 2004), and phosphorylation by other kinases such as Chk1 kinase, further stimulates the activity of CPC associated AURKB (Petsalaki et al., 2011). Moreover, the PP1 and PP2A counteract the AURKB kinase activity by dephosphorylating its target residues and thereby contribute to the co-ordination of mitotic progression (Foley et al., 2011; Liu et al., 2010). Apparently, residual CPC activity is also needed in interphase cells because transient inhibition of AURKB in interphase cells perturbs the fidelity of chromosome segregation (Hayashi-Takanaka et al., 2009).

2.4.4 Functions of CPC

The AURKB kinase activity is crucial for the error correction at the kinetochore-microtubule interface. The correction process is a complex chain of events that needs timely co-operation by many above-mentioned proteins and protein complexes. One of the key reactions is the AURKB-mediated phosphorylation of several residues at the N-terminus of Hec1 that decreases the binding affinity of the Ndc80 complex with the microtubules (Cheeseman et al., 2006; De Luca et al., 2006; Zaytsev and Grishchuk, 2015; De Luca et al., 2011). Another regulatory step occurs in the Ska complex. In eukaryotes, Ska complex maintains the binding of kinetochores with the depolymerizing microtubule ends (Welburn et al., 2009). The phosphorylation of Ska complex by AURKB negatively affects its binding to KMN network and thus leads to destabilization of incorrectly attached microtubules (Chan et al., 2012; Tien et al., 2010). Furthermore, AURKB phosphorylates the Mitotic Centromere-Associated Kinesin (MCAK) to induce its kinetochore localization and microtubule depolymerase activity (Lan et al., 2004; Andrews et al., 2004). The enrichment of AURKB and MCAK at merotelically attached kinetochores underlines the importance of this regulation for accurate error correction (Knowlton et al., 2006). Finally, I wish to mention the AURKB-mediated control of kinetochore localization of the Centrosome-associated protein E (Cenp-E) (Ditchfield et al., 2003), which serves as an important early event for the generation of lateral kinetochore-microtubule attachments that supports the formation of correct amphitelic attachments (Magidson et al., 2011). Once moved to the central spindle the CPC performs many important functions to mediate cytokinesis and reformation of the nuclear envelope in the progeny cells (Ramadan et al., 2007). Importantly, AURKB facilitates cleavage furrow ingression (Ahonen et al., 2009) but for the abscission, the kinase activity must be suppressed (Steigemann et al., 2009).

2.4.5 CPC and SAC

The kinetochore-microtubule interactions and the physical pulling forces created by the attached microtubules communicate with the SAC to control the start of anaphase. As discussed above, CPC has an important role in the correction of abnormal microtubule attachments at the kinetochores and thereby it participates in the SAC induction (Vader et al., 2007). For example, chemical inhibition of AURKB activity by Hesperadin (Hauf et al., 2003) and ZM447439 (Ditchfield et al., 2003), or introduction of function-blocking anti-AURKB or anti-INCENP antibodies into the dividing cells (Kallio et al., 2002; Ahonen et al., 2009), renders the SAC ineffective in response to microtubule drug-induced cell cycle ar-

rest. The cells slip out of mitosis prematurely in the absence of CPC function, which is a direct indication that normal CPC function is essential for the maintenance of SAC activity in the presence of unattached kinetochores.

The major role for AURKB activity in the maintenance of SAC is attributable to the AURKB mediated recruitment of Mps1 kinase to the unattached kinetochores, which is an early event in the SAC activation (Vigneron et al., 2004; Saurin et al., 2011; Nijenhuis et al., 2013). Besides causing destabilization of incorrect kinetochore-microtubule attachments via phosphorylation of the Hec1, AURKB at the same time increases Hec1 binding to Mps1 leading to the recruitment of Mps1 to the kinetochores (Zhu et al., 2013). Also, an important feedback mechanism exists in which the Mps1 facilitates AURKB centromere localization by inducing phosphorylation of Thr120 of H2A (Van Der Waal et al., 2012). This is one example about the complexity of communication between kinetochore-microtubule interface and SAC machinery. Finally, it should not be forgotten that the enrichment of many SAC proteins like Mad1, Mad2, BubR1, Bub1, Bub3, Zw10, and ROD on the unattached kinetochores is dependent on normal AURKB activity (Vigneron et al., 2004; Famulski and Chan, 2007; Ditchfiled et al., 2003).

2.5 Mitotic arrest deficient 2 (Mad2)

As reviewed in Chapter 2.3, the Mad2 protein has a central role in generation of the MCC and co-ordination of the early mitotic events with the SAC activity. Mitotic arrest deficient (Mad) proteins namely Mad1, Mad2 and Mad3 (BubR1 in humans) were first identified in a yeast genetic screen to be important regulators of mitotic checkpoint in response to the spindle abnormalities caused by microtubule destabilizing drugs (Li and Murray, 1991). Later the functional conservation of Mad proteins from yeast to humans was confirmed (Li and Benezra, 1996).

2.5.1 Structural conformations of Mad2

Mad2 was originally reported to exist as a monomer and dimer (Fang et al., 1998). As mentioned above, later Mad2 was found to exist *in vivo* in two conformations known as C-Mad2 and O-Mad2. The C-Mad2 is critical for SAC activity during mitosis as it interacts with Mad1, Cdc20, O-Mad2, BubR1 and p31 comet.

O-Mad2 is able to dimerize with C-Mad2. The C-Mad2 catalyzes the conversion of O-Mad2 to C-Mad2. This is an important catalytic step in defining the SAC activity, as O-Mad2 cannot act as an endogenous APC/C inhibitor. The structural properties of O-Mad2 do not permit the formation of O-Mad2:O-Mad2 homodimer and O-Mad2:Cdc20 heterodimer. Conversely, C-Mad2 is able to form a homodimer with C-Mad2 and heterodimers with O-Mad2, Cdc20 and Mad1. Importantly, C-Mad2 protein presents the same binding region for both Mad1 and Cdc20, which leads to competition between the two proteins for C-Mad2 binding (Luo et al., 2000; 2002 and 2004; Mapelli et al., 2007; Yang et al., 2008). Typically, the C-Mad2 associates with its binding partners Mad1 and Cdc20 to form a tight 'safety belt' where C-Mad2 is wrapped around Mad1 and Cdc20 (Luo et al., 2002; Sironi et al., 2002). Also, O-Mad2, BubR1 and p31comet have been observed to compete with each other for the C-Mad2 binding (Tipton et al., 2011; Mariani et al., 2012; Chao et al., 2012).

2.5.2 Mad2 localization

In interphase cells, a fraction of Mad2 protein is located at the nuclear envelope together with Mad1 (Campbell et al., 2001). During mitosis, Mad2 is observed at the unattached kinetochores, spindle microtubules and spindle poles (Howell et al., 2000). Moreover, a large fraction of Mad2 remains soluble in the cytoplasm for the entire cell cycle.

During mitosis, the recruitment of Mad2 to the unattached kinetochores is dependent on Mad1 (Chen et al., 1998) and the presence of Mad1 bound C-Mad2 at the kinetochores determines the rate of MCC formation (De Antoni et al., 2005). It is estimated that a single unattached kinetochore can possess up to one thousand binding sites for Mad2 proteins, which would be essential for maximum MCC production (Howell et al., 2000). According to the current model, in mitotic cells, Mad2 is a dynamic protein that traffics between the cytosol and kinetochores. More precisely, about half of protein in the pool turns over slowly (the Mad2 bound with Mad1) and the other half rapidly (the O-Mad2 and C-Mad2 forms) at the kinetochores (Shah et al., 2004). Finally, before the onset of anaphase, the Mad1 and Mad2 are transported from the kinetochores to the spindle poles along the microtubules in a dynein-mediated process (Howell et al., 2004; Shah et al., 2004).

2.5.3 Mad2 function

In mitosis, the Mad1 dependent kinetochore localization of Mad2 is needed to produce high amounts of C-Mad2 and MCC that prevent chromosome segregation even in the presence of a single unattached kinetochore. Furthermore, Mad2 controls the mitotic timing (Meraldi et al., 2004) and stability of the kinetochore-microtubule attachments (Kabeche and Compton, 2012) in a manner that does not require its kinetochore localization. It should be noted that also in interphase cells, the Mad1:C-Mad2 complex forms at the nuclear envelope to produce Cdc20 inhibitor complex during interphase (Rodriguez-Bravo et al., 2014). The erroneous inactivation of the Cdc20 inhibitory complex during interphase initiates the premature degradation of Cyclin B and securin that upon entry to M phase leads to precocious anaphase (Rodriguez-Bravo et al., 2014; Malureanu et al., 2009; Maciejowski et al., 2010).

Under normal growth conditions, functional inactivation of Mad2 induces cellular senescence (Prencipe et al., 2009; Yun et al., 2009; Lentini et al., 2012) and causes defects in the cells' response to DNA damage (Fung et al., 2008; Lawrence et al., 2015). In addition, the deregulation of Mad2 generates significant mitotic anomalies. For example, cells deficient of Mad2 function exhibit faster progression of mitosis; the cells separate sister chromatids before the chromosome congression to the spindle equator is completed (Gorbsky et al., 1998; Meraldi et al., 2004). On the other hand, overexpression of Mad2 causes many defects such as mitotic delay, the formation of hyperstabilized and incorrect kinetochore-microtubule attachments, lagging chromosomes and induction of aneuploidy in the daughter cells (Howell et al., 2000; Kabeche and Compton, 2012).

2.5.4 Regulation of Mad2 expression

Mad2 expression shows a cell cycle-dependent variation; the Mad2 protein is less produced in G1 phase while the production is increased during the S phase and reaches maximum at M phase (Hernando et al., 2004; Jeong et al., 2004). Importantly, the cell cycle-dependent expression of Mad2 is regulated by transcriptional, post-transcriptional and post-translational mechanisms. In normal cell cycle, the *MAD2* transcription is negatively regulated by the repressor-element-1-silencing transcription factor (REST) at G2 phase. Later, an interaction of REST with the ubiquitin ligase SCF (beta-TrCP) facilitates REST degradation that uplifts the *MAD2* repression (Guardavaccaro et al., 2008). However, several mechanisms exist to keep the Mad2 protein levels at normal physiological concentrations to facilitate ordered mitosis and protect cells from genomic imbalance via altered Mad2 expression.

In humans, the *MAD2* gene is mapped on Chr4q27 and a pseudogene on Chr14 (Krishnan et al., 1998). *MAD2* gene promoter presents the binding sites for at least three transcription factors, namely E2F transcription factor 1 (E2F1) (Hernando et al., 2004), Breast-Cancer-Associated-gene 1 (BRCA1) (Wang et al., 2004) and c-MYC (Menssen et al., 2007) whose increased expression positively correlates with elevated *MAD2* expression. On the other hand, the *MAD2* has also been observed to be downregulated in cancer cells due to e.g. promoter hypermethylation as was the case in hepatocellular cancer cells (Jeong et al., 2004). At the post-transcriptional level, the *MAD2* gene expression is regulated by microRNAs (miRNAs; discussed in chapter 2.6) that add an important control over the Mad2 dependent cellular events. For example, miR-433 and miR-28-5p have been validated to bind to the *MAD2* mRNA and downregulate the Mad2 protein levels when present in excess and *vice versa* (Furlong et al., 2012; Hell et al., 2014; Schneider et al., 2014). The post-translational modifications of Mad2 contribute to its function. First, phosphorylation of Mad2 at serines170, -178 and -195 needs to be minimal to allow the association of the protein with Mad1 and MCC (Wassman et al., 2003; Kim et al., 2010). The Mad2 phoshorylation increases after the SAC is satisfied (Wassman et al., 2003). Another level of Mad2 regulation is at the control of Mad2 protein stability; the HECT ubiquitin ligase Smurf2 protects Mad2 from proteasome mediated degradation (Osmundson et al., 2008). The human cells depleted of Smurf2 by RNAi exhibit impaired SAC due to enhanced degradation of Mad2 protein (Osmundson et al., 2008).

2.6 Micro-RNAs (miRNAs)

In animals, the family of small non-coding RNA molecules includes three primary classes; micro-RNAs (miRNA), small interfering RNAs (siRNA) and PIWI-interacting RNAs (piRNA).

miRNAs are evolutionary conserved tiny (18-25 nucleotides in length) non-protein coding RNA molecules that regulate gene expression at post-transcriptional level (reviewed in Ha and Kim, 2014). The first miRNA to be discovered was lin-4 that was shown to negatively regulate the gene expression of *LIN-14* and affect the development of *C. elegans* (Lee et al., 1993; Wightman et al., 1993). Importantly, these studies showed that an intact 3'UTR within the target mRNA was required for the miRNA to act as post-transcriptional gene silencer (Lee et al., 1993; Wightman et al., 1993). Since then a large group of miRNAs has been identified in various species and studies have verified that the miRNAs are *bona fide* members of the genome. For example, in humans, there are 1881 miRNA precursors and 2588 mature miRNAs known to date (http://www.mirbase.org/cgi-bin/browse.pl?org=hsa) (5.3.2016). The miRNAs essen-

tially fine tune gene expression and regulate a wide spectrum of cellular pathways ranging from cell survival and apoptosis to development and differentiation.

2.6.1 miRNA genomics

miRNA precursor sequences are positioned as intergenic or intragenic in the genome. Intergenic miRNA exists as an independent transcriptional unit whereas intragenic miRNA lies within an intron of a gene (usually a protein-coding gene). Importantly, the production of both miRNA types is facilitated by RNA-polymerase II-mediated gene transcription (Lee et al., 2004). Moreover, in most cases, the transcription of the intragenic miRNA coincides with the transcription of the host gene (Baskerville and Bertel, 2005; Rodriguez et al., 2004). However, a small number of intragenic miRNAs can also be produced independently of their host gene (Monteys et al., 2010).

A group of miRNAs that originate from the common ancestor gene are categorized together as a miRNA family. Typically, the individual miRNAs within a particular family possess similar seed sequence, which is a region consisting of nucleotides 2 to 8 at the 5'end of a miRNA (e.g. miR-17~92 family). However, some exceptions exist in which the seed sequence slightly varies within a miRNA family (e.g. miR-141 and miR-200c that both belong to miR-200 family) (Kim et al., 2013). Gene duplication during evolution is thought to be one reason for the existence of these miRNA paralogs (Hertel et al., 2006; Yuan et al., 2011). It should be noted that the miRNAs composing a family are not always located together within the same genomic region but can be placed in even different chromosomes (Bartel, 2009). Similarly, not all miRNAs located at the same genomic region, lined-up one after the other, belong to the same miRNA family, but rather are classified as clustered miRNAs (e.g. Chr14q32 miRNA cluster).

Importantly, the miRNAs having a similar seed sequence, but different length and 3'end, can be produced from the same miRNA locus and are known as isomiRs (Marti et al., 2010; Landgraf et al., 2007; Lee et al., 2010). Additionally, separate genomic loci can contain miRNAs with exactly the same mature sequence (miR-125b-1 on chr11 and miR-125b-2 on Chr21). The isomiRs can coordinate their canonical miRNAs to regulate similar signaling pathways (Cloonan et al., 2011).

2.6.2 miRNA biogenesis

A single stranded mature miRNA is the final product of miRNA biogenesis pathway (Fig. 5). The canonical pathway to produce mature miRNA is a step-wise maturation process that starts from the production of a primary-miRNA (Pri-miRNA). Pri-miRNA is synthesized by transcription of an intergenic miRNA or the host gene of an intragenic miRNA. The Pri-miRNA is several hundred nucleotides long and it undergoes further cleavage mediated by a RNAase III enzyme called Drosha (160kDa) and RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8) (90kDa). The cleavage product is preliminary-miRNA (Pre-miRNA) that is a hairpin loop structure of ~60-70 nucleotides long (Lee et al., 2003; Han et al., 2006). These processes take place in the nucleus, but for further maturation, the Pre-miRNA is exported to the cytoplasm by Exportin-5-Ran-GTP (Yi et al., 2003; Lund et al., 2004). In the cytoplasm, the Pre-miRNA is recognized by another RNase III enzyme called Dicer (200kDa) (Hutvagner et al., 2001; Bernsteine et al., 2001) and a Transactivation-responsive RNA binding protein (TRBP) that turn the hairpin loop into a miRNA duplex of ~22 nucleotides in length (Chendrimada et al., 2005). Furthermore, the miRNA duplex is loaded onto regulatory proteins called Argonaute (AGO, four different family members exist, AGO1-4) to form a miRNA-induced silencing complex (miRISC) together with Glycine tryptophan repeat-containing protein of 182 kDa (GW182). Importantly, prior to the recognition of a target mRNA, the AGO proteins processes the miRNA duplex to produce a mature single stranded miRNA (Hammond et al., 2001; Song et al., 2004; Schirle and MacRae, 2012). The removal of passenger strand takes place either by cleavage or unwinding that is mediated by AGO2 and AGO1, 3 or 4 respectively (Kawamata et al., 2009; Gregory et al., 2005). However, this is not the only function of AGO proteins present in the miRISC as they also take an active part in the silencing of target mRNA.

Importantly, a small portion of miRNAs are also produced via alternative mechanisms. For example, mirtrons (Pre-miRNAs from introns) can skip the Drosha and DGCR8 mediated cleavage but require processing by Dicer in the cytoplasm (Ruby et al., 2007; Berezikow et al., 2007). Moreover, certain miRNAs such as miR-451 are processed by AGO2 instead of a Dicer-dependent mechanism (Cheloufi et al., 2010; Cifuentes et al., 2010). Furthermore, small RNA molecules that are produced from other non-coding RNAs like t-RNAs and small nucleolar RNA (sno-RNA) also evade the Drosha-mediated canonical mechanism but require Dicer processing for their maturation (Babiarz et al., 2008; Ender et al., 2008).

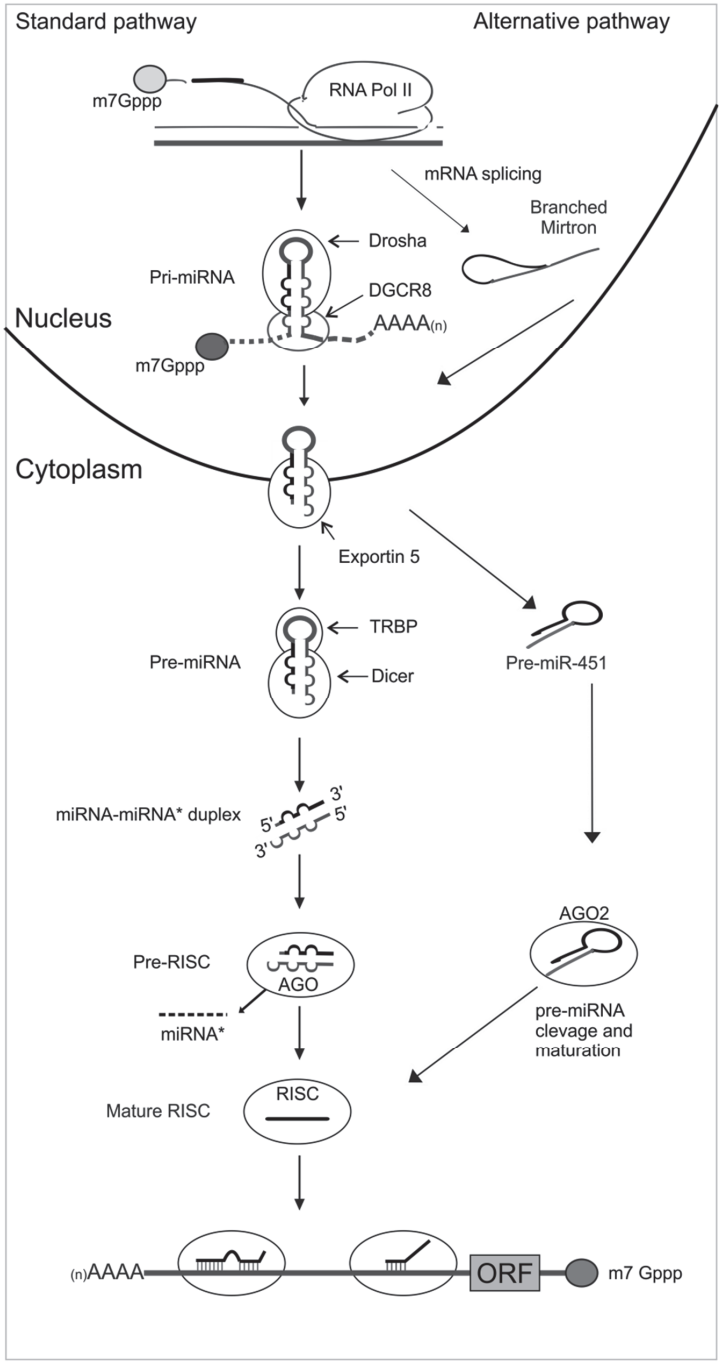

Figure 5: miRNA biogenesis pathways (see text for details). Modified from Ameres and Zamore, 2013. m7Gppp= 7-methylguanosine cap, RNA pol II= RNA polymerase II, AAAA (n)= poly A-tail, ORF= open reading frame. Branched mirtron is a Pri-miRNA generated from intron after mRNA splicing.

2.6.3 miRNA function

Once in miRISC, the miRNA determines the identity of the target mRNAs via complementary binding, whereas the protein components AGO and GW182 executes the silencing of that particular mRNA (Eulalio et al., 2008; Takimoto et al., 2009). The miRISC mediated silencing of target mRNAs is achieved either by repression of translation without affecting the expression levels of the mRNA or by inducing mRNA decay that results in the reduction of total mRNA levels.

Mechanistically, GW182 interacts with AGO proteins that recruit it to the miRISC (Takimoto et al., 2009; Lian et al., 2009). When a miRNA has guided the miRISC to the mRNA, GW182 recruits CCR4–NOT and PAN2-PAN3 deadenylase complexes to the mRNA that mediates the removal the poly-A tail and trigger the mRNA destabilization (Chekulaeva et al., 2011; Braun et al., 2011; Fabian et al., 2011). In addition, the CCR4–NOT complex also regulates the translational repression independent of deadenylation (Cooke et al., 2010). On the other hand, if the miRNA in the miRISC attains perfect complementary binding to the target mRNA then AGO2 mediates the mRNA cleavage (Liu et al., 2004; Yekta et al., 2004). The translational repression by miRISC involves reduced rate of translation initiation and destabilization of the translational machinery (Mathonnet et al., 2007). It should, however, be noted that many details of these processes are still poorly understood. Interestingly, some mRNAs can undergo both translational repression as well as decay, and in such cases, the translational inhibition precedes the mRNA decay (Bazzini et al., 2012).

The two miRNAs, miR-378a-5p and miR-493-3p, that were investigated in this thesis for their role in mitosis are described below.

2.6.4 miR-378

miR-378a is an intragenic miRNA located on Chr5q where it resides within the first intron of its host gene Peroxisome proliferator-activated receptor gamma coactivator 1 beta *PPARGC1B* also known as *PGC-1β*. In human cells, miR-378 is present as different variants (miR-378- a-j) that all have the same seed sequence but are different individual miRNAs. These miR-378 variants are present at different genomic locations and exist either as intergenic or intragenic miRNAs (Krist et al., 2015). The miR-378a, which represents the most commonly expressed form of miR-378, gives rise to either miR-378a-3p or miR-378a-5p mature miRNA.

Function

The miR-378a and its host gene *PGC-1β* expression is co-regulated and show higher expression in heart, skeletal muscle and brown adipose tissue (Carrer et al., 2012). *PGC-1β* is an important regulator of energy metabolism (Lin et al., 2005) and its expression has been found to increase in response to exercise, hypoxia, caloric restriction and ageing (reviewed in Patten and Arany, 2012). Interestingly, miR-378a and PGC-1β are implicated in the same signaling pathways. For example, in glucose and fatty acid metabolism miR-378a counteracts the activity of its host gene (Carrer et al., 2012). In cancer cells, the miR-378a-5p mediates the Warburg effect to shift the cancer cell metabolism from oxidative to glycolytic metabolic pathway; the miRNA regulates the activity of PGC-1β by targeting two of its binding partners, namely the ERRγ and GA-binding protein-α, for decay (Eichner et al., 2010). Moreover, miR-378a-5p is implicated in angiogenesis where it positively controls the expression of vascular endothelial growth factor-A *VEGF-A* (Hua et al., 2006; Lee et al., 2007). miR-378a-5p competes for binding to the *VEGF-A* mRNA-3'UTR with another miRNA, miR-125a. This way miR-378a-5p prevents translation inhibition of *VEGF-A* mRNA by miR-125a resulting in increased VEGF-A production (Hua et al., 2006). Additionally, miR-378a-5p downregulates SuFu (Lee et al., 2007) and can activate sonic hedgehog (SHH) pathway. Importantly, also the activation of SHH pathway is associated with increased production of VEGF-A (Pola et al., 2001; Nagase et al., 2005).

2.6.5 miR-493

miR-493 is an intergenic miRNA located in locus chr14q32.2. Importantly, this locus houses more than 60 miRNAs representing one of the largest miRNA clusters in the human genome identified thus far (Seitz et al., 2004). In addition, several paternally and maternally imprinted genes are present in this locus. For example, *DLK1* and *RTL1* represent the paternally expressed protein-coding genes; while *MEG3*, *MEG8*, *RTL1as*, *snoRNAs* represent maternally expressed non-coding genes (Kagami et al., 2008; da Rocha et al., 2008).

Function

miR-493-3p has been shown to control the cell adhesion, migration and invasion by negatively regulating the gene expression of Ras homolog family member C *RhoC*, Frizzled class receptor 4 *FZD4* (Ueno et al., 2012), Mitogen-activated protein kinase kinase 7 *MKK7* (Sakai et al., 2014), *E2F1* (Gu et al., 2014), insulin-like growth factor 1 *IGF1R* (Okamoto et al., 2012) and Dickkopf-1 *DKK1* (Jia et al., 2016). A recently published study by Jia and colleagues show that miR-493-

3p downregulates DKK1 and promote the cell proliferation and invasion in gastric cancer cells *in vitro* and *in vivo* (Jia et al., 2016). This finding is in contrast with the previous reports where miR-493-3p was found to inhibit cell migration and invasion especially in gastric cancer cells (Ueno et al., 2012) and lung cancer cells (Gu et al., 2014). Also in terms of the miR-493-3p levels two studies have shown contrasting results; either upregulation or downregulation of the miRNA in gastric cancer cells (Ueno et al., 2012; Jia et al., 2016). The reason for these opposite results is not yet clear.

2.7 Dual Specificity Phosphatases (Dusps)

Protein phosphatases counteract the activity of protein kinases to regulate various cellular events and signaling pathways. In eukaryotes, the kinases modify typically the tyrosine (Tyr), serine (Ser) and threonine (Thr) residues in the substrate protein by addition of a covalently bound phosphate group. This phosphorylation reaction alters the confirmation of the substrate protein causing e.g. its activation. Protein phosphatases can remove the phosphate group and thereby reverse the reaction causing e.g. functional inactivation of the substrate protein.

2.7.1 Dusp family of phosphatases

The Dual specificity phosphatases (Dusps) compose one subcategory in the protein tyrosine phosphatase (PTPs) superfamily. Dusps consist of 63 proteins that are further classified into seven sub-groups, namely, the mitogen-activated protein kinase (Mapk) phosphatases (MKPs), atypical dual-specificity phosphatases (A-Dusps), phosphatases of regenerating liver (PRLs), slingshots phosphatase, phosphatase and tensin homologs (PTENs), myotubularins and cell division cycle 14 homologues (Cdc14s) (Duan et al., 2015; reviewed in Pavic et al., 2015).

The members of the Dusps possess a unique ability to dephosphorylate both the phosphorylated Ser/Thr and tyrosine residues that the other members of the PTP family cannot perform. This characteristic is based on a shallow and broad catalytic cleft that allows access to biphosphorylated substrate molecules. It is therefore not surprising that the Dusps have been observed to regulate a diverse set of cellular molecules including peptides, mRNA, phosphoinositide and glycans (reviewed in Pavic et al., 2015). Importantly, the Dusp family members share a similar catalytic mechanism in which the cysteine (Cys) in the phosphate-binding loop and aspartate (Asp) in the WPD-loop contribute to their enzymatic activity (Tonks, 2013). As a result, the MKPs and A-Dusps have several common sub-

strates; however, the A-Dusps possess an overall more diverse set of substrates. The MKPs and A-Dusps are the most widely studied Dusps.

2.7.2 Mapk phosphatases (MKPs)

The MKPs are well-known regulators of the Mapks extracellular signal-regulated kinase 1 and 2 (Erk1/2), c-Jun N-terminal kinase (Jnk) and p38. The MKP family consist of 10 members namely Dusp1, 2, 4, 5, 6, 7, 8, 9, 10 and 16. The presence of N-terminal Cdc25 homology (CH2) domain and the Mapk binding (MKB) domain facilitate the MKP's substrate specificity (Caunt and Keyse, 2013). Importantly, MKPs are required for the early response of a cell to the extracellular growth and stress stimuli generated by cytokines, serum and/or growth factors (Caunt and Keyse, 2013).

2.7.3 Atypical Dusps (A-Dusps) and Dusp3

The A-Dusp family consists of 23 members and represents the largest subgroup in the Dusp family. In general, A-Dusps lack the N-terminal CH2 homology and MKB domains and are smaller in size as compared to other proteins in the Dusp family. Dusp3/Vhr (20kDa) was the first Dusp protein to be crystallized (Yuvaniyama et al., 1996). Later it became evident that Dusp3 dephosphorylates both the Ser/Thr and Tyr-phosphorylated residues, and exhibits strong catalytic activity towards biphosphorylated residues as compared to the monophosphorylated ones (Schaumacer et al., 2002). In addition, Dusp3 favors phosphorylated Tyr residues over the phosphorylated Ser/Thr residues (Luechapanichkul et al., 2013). Although Dusp3 has a low sequence similarity with the PTPs and MKPs, the Cys and Asp residues critical for the catalytic activity are conserved.

Dusp3 was first discovered to target Tyr-phosphorylated growth factor receptors for insulin, Pdgf, Egf and Kgf and Ser-phosphorylated casein kinase (Ishibashi et al., 1992). Later, the growing interest towards the Dusp3 led to the identification of several other substrates such as the Mapk's Erk1/2 (Todd et al., 1999) and Jnk (Todd et al., 2002), as well as erythroblastic leukemia viral oncogene homolog 2 (ErbB2) (Wang et al., 2011), signal transducer and activator of transcription-5 (STAT5) (Hoyt et al., 2007) and protein kinase C (Pkc) (Amand et al., 2014). Two decades of research has implicated Dups3 in diverse cellular processes including cell proliferation, apoptosis, immune response and angiogenesis.

2.7.4 Dusp3 regulates Mapk pathway

The Dusp3 has emerged as an important phosphatase to regulate Erk1/2 and Jnk, especially in the context of normal and malignant cell growth (Rahmouni et al., 2006; Hao and ElShamy, 2007; Wagner et al., 2013) (Fig. 6).

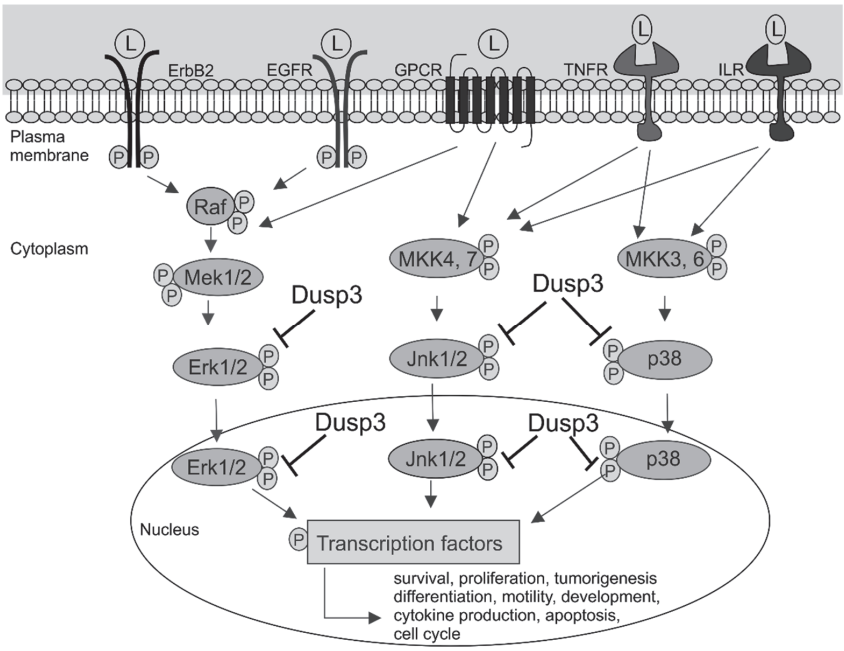

Figure 6: The reported physiological substrates of Dusp3 in the Mapk pathway. Modified from Pavic et al., 2015. L= hormones, growth factors, cytokines, interleukins, stress stimuli, lipopolysaccharide, mitogens, GPCR activation; ILR= interleukin receptor; TNFR= tumor necrosis factor receptor; GPCR= G-protein-coupled receptor, MKK= Mitogen-activated protein kinase kinase.

Although *DUSP3* is not an early response gene to the Mapk pathway activating stimuli (Alonso et al., 2001) and it lacks the MKB domain, it was found to control cell cycle transitions at the G1-S and G2-M phases in an Erk1/2 and Jnk dependent manner (Rahmouni et al., 2006). However, the regulation of Erk1/2, Jnk and p38 Mapk by Dusp3 has been controversial and it appears to have cell line specific characteristics. For example, in COS-1 cells, the Dusp3 negatively regulated the Erk activity with only a minor impact on Jnk and p38 activities (Todd et al., 1999). In another study where a non-small cell lung cancer (NSCLC) cell lines were investigated, the Dusp3 was shown to induce dephosphorylation of both Erk1/2 and Jnk1/2 in the H1792 cells, while in the H460 cells the phospha-

tase targeted only Erk1/2 (Wagner et al., 2013). Furthermore, in B cells, T cells, macrophages and platelets derived from a Dusp3$^{-/-}$ mouse the activity of Erk1/2 and Jnk was not affected by the loss of the phosphatase (Amand et al., 2014). These findings can be explained by co-factor variations, functional redundancy and cell line specific reactions in the Dusp-Mapk pathway.

2.7.5 Regulation of Dusp3 protein levels and activity

The details concerning the extent and control of Dusp3 expression are very limited. In HeLa cells, Dusp3 protein is expressed in a cell cycle-dependent manner with the lowest expression at G1 and maximal at G2/M phase (Rahmouni et al., 2006). The same study shows that Dusp3 is less stable at G1 phase in comparison to S and G2/M phases. To date, only two negative regulators of Dusp3 expression, a histone lysine demethylase (KDM2A) and BRCA1-IRIS, have been reported to operate in NSCLC cell lines (Wagner et al., 2013) and breast cancer cells (Hao and ElShamy, 2007), respectively. The activity of Dusp3 is also controlled at post-translational level. For example, activation of T-cell receptor signaling enhances Dusp3 phosphorylation at Tyr138 by ZAP-70 kinase. The Tyr138 phosphorylated Dusp3 is functionally more active than the non-phosphorylated phosphatase (Alonso et al., 2003). Moreover, the mutation of Cys124 to Ser within the active site of the Dusp3 renders it functionally inactive (Todd et al., 2002; Alonso et al., 2001; Ishibashi et al., 1992). Furthermore, Dusp3 is able to form a homodimer and alter its own catalytic activity (Pavic et al., 2014). In addition, the association of Dusp3 and VRK3, which is another Dusp protein, enhances the catalytic activity of Dusp3, at least towards the Erk1/2 (Kang and Kim, 2006).

2.7.6 Localization of Dusp3 and Erk1/2 during mitosis

Both Dusp3 and non-phosphorylated Erk1/2 are cytosolic in early mitotic mammalian cells with some concentration within the area occupied by the spindle apparatus. In late mitotic cells, Dusp3 accumulates to the midbody while the majority of non-phosphorylated Erk1/2 remains cytosolic with little midbody concentration (Rahmouni et al., 2006; Willard and Crouch, 2001). The studies regarding the quantity and spatial distribution of active phosphorylated Erk1/2 at M phase show controversial results that are possibly due to the technical challenges in the detection of active forms of Erk1/2 at the level of mitotic organelles (Shapiro et al., 2008; Shinohara et al., 2006). Lack of information about the Dusp-Mapk axis

has left a gap in understanding the function of the phosphatase, especially in the mitotic cells.

2.8 Cancer and Genomic balance

2.8.1 Aneuploidy

Cancer evolves from normal cells as a result of defects in one or more regulatory signaling pathways. Typically, the development of cancer is a multi-step process and involves selection of cell clones that have acquired growth and survival advantage, which helps them to proliferate in an unregulated way and to bypass normal tissue homeostasis (Hanahan and Weinberg, 2011). One of the hallmarks of cancer is aneuploidy, which can arise either by gain or loss of individual chromosomes (chromosomal aneuploidy) or by chromosome rearrangements such as translocations, inversions, amplifications and deletions (segmental or structural aneuploidy) (Janssen et al., 2011; Liu et al., 2011). The tendency of cells to undergo unfaithful chromosome segregation is referred to as chromosomal instability (CIN). Every cell with CIN can be aneuploid whereas not every aneuploid cell is genomically unstable (Gordon et al., 2012). For example, a person with the Down syndrome has an extra copy of chromosome 21 (Chr21) in each cell of the body, or to a much lesser extent structural aberrations in Chr21 or a mosaic cell population where both the normal and Chr21 trisomic cell lines are present. However, the cells are not chromosomally unstable as they do not show further defects in chromosome segregation postnatally.

Importantly, a cell is equipped with cell cycle checkpoints (see chapter 2.1) and other safeguard mechanisms such as tumor suppressor genes *p53* and *retinoblastoma* (Rb) that aim to the maintenance of genomic balance and faultless DNA (Davoli and de Lange, 2011). However, these signaling pathways can be hijacked by cancer cells to stimulate tumorigenesis and provide growth advantage and/or selection power for tumor cells.

The pioneering work by David Paul Von Hansemann (von Hansemann, 1890) and Theodar Boveri (Boveri, 1914) more than a century ago led to a hypothesis that aberrant mitosis would generate cells with different chromosome content that might contribute to tumorigenesis. In line with their observations, chromosomal aneuploidy is frequently detected in tumor cells (Lengauer et al., 1997) and defective cell division is believed to be the main driver for this abnormality (reviewed in Holland and Cleveland, 2009). During mitosis, the defects in SAC signaling, sister chromatid cohesion, kinetochore-microtubule attachments and

spindle assembly (Thompson and Compton, 2008; Barber et al., 2008; Weaver et al., 2007; Baker et al., 2009) can directly or indirectly contribute to chromosome missegregation (Fig. 7).

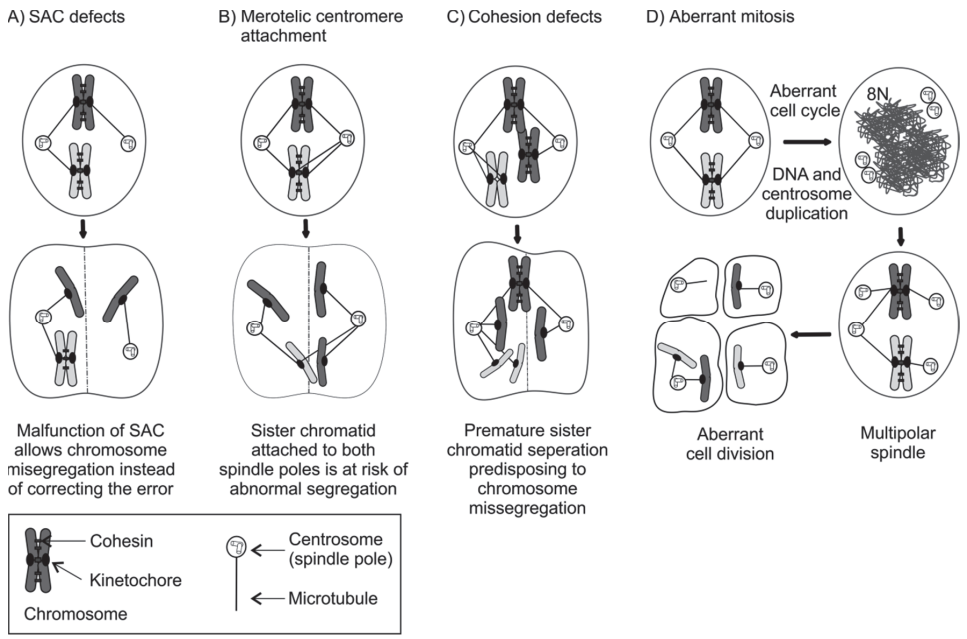

Figure 7: The roads to aneuploidy. Modified from Kops et al., 2005.

2.8.1.1 SAC defects lead to aneuploidy

Several studies have reported induction of aneuploidy and tumor formation in cell culture and mouse models as a consequence of deregulation of SAC proteins. For example, the reduced levels of Cenp-E protein (Weaver et al., 2003; 2007) and haploinsufficiency of *BUBR1* and *MAD2* (Dai et al., 2004; Michel et al., 2001) have been shown to cause faulty SAC and increased rate of chromosome missegregation in cell culture. In mouse models, reduced levels of Cenp-E, BubR1 and Mad2 (Weaver et al., 2007; Dai et al., 2004; Michel et al., 2001) have been reported to increase the frequency of aneuploidy and cause formation of tumors *in vivo*. In addition, Mad2 upregulation mediates CIN and tumor progression after the loss of Rb and p53 inactivating mutation (Hernando et al., 2004; Sotillo et al., 2010; Schvartzman et al., 2011). It is important to note that the partial loss of Mad2 is implicated in tumorigenesis while the complete loss is lethal even for cancer cells (Michel et al., 2001). Interestingly, high expression of

BubR1 exerts protective functions by delaying the aneuploidy induction in the mice, which extends their lifespan (Baker et al., 2013).

In human tumors, mutations in *BUBR1* (Hanks et al., 2004) have been linked to induction of mosaic variegated aneuploidy (MVA), a rare disorder due to constitutional aneuploidy that predisposes these individuals to develop certain cancers. Experiments with MVA patient-derived cell lines have shown that these cells exhibit reduced BubR1 levels, which was associated with low SAC activity and defects in chromosome alignment (Suijkerbuijk et al., 2010). Several other SAC proteins, as for example AURKB, MCAK, Kif2b, Ndc80 complex, are important for establishment and stability of normal kinetochore-microtubule attachments (Hauf et al., 2003; Knowlton et al., 2006; Bakhoum et al., 2009; DeLuca et al., 2006; Kabeche and Compton, 2012; Elowe et al., 2007). Moreover, deregulation of these proteins in cancer cell lines can lead to incorrect chromosome segregation, which provides one potential explanation for the aneuploidy induction in cancer cells.

2.8.1.2 Merotelic kinetochore-microtubule attachments give rise to aneuploidy

SAC monitors kinetochore-microtubule attachments to prevent aneuploidy. However, merotelic attachments are not detected by the checkpoint and therefore the lagging chromatids/chromosomes created by merotely during anaphase increase the risk of chromosomal aneuploidy (Cimini et al., 2001; 2002). The defects in condensin (Samoshkin et al., 2009) and cohesion functions (Loncarek et al., 2007), induction of multipolar spindles (Ganem et al., 2009; Silkworth et al., 2009), and hyperstable kinetochore-microtubule interactions (Kabeche and Compton, 2012) can all generate merotelic attachments.

2.8.1.3 Defects in cohesion pathway lead to aneuploidy

The cohesin complex is needed for chromosome condensation, sister chromatid cohesion, DNA damage response and DNA recombination, and thus is implicated in control of genomic stability (Kim et al., 2002; Yazdi et al., 2002; Jessberger et al., 1996). The deregulation of cohesion complex mainly results in premature separation of sister chromatids, which can lead to aneuploidy in the daughter cells. Inactivation of proteins working in the cohesion pathway such as SMC1A, SMC3 and STAG2 has been reported to cause aneuploidy (Barber et al., 2008; Solomon et al., 2011). Moreover, other members of the cohesion pathway as for example WALP (Oikawa et al., 2004), separase (Zhang et al., 2008) and securin (Zou et al., 1999) are found overexpressed in many cancers.

2.8.1.4 Abnormal spindle assembly leads to aneuploidy

The spindle poles contribute to the formation of a bipolar mitotic spindle in early mitosis. Cancer cells can harbor multiple spindle poles that may arise by means of centriole overduplication, cytokinesis failure, cell fusion and/or *de novo* centriole assembly. Importantly, multipolar mitotic spindles can also be generated without centrosome amplification by a process that involves loss of centrosome integrity to cause centriole disengagement and/or PCM fragmentation (reviewed in Maiato and Logarinho, 2014).

Experiments with cultured cells have shown that progression of mitosis in the presence of multiple spindle poles leads to defects in chromosome segregation (Ganem et al., 2009; Silkworth et al., 2009; Maiato and Logarinho, 2014). Interestingly, multipolar mitotic cells do not always generate multipolar anaphases (Quintyne et al., 2005; Kwon et al., 2008). This fact points to the ability of certain cells to cluster multiple centrosomes into two "poles" to undergo bipolar cell division. Nevertheless, in these cells merotelic attachments are frequent and the consequent lagging chromosomes cause low-level aneuploidy (Ganem et al., 2009; Silkworth et al., 2009). Moreover, mitotic spindles can assemble without the centrosomes, but these cells may undergo defective cytokinesis (Piel et al., 2001; Khodjakov and Rieder, 2001) and exhibit chromosome segregation errors (Sir et al., 2013) and cell cycle arrest (Wong et al., 2015; Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001).

2.8.2 Defects in mitosis contribute to induction of segmental aneuploidy

Besides causing chromosomal aneuploidy, mitotic mistakes can also give rise to structural chromosome aberrations (Janssen et al., 2011; Liu et al., 2011; Ganem and Pellman, 2012). For example, the Mad2 overexpression in a mouse model led to the induction of loss or gain of whole chromosomes, as well as caused structural defects in the chromosomes and initiated tumorigenesis (Sotillo et al., 2007). In another example, the cytokinesis failure in p53-null (p53-/-) mouse mammary epithelial cells generated tetraploidy, as well as chromosomal rearrangements and these cells, formed tumors upon transplantation into nude mice (Fujiwara et al., 2005).

In addition, the prolonged mitotic arrest is also a source of DNA damage. For example, treatment of human cancer cells and primary fibroblasts with microtubule drugs induces a prolonged mitotic arrest. However, the treatments can also increase DNA breaks detected by the presence of chromosome fragments and increased γ-H2AX staining specifically during mitosis (Dalton et al., 2007; Qui-

gnon et al., 2007). Moreover, a similar phenotype was observed after depletion of Cenp-E by RNAi in colon cancer cells; the cells exhibited a transient mitotic arrest and DNA breaks (Dalton et al., 2007).

The presence of damaged DNA and chromosome breakage, or lagging chromosomes may result in the formation of aberrant nuclear structures called micronuclei (Cimini et al., 2001; Guerrero et al., 2010). Micronuclei can contain a chromosomal fragment or an entire chromosome. The content of a micronucleus is predisposed to undergo further DNA damage and chromosomal rearrangements due to asynchronous and defective DNA replication (Crasta et al., 2012; Zhang et al., 2015). Specifically, the DNA replication is hampered due to reduced recruitment of replication factor Cdt1, and DNA helicase complex components Mcm2 and Mcm3 (Crasta et al., 2012). Moreover, the micronucleus is not as efficient as the primary nucleus in recruiting the DNA damage repair proteins, and show a slower rate of DNA repair (Crasta et al., 2012). In addition, another mode for the induction of DNA damage in the missegregated chromosomes is through the cleavage furrow generated-forces during cytokinesis. For example, cells treated with Eg5 or Mps1 inhibitors have been shown to exhibit DNA damage due to breakage of chromatids by the constricting cleavage furrow during cytokinesis (Janssen et al., 2011). Importantly, the rescue mechanisms that aim to reduce insults on mitotic chromosomes can themselves jeopardize genomic stability. For example, the activation of DNA damage repair process during mitosis, specifically the double strand break repair (DSB), can cause aneuploidy by induction of telomere end-to-end fusion (Orthwein et al., 2014).

2.8.3 Polyploidy

Polyploidy refers to the gain of copies of the whole chromosome set. Besides polyploidy observed in tumor cells, the same process is implicated in certain normal cells of the human body. For example placental trophoblast giant cell (TGC), megakaryocytes, hepatocytes, osetoclasts and skeletal muscle cells are polyploid (Davoli and de Lange, 2011). The cellular process of becoming polyploid is in principle similar in both normal and cancer cells; the cells may skip over mitosis (as in the case of TGC's) or undergo defective cytokinesis (megakaryocytes and hepatocytes) or cell fusion (skeletal muscle cells and osteoclasts) (Davoli and de Lange, 2011).

At the molecular level several mechanisms have been found to facilitate induction of polyploidy. The cancer cells can undergo rounds of cell growth and DNA replication without intervening mitosis; this condition is called endoreduplication. For example, the cells with persistent DNA damage response can skip mito-

sis if the Cdk1/Cyclin B becomes suppressed by ATM/ATR and Chk1/Chk2 kinases (Davoli et al., 2010). As a result, the cells go through two rounds of DNA replication without intervening chromosome segregation and end up polyploidy (Davoli et al., 2010). In addition, viruses, such as the Human Papiloma Virus (HPV) implicated in cervix malignancies, have been reported to cause intermediates of tetraploidy, e.g. formation of binucleate cells via induction of cell fusion (Duelli et al., 2007; Hu et al., 2009). Moreover, loss-of-function of AURKB (Delaval et al., 2004; Steigeman et al., 2009) and overexpression of Mad2 (Sotillo et al., 2007), Emi1 (Lehman et al., 2006) and AURKA (Meraldi et al., 2002) have all been reported to lead to formation of tetraploid cells as a result of defective cytokinesis.

A polyploid cell can lead to the generation of aneuploidy. For example, a tetraploid cell with 4N DNA content has multiple centrosomes that can give rise to multipolar mitotic spindle and therefore lead to chromosome missegregation (Ganem et al., 2007). In another mechanism, a chromosomally unstable tetraploid cell is able to produce aneuploid daughter cells with hypertriploid and hypotetraploid genome (Shackney et al., 1989; Galipeau et al., 1996; Andreassen et al., 2001; Meraldi et al., 2002).

2.8.4 Impact of genomic imbalance

The germline aneuploidy due to defects in meiosis is in most cases lethal to the developing embryo and leads to spontaneous abortion (Hassold et al., 1980; reviewed in Hassold and Hunt, 2001; 2007). In humans, the most common sporadic chromosomal abnormality observed is trisomy, accounting for about 30% of all miscarriages. Trisomy is primarily a result of meiotic errors related to increased maternal age (Hassold and Hunt, 2001). However, trisomy does not always lead to the death of the fetus; individuals with Down syndrome (trisomy 21), Edwards syndrome (trisomy 18) and Pateu syndrome (trisomy 13) are born alive but these people suffer from impaired mental and physical abilities (Nicolaidis and Petersen, 1998). In the patients with MVA, constitutional aneuploidy impairs normal course of development, as well as predisposes these individuals to the development of cancer (Hanks et al., 2004; Snape et al., 2011). In addition the aneuploidy caused during the post-natal development stage also predisposes the organism to pathological conditions such as cancer (Weaver et al., 2007; Silk et al., 2013; Lengaeur, 1997). Aneuploidy has adverse effects on cell proliferation due to the induction of cell cycle arrest and cell death (Thompson and Compton, 2008; Silk et al., 2013). Different characteristics of the chromosomal aneuploidy present in the tumor cells contribute to the malignant phenotype; does the aneuploidy involve loss or gain of a chromosome, which chromosome is in question,

and how many chromosomes are missegregated. For example, cells with trisomy 21 are less likely to develop cancer (Hasle, 2001) whereas cells with trisomy 8 are likely to promote the development of cancer (Paulsson and Johansson, 2007). Moreover, mice carrying haploinsufficient *CENP-E* exhibit aneuploidy that can either be pro-tumorigenic or anti-tumorigenic depending on the age of mice, tissue in question and status of tumor suppressor gene *p19ARF* (Weaver et al., 2007). Furthermore, increasing the rate of aneuploidy by depletion of both Mad2 and Cenp-E together, led to the suppression of tumors due to increased cell death (Silk et al., 2013). Importantly, the ability of aneuploid cells to survive and contribute to cancer largely depend on the status of tumor suppressor gene *p53* (Thompson and Compton, 2008; Baker et al., 2009; 2010), which can be lost or inactivated in cancer cells.

2.9 Mitosis as a therapeutic opportunity

2.9.1 Microtubule-targeting agents (MTAs)

Cancer often involves uncontrolled proliferation of cells. Thus, mitosis provides an attractive therapeutic target to intervene the cancer progression and tumor development. Microtubule-targeting agents (MTAs) such as taxanes, epithilones and vinca alkaloids possess proven clinical efficacy in killing several different cancer cell types. At the moment these agents are approved for the treatment of several types of cancers, for example locally advanced or metastasized cancer of the breast, ovary, lung, prostate, adenocarcinoma of stomach, squamous cell carcinoma of head and neck; and Kaposi's sarcoma, Hodgkin lymphoma, Non-Hodgkin lymphoma. All these drugs impair tubulin polymerization and perturb microtubule dynamics *in vitro* and *in vivo*. In cultured cells, high MTA concentrations (10-200 nM) suppress cell proliferation by causing an M phase arrest and cell death (during mitosis or post-mitosis). The cell death at mitosis is a result of prolonged mitotic arrest induced by activated SAC and accumulation of pro-apoptotic signals (Topham et al., 2015; Gascoigne and Taylor, 2008). On the other hand, MTAs induced post-mitotic cell death or cell cycle arrest *in vitro* is a result of cellular stress generated by severe aneuploidy in these cells (Zasadil et al., 2014). However, the detailed molecular level mechanism of action of MTAs *in vivo* is under debate. The intracellular MTA concentrations in the tumor cells of treated patients have not been determined. For this reason, it is not known if the concentrations are high enough to inhibit mitosis or if the growth suppression involves other mechanisms. In this context, it should be noted that these drugs also disrupt normal cell signaling and cargo transport via the microtubules, which

in fact is believed to contribute to their anti-proliferative effects (Zhu et al., 2010; Giannakakaou et al., 2002). Finally, many of the MTAs also have antivascular actions due to their impact on endothelial cells, which are thought to improve their clinical efficacy, again specifically at low therapeutic concentrations (Schwartz, 2009).

Paclitaxel and docetaxel (or taxol in general) are the most clinically utilized MTAs (Zasadil et al., 2014) with total annual sales exceeding US$1.0 billion. *In vitro*, paclitaxel binds to the beta-tubulin in the microtubule structure. At low concentrations taxol stabilizes the microtubules, while at higher concentration it increases microtubule polymerization (Schiff et al., 1979; Jordan et al., 1993). In various cultured human cells taxol treatment leads to induction of SAC and prolonged mitotic arrest followed by cell death (Kelling et al., 2003). The *in vitro* studies with normal and cancer cell lines indicate a variable response to taxol (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). For example, cells can either undergo cell death at mitosis (Panvichian et al., 1998) or at interphase (Jordan et al., 1996), and the cell death can occur in a caspase-dependent (Panvichian et al., 1998) or independent (Niikura et al., 2007) manner. Moreover, cells can exit mitosis in the presence of taxol either as a single cell, or undergo bipolar or multipolar cell division (Jordan et al., 1996; Chen and Horwitz, 2002). Similarly, *in vivo* studies with mouse models (Milross et al., 1996) and reports from the clinics (Symmans et al., 2000) point to the fact that tumor regression observed upon taxol treatment does not always co-relate with increased mitotic index (Zasadil et al., 2014).

A therapeutic issue with MTAs, and most of the other anti-mitotic drugs in development (reviewed in Salmela and Kallio, 2013), is their high cytotoxicity on normal cells. These agents affect the proliferation of normal cells and perturb cellular reactions in even highly differentiated non-cycling cell types, which cause many adverse effects such as myelosuppression and neuropathy (Rowinsky et al., 1993). Unfortunately, the treatment side effects are not the only clinical issue, since several cancer patients do not benefit from MTA therapy at all and many times tumors recur after the treatment. For example, up to 60% of ovarian cancer patients initially respond to the combined surgery-chemotherapy (paclitaxel-platinum treatment as the first adjuvant therapy) but even as many as 70% of them relapse at a median of 15 months from the original diagnosis (McGuire et al., 1989). In general, this inefficacy is related to intrinsic and acquired drug resistance that can arise by multiple independent mechanisms. First, the drug uptake and retention can vary even within one cell population (Jang et al., 2001; Breuninger et al., 1995; Huisman et al., 2005). This often involves enhanced expression of ATP binding cassette (ABC) transporter proteins (e.g. P-glycoprotein) (Breuninger et al., 1995; Huisman et al., 2005), mutations in the β-

tubulin and altered expression of β-tubulin isotypes that negatively affects the binding of MTAs to the tubulin/microtubules (Kavallaris, 2010). Secondly, defects in cell death signaling can support cancer cell survival (Panvichian et al., 1998) and loss-of-function of p53 can allow the proliferation of tetraploid progeny cells (Lanni and Jacks, 1998; Andreassen et al., 2001). Thirdly, deregulation of SAC proteins can increase the viability of MTA treated cancer cells (Wang and Burke, 1995; Wang et al., 2002). Lastly, recent studies have identified altered expression of miRNAs as an important determinant of cancer cells sensitivity towards MTAs (reviewed in Kanakkanthara and Miller, 2013).

2.9.2 Deregulation of miRNAs in cancer

The miRNAs that are upregulated in cancer and possibly support the malignant development are termed as oncomiRs, while the miRNAs that are downregulated in cancer and prevent malignant development are termed as tumor suppressor miRNAs. Importantly, the deregulated miRNA levels in cancer can initiate and stimulate tumorigenesis as well as affect the cells response to chemotherapy. The copy number changes of miRNAs observed in cancer cells occur due to gene deletions, translocations and/or amplifications (Calin et al., 2002). In addition, mutations in the miRNA genes can yield a non-functional miRNA precursor that is not processed into a mature miRNA (Kotani et al., 2010). Moreover, functional inactivation of a miRNA can occur as a result of mutations in the miRNA seed sequence or its binding sequence in the target mRNA. Moreover, the deregulated transcription factors such as p53 and Myc can lead to altered expression of certain miRNAs, such as the miR-34 family (Chang et al., 2007; Bommer et al., 2007) and miR-17-92 family members (O'Donnell et al., 2005; Dews et al., 2006), respectively. Finally, the hyper-methylation of DNA and post-translational modifications of histone proteins affect the transcription of miRNA genes and/or their host genes (Lujambio et al., 2008; Guil and Esteller, 2009). Also, the deregulation of DROSHA, DGCR8, DICER and TRBP can affect the miRNA levels globally in a cell (Lin and Gregory, 2015).

2.9.3 miRNAs as determinants of taxol sensitivity in cancer cells

Several miRNAs have been identified to influence the cells' response to taxol treatment. For example, miR-125b negatively regulates expression of BAK1, a pro-apoptotic protein, and thus breast cancer cells with excess miR-125b do not undergo cell death in response to taxol (Zhou et al., 2010). Similarly, miR-34a negatively regulates Bcl-2, an anti-apoptotic protein, and therefore downregula-

tion of miR-34a can confer resistance to taxol (Kastl et al., 2012). In another mechanism, miRNA-let7g and miR-27a are shown to positively and negatively regulate the expression of Multi Drug Resistance 1 (*MDR1*) gene, respectively. The downregulation of miR-let7g and overexpression of miR-27a can prevent taxol-mediated cell death in ovarian cancer cells (Boyerinas et al., 2012; Li et al., 2010). In addition, downregulation of miR-200 family in ovarian cancer cells, and miR-100 in MCF-7 breast cancer cells increases the expression of β-tubulin-III isotype leading to the reduced sensitivity of cancer cells to taxol treatment (Cochrane et al., 2010; Leskela et al., 2010; Lobert et al., 2011). Importantly, SAC regulating miRNAs such as miR-433 and miR-125b downregulates Mad2 and Mad1, respectively, and confers ovarian cancer cells resistant to taxol treatment (Furlong et al., 2012; Bhattacharjya et al., 2013). miR-100, which targets Plk1, has been observed to be downregulated in taxane-resistant human lung adenocarcinoma cells SPC-A1/DTX in comparison to the drug-sensitive parental SPC-A1 cells (Feng et al., 2012). miR-34 induces cell cycle arrest at G1 phase by downregulating cyclin D1 and thereby prevents breast cancer cells from entering mitosis, which impedes the cytotoxic effects of taxol (Kastl et al., 2012).

Restoration of reduced levels of specific miRNAs back to normal is one intriguing future opportunity to improve the therapeutic efficacy of MTAs and other drugs in situations where the drug resistance involves altered expression of these miRNAs. The results from a xenograft assay in which the levels of miR-26a were exogenously elevated to counteract the loss of the endogenous miRNA expression in hepatocellular carcinoma cells are encouraging; upon restoration of the miR-26a expression cancer cell proliferation became suppressed and the cells underwent apoptotic cell death that led to a dramatic protection from disease progression without toxicity (Kota et al., 2009). On the other hand, antisense-miRNAs (Krutzfeldt et al., 2007) or miRNA-specific sponges are used to inhibit the upregulated miRNAs (Ebert and Sharp, 2007). These strategies have worked effectively in cell culture and animal models (Krutzfeldt et al., 2005; Elmen et al., 2008; Kota et al., 2009; Johnson et al., 2007). Importantly, a miRNA based therapeutic drug called Miravirsen, an antisense-miRNA against miR-122, has been successfully used in the treatment of Hepatitis C-virus (HCV) infection in chimpanzees (Lanford et al., 2010) and has advanced to phase-II clinical trials (Janssen et al., 2013). However, till date no miRNA-based therapy has been approved to treat cancer, and much work has to be done to achieve specific delivery of the miRNA-based therapeutics into the tumors and to minimize the risk factors associated with miRNA therapeutics before they can be approved for clinical use (reviewed in Li and Rana, 2014). Parallel to the development of miRNA-based therapies specific miRNAs are tested for their potency to serve as biomarkers for cancer diagnostic and prognostic (Lu et al., 2005), i.e. as predictors of tumor cells' response to chemotherapy.

2.9.4 miRNAs as biomarkers

The analysis of miRNA expression levels in hematological cancer and solid tumors is one strategy to identify deregulated miRNAs in these malignancies (Calin et al., 2002; Johnson et al., 2005; O'Donnell et al., 2005; He et al., 2005). In this context, the miRNA profiling can assist in the cancer subtyping and therefore in the selection of proper chemotherapy. However, at this time point, the disease may have already spread. An earlier diagnostic may be possible to achieve by identification and analysis of circulating miRNAs (c-miRNA) from body fluids (Pritchard et al., 2012; Nair et al., 2012) such as plasma, urine and saliva (Mitchell et al., 2008; Hanke et al., 2010; Michael et al., 2010). C-miRNAs are believed to facilitate distant cell-to-cell communication and influence target gene translation in the recipient cells (Zhang et al., 2010). Moreover, in a given disease the amount of specific c-miRNAs in e.g. plasma can be significantly altered from the normal levels allowing disease diagnostics. One important feature of c-miRNAs is their surprising stability; they are packaged inside microparticles such as exosomes, small vesicles and lipoprotein complexes, which protects them from degradation (Valadi et al., 2007; Vickers et al., 2011). The signaling mechanisms and cellular release of c-miRNAs are currently poorly understood but under intense research because of the diagnostic potency.

The miRNA profiling from the body fluids is technically very challenging but some encouraging results have been reported. For example, a signature of 34 miRNA's from the serum of early stage NSCLC patients was identified that could facilitate early detection of the disease (Bianchi et al., 2011). Similarly for breast, colorectal and prostate cancer the circulating miRNA signatures have been reported (Redova et al., 2013). Volinia and colleagues have undertaken large-scale screening of normal and cancer tissue samples from thousands of patients and identified a set of miRNAs that were expressed differently in sick and healthy people (Volinia et al., 2010). However, one should be cautious when interpreting results from these studies. First, the miRNA profiling is not yet fully validated technology and variations between different detection methods and measurement platforms have been observed. Moreover, the miRNAs present, for example, in the blood cells can cross-contaminate the pool of c-miRNAs in the plasma and lead to misinterpretation of the results (Pritchard et al., 2012b). Finally, it should be kept in mind that a phenotype associated with deregulation of a specific miRNA potentially involves many pathways in which the target genes of the miRNA work. In order to obtain a more comprehensive understanding of the diagnostic utility of c-miRNAs years of basic and clinical research as well as technology development is needed.

3 AIMS OF THE STUDY

The research work presented in this thesis is aimed at the identification of previously unknown regulators of mitosis signaling, with emphasis on miRNAs and a protein phosphatase.

Objective I

To identify novel mitosis regulating miRNAs and their target genes.

Objective II

To study the cellular consequences associated with deregulated levels of miR-378a-5p and miR-493-3p in cancer cell lines *in vitro* and retrospectively in tumor samples derived from breast and ovarian cancer patients.

Objective III

To evaluate the contribution of miR-378a-5p and miR-493-3p in cells' response to microtubule-targeting drugs *in vitro* and *in vivo*.

Objective IV

To explore the roles of Dual specificity protein phosphatase 3 (Dusp3) in the regulation of mitosis

4 MATERIALS AND METHODS

The original publications (I-III) contain a detailed description of the materials and methods listed below.

4.1 Methods

Method	*Used in*
Cell based high-throughput screen (HTS)	I, II
Cell culture	I, II, III
Cell cycle synchronization	I, II
Chromosome spreads	II
Clinical data analysis	II, III
Cloning	I
Enzyme-linked immunosorbent assay (ELISA)	I
Flow cytometry	I
Fluorescence *in situ* hybridization (FISH)	I, II
Image acquisition and analysis	I, II, III
Immunofluorescence	I, II, III
Live cell imaging	I, II, III
Luciferase assays	I, II
Microscopy	I, II, III
RNA isolation and qRT-PCR	I, II, III
RTK phosphorylation and kinase phosphorylation array	I
Senescence assay	II
Statistical analysis	I, II, III
Transfections	I, II, III
Western blotting	I, II, III

4.1.1 Luciferase assay to detect AURKB promoter activity (I, unpublished)

The HeLa cells were transfected with miR-control and miR-378a-5p using Hiperfect reagent (reverse transfection) in a 96-well plate. The next day (24h later) these cells were transfected (forward transfection) with promoter-luciferase reporter constructs (which contained empty promoter or GAPDH promoter or AURKB promoter to drive luciferase gene expression) using FuGENE HD transfection reagent, according to the manufacturer's protocol. The promoter-luciferase reporter constructs were purchased from SwitchGear Genomics. The

empty promoter vector was used to measure the background signals, the GAPDH promoter served as housekeeping gene control to normalize the luciferase signals and AURKB promoter was used as test promoter to analyze the effects of miRNA on its activity. The FR180204 and DMSO was added for ~16h. The luciferase signals were measured using EnVision 2100 plate reader (Perkin Elmer Inc., Wellesley, MA, USA) as indicated in the manufacturer's protocol.

4.2 Cell Lines

Cell line	*Description*	*Used in*
CAOV-3	Ovarian adenocarcinoma	III
HCT-116	Colorectal carcinoma	I, II
HeLa	Cervical adenocarcinoma	I, II, III
HeLa-H2B-GFP	HeLa cells stably expressing H2BGFP	I, II
MCF-7	Breast adenocarcinoma	I, II
OVCAR-8	Ovarian adenocarcinoma	II

4.3 Chemicals

Chemical	*Supplier, Catalogue number*	*Concentration*	*Used in*
Doxorubicin	Sigma	50nM	II
FR180204	Tocris Bioscience 3706	25µM	I, III
Jnk inhibitor-II	Callbiochem, 420119	1µM	III
Nocodazole	Sigma, M1404	150-300 nM	I, II, III
PD98059	Tocris Bioscience	25µM	III
Taxol	Sigma, T7191	100nM	I, II, III
Thymidine	Sigma, T9250	2mM	I, II
TPA (12-OTetradecanoylphor bol-13-acetate)	Sigma, P1585	200nM	I

4.4 Transfection Reagents

Reagent	*Supplier*	*Used in*
DharmaFECT	Dharmacon	I
FuGENE HD	Promega	I, III
HiPerfect	Qiagen	I, II, III
Lipofectamine 2000	Invitrogen	I, II
Lipofectamine 3000	Invitrogen	III
siLentFect	Bio-Rad	I, II

4.5 miRNA precursors and inhibitors

Type	*Supplier*	*Used in*
Pre-miR™ miRNA Precursor hsa-miR-378a-5p	Ambion	I
Pre-miR™ miRNA Precursor hsa-miR-493-3p	Ambion	II
Pre-miR™ miRNA Precursor negative control #1	Ambion	I, II
Pre-miR™ miRNA Precursor negative control #2	Ambion	I, II
Anti-miR™ miRNA Inhibitor for hsa-miR-493-3p	Ambion	II
miRCURY LNA™ microRNA Target Site Blockers	Exiqon	II

4.6 Primary Antibodies

Antigen	*Species*	*Supplier, Catalogue number*	*Application, Dilution*	*Used in*
Aurora B	rabbit	Abcam, ab2254	WB, 1:800	III
Aurora B (AIM1)	mouse	BD Biosciences, 611083	WB, 1:200, 1:250 IF, 1:1000	III
Bub1	rabbit	Abcam, ab9000	IF, 1:150	II
CREST (human autoimmune serum)	human	Antibodies incorporated	IF, 1:200	I, II
c-Jun	rabbit	Cell Signaling, 9165	WB, 1:1000	III
Cyclin B	mouse	BD Bioscience-Pharmingen, 554178	WB, 1:500	II
Dusp3	mouse	BD Bioscience, 610546	WB, 1: 1:300	III
E2F1	mouse	Santa Cruz, sc-251	WB, 1:500	II
GAPDH	mouse	Advanced ImmunoChemical Inc., mAb 6C5 or Hytest Ltd.	WB, 1:30000, 1:50000	I, II, III
HA-tag	rabbit	Cell Signaling, 37245	IF, 1:700	III
INCENP	rabbit	gift from E.Nigg	IF, 1:1000	I
Mad2	mouse	Abcam, ab10691	WB, 1:500, 1:1000 IF, 1:75	II
	mouse	SantaCruz, sc-65492		II
Mek1/2	rabbit	Cell Signaling, 9122	WB, 1:1000	III
p44/42 MAPK	rabbit	Cell Signaling, #4695	WB, 1:1000	I
	rabbit	Cell Signaling, #9102	WB, 1:1000	III
Pericentrin	rabbit	Abcam, ab4448	IF, 1:500, 1:1000	I, III
phospho-Cenp-A (Ser7)	rabbit	Upstate, 05-792	IF, 1:1000	I
phospho-c-Jun (Ser73)	rabbit	Cell Signaling, 9164	WB, 1:1000	III
phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	rabbit	Cell Signaling, #9101	WB, 1:1000	I, III
phospho-PDGFR-β (Tyr857)	goat	SantaCruz, sc-12907	WB, 1:1000	I
phospho-Sapk/Jnk	rabbit	Cell Signaling, 9251	WB, 1:1000	III
Securin	mouse	Abcam, ab3305	WB, 1:250	II
Survivin	rabbit	Abcam, ab469	IF, 1:300	I
α-tubulin	rat	Abcam, ab6160	IF, 1:200, 1:500	I
β-tubulin (SAP4G5)	mouse	Sigma, T7816	IF, 1:500	III

4.7 Secondary Antibodies

Antibodies	*Supplier*	*Application, dilution*	*Used in*
anti-mouse, anti-rabbit, anti-human (Alexa fluor 488, 555, 647)	Invitrogen	IF, 1:500	I, II, III
anti-mouse, anti-rabbit (HRP linked)	Cell Signaling	WB, 1:5000	II, III
anti-mouse, anti-rabbit and anti-goat 680 (Alexa Fluor)	Invitrogen	WB, 1:5000	I, II
anti-mouse and anti-rabbit 800 (IR Dye conjugated)	Rockland Immuno-chemicals	WB, 1:5000	I, II

4.8 Primers used in qRT-PCR

Gene	*Forward*	*Reverse*	*Probe*	*Used in*
AURKB	ATTGCTGACTTCGG CTGGT	GTCCAGGGTGCCAC ACAT	#69	I
DUSP3	CTTCATTGAC-CAGGCTTTGG	GGGGAGCGGCTATAAC-CTT	#86	III
E2F1	TCCAAGAAC-CACATCCAGTG	CTGGGTCAACCCCTCA AG	#5	II
ERK1	CCCTAGCCCAGACAG-ACATC	TCATCTTCCAGGAGA-CAGCA	#16	III
ERK2	TCACGCTCACCAC-TCCTG	GGTCTGTTTTCCGAG-GATGA	#73	III
GAPDH	ACGACCAAATCCGT TGACTC	CTCTGCTCCTCCTG TTCGAC	#60	I, II, III
	AGCCACATCGCTCAG ACAC	GCCCAATACGAC-CAAATCC	#60	I, II, III
MAD2	CGCGTGCTTTT-GTTTGTGT	GCTGTT-GATGCCGAATGAGA	#32	II
VEGFA	CTACCTCCACCATG CCAAGT	CCACTTCGTGATGA TTCTGC	#29	I

Probe numbers are in accordance with Roche Universal probe Library.

4.9 Primers used for Luciferase Gene Constructs

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

4.10 Patient cohorts used in the study

Cohort	*miRNA profiling array*	*mRNA profiling array*	*References*	*Used in*
Cohort I *MICMA* *(Oslo, Norway)* 101 tumor samples (Breast carcinoma)	Agilent 8_15k 'Human miRNA Microarray Kit (V2)' with design ID 019118	Agilent 4x44K one-color oligonucleotide arrays	(Enerly et al., 2011); (Naume et al., 2001)	I
Cohort II *METABRIC* 1031 tumor samples (Breast carcinoma) 116 normal breast tissue samples	Agilent miRNA Complete Labeling and Hyb Kit (p/n 5190-0456) and MicroRNA Spike-In Kit (p/n 5190-1934)	Agilent Illumina HT-12 v3	(Dvinge et al., 2013)	I
Cohort III *(Oslo, Norway)* 12 HGSC, 9 CCC (Ovarian carcinoma) 9 normal ovary tissue samples	Affymetrix miRNA 2.0 Arrays	Affymetrix Human Genome U133 Plus 2.0	(Elgaaen et al., 2014; 2012; 2010)	II
Cohort IV *(TCGA)* 572 tumor samples (Ovarian carcinoma) 8 normal ovary tissue samples	Agilent 8x15K miRNA-specific arrays	Affymetrix Human Genome U133 Plus 2.0 Arrays	(Granzfried et al., 2013; Ovaska et al., 2010)	II
Cohort V *(Bergen, Norway)* 223 tumor samples (Breast carcinoma) (25 samples each for paclitaxel and epirubicin arm)	50bp single end sequencing using illumina HiSeq2500 instrument. Post-sequencing analysis using MiRDeep2 (v.2.0.0.5)	No data available	(Hayward et al., 1997 ; Chrisanthar et al., 2008; Friedlander et al., 2012; Kozomara, et al., 2014)	II

Approval for the retrospective analyses of human tumor materials was granted by the institutional review boards and all patient material is managed according to the instructions of ethical committees. Coded tissue materials were placed in locked rooms and patient data stored in a separate computer requiring a password for access.

5 RESULTS

5.1 miR-378a-5p regulates mitosis and is found deregulated in breast cancer tumors (I)

The regulation of mitosis by miRNAs was poorly understood and therefore we aimed at identifying and functionally characterizing novel mitosis regulating miRNAs. Further, we were interested to get insights on how the altered expression of these miRNAs would contribute to malignant proliferation of cancer cells and their sensitivity to microtubule-targeting drugs.

5.1.1 Excess of miR-378a-5p in cells causes resistance to taxol

We performed a high-throughput screen (HTS) where 810 different miRNAs were transfected individually to HeLa-H2B-GFP cells and the response to taxol was analyzed. It was observed that in miR-378a-5p overexpressing cell population the mitotic index was significantly reduced and the interphase cells showed abnormal nuclear structure (multiple lobed) (I, Fig. 1A). The live cell imaging confirmed that the miR-378a-5p overexpressing cells underwent forced exit from mitosis (I, Fig. 1D, E, Supplementary Movies 1 and 2). However, in drug-free conditions, the miR-378a-5p transfected cells exhibited larger interphase nucleus, which we referred to as polyploid cells (I, Fig. 1A, S3). Moreover, the spindle architecture was abnormal as 73.7±3.5% of mitotic cells with excess miR-378a-5p exhibited multiple spindle poles as compared to 23.0±7.1% in control cells, $p<0.01$ (I, Fig. 1F). Finally, the live cell imaging of cells released from double thymidine block revealed that excess miR-378a-5p induced a transient cell cycle delay in both interphase and mitosis (I, Fig. 1D). We identified miR-378a-5p as mitosis regulating miRNA whose excess levels resulted in mitotic aberrations and caused resistance to taxol-induced mitosis arrest.

5.1.2 miR-378a-5p regulates VEGFA and Mapk pathway and alters fate of mitotic cells

In order to get a mechanistic view on the mode-of-action of miR-378a-5p in the regulation of mitosis, we attempted to link malfunction of known target genes of

the miR-378a-5p with the observed mitotic phenotype. First, we independently repeated earlier findings by Hua and colleagues (Hua et al., 2006) that miR-378a-5p controls VEGF-A production. We showed that cells with excess miR-378a-5p exhibited significantly increased levels of VEGF-A mRNA ($p<0.01$) and in the culture medium significantly more VEGF-A protein was observed in comparison to the controls ($p<0.01$) (I, Fig. 2 A). VEGF-A is a known activating ligand for several receptor tyrosine kinases (RTKs) namely PDGFR-b, ErbB2, EphRA7 and VEGFR-2 (Ball et al., 2007). We used phospho-RTK dot-blot assay and detected increased phosphorylation of the above mentioned four RTKs in the miR-378a-5p overexpressing cells in comparison to control cells (I, Fig. 2B). In addition, the phospho-kinase dot-blot and Western blotting assay confirmed activation of the intracellular Mapk-Erk1/2 pathway in the cells transfected with miR-378a-5p (I, Fig. 2C). Next, we analyzed the mitotic behavior of cells having an excess of either miR-control or miR-378a-5p in cells cultured in the presence or absence of FR180204, an Erk1/2 inhibitor. In drug-free conditions, the miR-378a-5p overexpressing cells exhibited transient mitotic arrest, underwent mitotic slippage, and had increased percentage of polyploid cells in comparison to the miR-control cells. Importantly, FR180204 treatment partially rescued these abnormalities induced by excess miR-378a-5p, and also partially sensitized the miR-378a-5p overexpressing cells to taxol-mediated mitotic arrest ($p<0.05$) (I, Fig. 2D). We conclude that excess of miR-378a-5p aberrantly activates the Mapk-Erk1/2 pathway, which partially explains the mitosis-specific effects of miR-378a-5p overexpression.

5.1.3 miR-378a-5p regulates AURKB gene expression

The aberrant high activity of Mapk-Erk1/2 pathway was previously shown to downregulate the activity of AURKB (Eves et al., 2006). Therefore, we investigated the effect of miR-378a-5p overexpression on the production and activity of AURKB. We identified AURKB to be downregulated in cells with excess miR-378a-5p. The levels of AURKB mRNA and protein were reduced significantly after the overexpression of miR-378a-5p when compared to the controls ($p<0.01$) (I, Fig. 3A-B, S3). Moreover, FR180204 partially rescued the drop in AURKB protein induced by miR-378a-5p (I, Fig. 3E). However, results from the luciferase reporter assay, which was used to determine the binding between miR-378a-5p and AURKB mRNA, indicated that *AURKB* mRNA was not a direct target for miR-378a-5p (I, Fig. 3D). To gain further clarity on this indirect regulation of *AURKB* by miR-378a-5p we performed an assay to detect the *AURKB* promoter activity in cells with the excess miRNA. The AURKB promoter activity in miR-378a-5p transfected cells was reduced to almost half ($p<0.001$) in comparison to

the miR-control transfected cells (Fig. 8, *unpublished data*). This regulation appeared to be Erk1/2 independent as FR180204 did not rescue the inhibition of *AURKB* promoter activity by miR-378a-5p (Fig. 8, *unpublished data)*. Nevertheless, perturbation of AURKB protein by miR-378a-5p led to significantly diminished protein levels of AURKB ($p<0.001$) and other CPC members, namely survivin ($p<0.001$) and INCENP ($p<0.001$) at the centromere (I, Fig. S2). The amount of AURKB protein was reduced to a level that impaired the normal function of the kinase; the phosphorylation of AURKB substrate protein CenpA (Ser7) was significantly reduced at the centromere ($p<0.001$) (I, Fig. 3C). We conclude that miR-378a-5p negatively regulates the AURKB promoter activity and downregulates the AURKB gene expression.

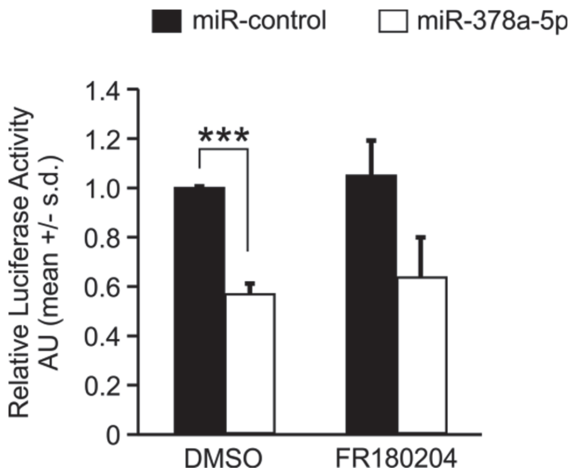

Figure 8: miR-378a-5p overexpression decreases AURKB promoter activity. The graph shows quantification of the relative activity of luciferase expressed under AURKB promoter. The activity of luciferase expressed under the GAPDH promoter was used for normalization, while that under empty vector was used to measure background signals. The asterisks denote statistical significance *** = $p<0.001$.

5.1.4 miR-378a-5p induces aneuploidy

It is known from previous studies that depletion of AURKB by RNAi leads to errors in chromosome segregation and induction of micronuclei (Hauf et al., 2003) as well as to cytokinesis defects that increase the frequency of polyploid cells (DeLaval et al., 2004). In addition, the polyploid cells harbor extra spindle poles, which can give rise to lagging chromosomes during anaphase (Ganem et al., 2007). To examine whether an excess of miR-378a-5p generates aneuploidy

we performed chromosome copy number analysis by using chromosome specific FISH probes in near-diploid HCT-116 colon cancer cells after transfections with miR-378a-5p and miR-control. The cells were analyzed 66h post-transfection for copy number changes of Chr12, Chr13 and Chr21. The results indicated that miR-378a-5p had a marked negative impact on faithful chromosome segregation as the proportion of cells with trisomy ($p<0.01$) and tetrasomy ($p<0.001$) was significantly elevated in the miR-378a-5p overexpressing cells in comparison to the controls (I, Fig. 4). However, it should be noted that the incidence of null or monosomy was not significantly increased in the miR-control or miR-378a-5p transfected cells (I, Fig. 4).

5.1.5 Specific breast cancer tumor subtypes have altered levels of miR-378a-5p

Changed expression of many individual miRNAs has been observed in context of abnormal growth of tissue. To determine the status of miR-378a-5p expression *in vivo* we retrospectively analyzed the miRNA expression in breast cancer cohorts. The analysis of tumor samples with different histological grade showed that miR-378a-5p levels were increased in high-grade tumor (grade 3) as compared to grade 1 ($p=0.03$) and 2 ($p=0.01$) (I, Fig. 5A). In addition, the samples with elevated miR-378a-5p levels correlated with tumors exhibiting higher cell proliferation classified by increased Ki67 score and mitotic index ($p<0.001$) (I, Fig. 5D). Based on molecular profiling, the breast cancer tumors are classified into five subtypes, namely Luminal A, Luminal B, basal like/triple negative, Her2 overexpression and normal-like. In our analysis we found that miR-378a-5p levels were increased in basal-like tumors as compared to Luminal A or B ($p<0.001$) (I, Fig. 5B). Similarly, the tumors negative for estrogen receptor exhibited higher miR-378a-5p levels when compared to estrogen positive tumors ($p<0.001$) (I, Fig. 5C). Interestingly, the miR-378a-5p levels were not significantly changed in tumor samples that represented different ploidy levels (diploid, triploid or tetraploid). Importantly, the gene expression pattern for miR-378a-5p and its host gene *PPARGC1-β* were similar across the tumor subtypes analyzed in this study (I, Fig. S4). We also obtained similar results with another breast cancer cohort consisting of ~1300 tumor samples (data not shown, METABRIC; Dvinge et al, 2013). We conclude that miR-378a-5p levels are deregulated in breast cancer tumorigenesis, and the expression of miR-378a-5p is increased with increasing tumor grade.

5.2 miR-493-3p regulates genomic balance and tumor cells' response to paclitaxel (II)

5.2.1 miR-493-3p overexpression in vitro causes override of SAC

miR-493-3p was identified from a HTS as a mitosis regulating miRNA (Winsel et al., 2012) based on its ability to reduce the mitotic index and to induce nuclear fragmentation in response to taxol. Further, we were interested to characterize the detailed mechanism of miR-493-3p mediated regulation of mitosis. The miR-493-3p overexpression was achieved by transfection of pre-miR-493 to cells in culture (II, Fig. 1A, B). It was observed that the majority of cells with excess miR-493-3p did not arrest in mitosis when cultured in the presence of taxol (p=0.002) or nocodazole (p=0.004). In fact, the cells underwent mitotic slippage in significant quantities while the miR-control cells exhibited a prolonged mitosis arrest followed by cell death (II, Fig. 1C, D). Furthermore, similar results were obtained from miRNA-transfected and double thymidine blocked cells that were released into a culture medium containing taxol or nocodazole; the mitotic index measured at various time-points after the thymidine washout was significantly lower in the miR-493-3p transfected cell populations as compared to controls (II, Fig. 1D). In drug-free conditions, duration of mitosis (NEBD to anaphase onset) was significantly shorter in cells with an excess of miR-493-3p in contrast to miR-controls (p=0.02) (II, Fig. 1E).

5.2.2 miR-493-3p regulates Mad2 gene expression

In order to identify the molecular targets of miR-493-3p, we performed a gene expression analysis from cells transfected with miR-control or miR-493-3p. This led to the identification of Mad2 as a candidate target gene of miR-493-3p (II, Table S1). The miRNA target prediction software supported this notion (II, Fig. 2A). We also validated biochemically that miR-493-3p directly interacted with Mad2 mRNA to cause its suppression (II, Fig. 2B). It was observed that miR-493-3p overexpressing HeLa cells had significantly reduced Mad2 mRNA and protein by 68 +/- 11% (p = 0.009) and 65 +/-5% (p = 0.002) respectively, as compared to control cells (II, Fig. 2C, D). Excess of miR-493-3p also led to decreased Mad2 protein production in HCT-116 (p=0.003) and MCF-7 cells (p=0.005) (II, Fig. S1). At prometaphase, mitotic HeLa cells with excess miR-493-3p showed diminished Mad2 protein (p=0.01) but no difference in Bub1 was detected when compared to miR-control transfected cells (II, Fig. 2E). Bub1 is an important SAC protein present at the unattached kinetochores. Therefore, we

used Bub1 as a control to highlight that the miR-493-3p mediated downregulation of SAC activity was primarily due to the reduction in Mad2 levels and not due to other SAC proteins like Bub1.

A previous study has validated miR-493-3p to target E2F1, which is one of the transcription factors of *MAD2* (Gu et al., 2014). Although we were able to confirm that miR-493-3p controls the levels of E2F1, we, however observed that depletion of E2F1 by RNAi did not change the *MAD2* expression (II, Fig. S2). Furthermore, we designed a competitive binding approach to rescue the Mad2 protein from inhibition of miR-493-3p. For this purpose, a synthetic oligonucleotide called as target-site blocker (TSB) was used to block the miR-493-3p binding site on Mad2 mRNA (termed as TSB-MAD2) (II, Fig. 2F). Importantly, in cells co-transfected with TSB-MAD2 and miR-493-3p the Mad2 protein levels were significantly rescued in comparison to cells co-transfected with TSB-control and miR-493-3p ($p=0.04$). In addition, TSB-MAD2 partially restored the SAC activity in the presence of miR-493-3p ($p=0.02$ in taxol and $p=0.04$ in nocodazole treated cells) (II, Fig. 2G, H). Moreover, the inhibition of endogenous miR-493-3p achieved after the introduction of antisense-miRNA (anti-miR-493-3p) into the cells led to a significant >2-fold increase in the levels of Mad2 protein ($p=0.03$) (II, Fig. 4A-C). Based on these results, we conclude that miR-493-3p negatively regulates Mad2 gene expression by directly targeting the Mad2-mRNA-3'UTR.

5.2.3 Excess of miR-493-3p causes defects in chromosome segregation

The previous studies have shown that the reduced levels of Mad2 result in accelerated mitosis, premature separation of sister chromatids and premature degradation of CyclinB1 and Securin (Gorbsky et al., 1998; Michel et al., 2004). Therefore, we wanted to investigate if excess of miR-493-3p would lead to a similar phenotype as downregulation of Mad2. As expected, miR-493-3p overexpression caused reduced levels of CyclinB1 and Securin, and premature separation of sister chromatids (II, Fig. 3B). In addition, based on the analysis of FISH signals for Chr12 and Chr21 in the interphase cells, it was clear that miR-493-3p overexpression caused gain or loss of chromosomes that elevated the frequency of aneuploidy in the progeny cells (II, Fig. 3C). Interestingly, TSB-MAD2 restrained the ability of miR-493-3p to generate aneuploidy as the cells co-transfected with miR-493-3p and TSB-MAD2 exhibited similar low levels of aneuploidy as the cells co-transfected with miR-control and TSB-control (II, Fig. 3C). In addition, cells, in which the endogenous levels of miR-493-3p were reduced by anti-miR-493-3p, exhibited significantly increased frequency of lagging chromosomes ($p=0.001$) and chromosome bridges ($p=0.03$) as a result of Mad2 overexpression

(II, Fig. 4D, E). Finally, the miR-493-3p overexpression also led to increased proportion of cells that were positive for β-gal staining, an indicator of cellular senescence (II, Fig. 3D). We conclude that altered levels of miR-493-3p hamper the faithful chromosome segregation and cause aneuploidy.

5.2.4 miR-493-3p and MAD2 expression inversely correlate with each other in ovarian cancer in vivo and in vitro

Our retrospective analysis of ovarian cancer cohort data indicated that the expression patterns of miR-493-3p and *MAD2* inversely correlated *in vivo* in ovarian cancer tumor samples. Specifically, the decreased levels of miR-493-3p and increased levels of *MAD2* were associated with high-grade serous ovarian cancer tumors (HGSC) when compared to the normal and a less aggressive tumor subtype clear cell carcinoma (CCC) (II, Fig. 5A). In another ovarian cancer cohort (TCGA cohort) similar findings were found for the *MAD2* but in the case of miR-493-3p the levels were not observed to change with the increasing tumor grade (II, Fig. 5B). In ovarian cancer cell lines OVCAR-8 and CAOV-3 an inverse correlation between *MAD2* and miR-493-3p was observed. In the OVCAR-8 cells, the expression of miR-493-3p was lower than in CAOV-3 cells while the expression of *MAD2* was the opposite (high in OVCAR-8 and low in CAOV-3 cells). This expression pattern concerned not only the mRNA levels of *MAD2* but also the protein levels (II, Fig. 6A-B). Moreover, overexpression of miR-493-3p in these cell lines caused a decrease in Mad2 protein levels (II, Fig. 6C) while transfection of cells with an anti-miR-493-3p led to a significant increase in the amount of Mad2 protein (II, Fig. 6E). Importantly, both these cell lines exhibited accelerated mitotic progression upon the transient transfection with miR-493-3p (II, Fig. 6D), which was in line with our earlier observation in HeLa cells (II, Fig. 1F). We conclude that in human ovarian cancer tumor samples *in vivo* and human ovarian cell lines *in vitro*, miR-493-3p and Mad2 expression negatively co-relate.

5.2.5 Efficacy of taxol treatment in vitro and in vivo is suppressed by high levels of miR-493-3p

In cell culture, the CAOV-3 and OVCAR-8 cells both resisted taxol treatment to varying extent. The majority of CAOV-3 (76.7 +/- 1.2 %) and to less extent OVCAR-8 cells (38.0 +/- 9.5 %) underwent mitotic slippage when cultured in the presence of taxol ($p=0.02$) (II, Fig. 7A). In addition, after transfection of these two cell lines with miR-493-3p and subsequent taxol treatment, the percentage of cells exiting mitosis was ~80 % for both cell lines (II, Fig. 7B). Based on these

findings, we hypothesized that expression of miR-493-3p in tumors could be an important determinant of the patients' response to paclitaxel chemotherapy. As paclitaxel is used to treat ovarian and breast cancer patients, we performed a retrospective analysis of tumor samples from HGSC (TCGA cohort) and breast cancer patients with primary stage-III disease (Bergen cohort). We found that high miR-493-3p levels were associated with reduced disease specific survival (DSS) and *vice versa* in both cohorts post-chemotherapy (II, Fig 7C-D). Interestingly, when the samples from the Bergen cohort were separated into groups of patients who received paclitaxel or epirubicin, we observed association of between reduced survival and high miR-493-3p levels only in paclitaxel therapy group (II, Fig 7E-F).

5.3 Dusp3 is required for bipolar spindle formation during mitosis (III)

5.3.1 Reduced levels of Dusp3 affects cell cycle progression and causes spindle multipolarity

Previous reports show that Dusp3 protein is critical for normal cell cycle progression (Rahmouni et al., 2006). However, the role(s) of the protein in M phase is unknown. We were interested to explore how the changed expression of Dusp3 may affect mitotic signaling. To this end, we used RNAi to reduce the endogenous Dusp3 protein levels in HeLa cells via transient transfections with two different Dusp3 targeting siRNAs (siR-Dusp3#1 or siR-Dusp3#2). As expected, the Dusp3-targeting siRNAs significantly decreased the Dusp3 mRNA and protein levels when compared to cells transfected with control siRNA (III, Fig. 1A-B). Next, the cellular phenotype of the Dusp3 silencing was investigated using live cell imaging. Analysis of time-lapse films indicated a cell cycle delay in the Dusp3 silenced cells in comparison to controls. Specifically, the time between two successive cell divisions was found to be 38.18 +/- 12.83 h for the siR-Dusp3#1, which was significantly longer ($p=0.004$) than the time taken by siR-control cells (22.00 +/- 4.51 h) (III, Figs. 1C, S1). In drug-free culture conditions, the mitotic duration calculated from NEBD to anaphase onset was significantly increased by the Dusp3 silencing compared to controls (III, Fig. 1D). Additionally, the fate of the cells cultured in the presence of taxol or nocodazole was changed by the Dusp3 RNAi; the Dusp3 silenced cells died significantly faster from the drug imposed M phase arrest in comparison to the siR-controls (III, Fig. 3).

Lastly, microscopic analysis of the mitotic spindle morphology after immunostaining with β-tubulin and pericentrin antibodies indicated elevated multipolarity by Dusp3 RNAi; in the siR-Dusp3#1 and siR-Dusp3#2 transfected cell populations 29.46 +/- 5.79 % (p= 0.03) and 29.16 +/- 2.12 % (p= 0.01) of the mitotic cells exhibited significantly more spindle poles, respectively in comparison to 4.01 +/- 3.25% in control cells (III, Figs. 2, S2B-C). We conclude that reduced levels of Dusp3 cause cell cycle delay in interphase as well as in mitosis, and induce multipolar spindle poles in dividing cells.

5.3.2 Dusp3 negatively regulates Erk1/2 and Jnk pathway

The regulation of Mapk pathway by Dusp3 is surrounded by controversial reports (Todd et al., 1999; Wagner et al., 2013; Amand et al., 2014). Different cell lines appear to exhibit dissimilar communication between the kinase and the phosphatase. We observed that in our HeLa cell line the cells exhibited elevated signals for phospho-Erk1/2, phospho-Jnk, c-Jun and phospho-c-Jun after transfection with Dusp3 siRNAs as compared to the control cells (III, Fig. S3). In addition, the overexpression of Dusp3 protein resulted in reduced phospho-Erk1/2 signals as compared to the cells expressing control plasmid (III, Fig. 5B).

5.3.3 Erk1/2 pathway mediates the formation of multipolar spindles in cells with reduced amount of Dusp3

Next, we sought to dissect the molecular pathway responsible for the formation of multipolar spindles in the siR-Dusp3 transfected cells. To this end, we treated the siR-control and siR-Dusp3#2 transfected cells with the chemical inhibitor of Erk1/2 (FR180204), Mek (PD98059) or Jnk (Jnk inhibitor II). As expected, the inhibition of Erk1/2, Mek1/2 and Jnk did not result in the induction of multipolar mitotic cells (III, Fig. 4A), which was in line with a previous report (Shinohara et al., 2006). Interestingly, the siR-Dusp3#1 and siR-Dusp3#2 transfected cell populations had less multipolar mitotic cells if the cells were treated with PD98059 or FR180204 in comparison to the DMSO treated Dusp3 silenced cells (III, Fig. 4A). Conversely, the inhibition of Jnk did not rescue the percentage of multipolar mitotic cells observed after Dusp3 silencing (III, Fig. 4A). Moreover, the co-transfection of siR-Dusp3#2 and siR-Erk1, or siR-Dusp3#2 and siR-Erk2 did not rescue the multipolar phenotype (III, Fig.4B). However, co-depletion of Dusp3 and both Erk1 and -2 led to significant reduction in the percentage of multipolar mitotic cells (III. Fig. 4B). We conclude that the formation of multipolar

spindles in cells with reduced levels of Dusp3 is dependent on the increased activity of either Erk1 or Erk2.

5.3.4 Dusp3 overexpression rescues spindle multipolarity in Dusp3 silenced cells

We hypothesized that reintroducing the WT Dusp3 in the Dusp3 silenced cells would restore the normal spindle architecture in these cells. To investigate this, we first characterized the Dusp3 silencing by another Dusp3 siRNA (siR-Dusp3#3) that targeted a sequence within the 3'UTR of Dusp3. The siR-Dusp3#3 was equally potent to reduce the Dusp3 protein expression (III, Fig. 5C) and induce multipolar mitotic cells, as seen before with siR-Dusp3#1 and #2 (III, Fig. 1B). Next, we produced a HA-tagged WT-Dusp3 that was resistant to the siR-Dusp3#3; the pEF/HA-Vhr^{WT} plasmid contained the open reading frame of Dusp3 but was devoid of Dusp3-3'UTR. The overexpression of HA-tagged-WT-Dusp3 was confirmed by immunofluorescence and Western blotting (III, Fig. 5A-B). Interestingly, the co-transfection of siR-Dusp3#3 and pEF/HA-Vhr^{WT} plasmid resulted in a significant reduction in the frequency of multipolar mitotic cells (10.78 +/- 3.74 %) when compared to the co-transfection of siR-Dusp3#3 and control-plasmid (18.73 +/- 3.92%) ($p=0.008$) (III, Fig. 5D). Therefore, we conclude that Dusp3 protein is needed to maintain bipolar spindle architecture during mitosis.

6 DISCUSSION

6.1 miR-378a-5p emerged as a novel mitosis regulating miRNA (I)

Our study led to the identification of miR-378a-5p as a new mitosis controlling element. We report that miR-378a-5p regulates mitosis at least by two routes: through activation of Mapk pathway and by suppression of AURKB.

The suppression of AURKB observed after overexpression of miR-378a-5p provides one explanation for the occurrence of mitotic defects in the cells with excess miR-378a-5p. AURKB is a key SAC protein, and its loss of function leads to defective SAC signaling and abnormal cytokinesis (Kallio et al., 2002; Ditchfield et al., 2003; Hauf et al., 2003). Importantly, miR-378a-5p did not influence the expression of luciferase gene harboring the *AURKB*-mRNA or *AURKB*-3'UTR sequence, which indicates that miR-378a-5p does not directly bind to the *AURKB* mRNA. However, miR-378a-5p transfected cells showed a partial increase in the AURKB protein levels after treatment with an Erk1/2 inhibitor. This supports a notion that miR-378a-5p regulates AURKB indirectly, possibly via Erk1/2 pathway. The literature presents contrasting views on Erk1/2 mediated regulation of AURKB. For example, the activation of Raf1-Mek-Erk cascade due to inhibition of Raf kinase inhibitory protein (RKIP) reduces AURKB activity in cultured HeLa cells (Eves et al., 2006) and mRNA levels in HEK-293 cells (al-Mulla et al., 2011). In contrast, activation of Erk1/2 can also upregulate AURKB protein (Bonet et al., 2012) that is a likely consequence of increase in the levels of FOXM1 (Bonet et al., 2012; Lok et al., 2011), a transcription factor for AURKB (Wang et al., 2005). Additionally, we observed that AURKB promoter activity is decreased in the miR-378a-5p transfected cells and this was not rescued by inhibition of Erk1/2 (*unpublished data*). Furthermore, our attempts to rescue the miR-378a-5p induced mitotic errors by overexpression of AURKB did not succeed as the excess levels of AURKB proved toxic to the cells. Therefore, we cannot exclude the possibility that there are other gene targets of miR-378a-5p whose suppression directly contributes to the mitotic phenotype observed in the miR-378a-5p overexpressing cells. Taken together, miR-378a-5p is a novel mitosis controlling miRNA that downregulates the expression and activity of AURKB indirectly possibly *via* stimulation of the RTK-Mapk-Erk1/2 pathway.

Altered activity of AURKB has been linked with induction of aneuploidy (Ota et al., 2002; Hauf et al., 2003; Hontz et al, 2007; Honma et al., 2014). In cell cul-

ture, the miR-378a-5p overexpressing cells showed an increase in the percentage of tri- and tetraploid cells. Based on the previous studies it is anticipated that miR-378a-5p induced polyploidy and spindle multipolarity may give rise to the aneuploid cells. However, *in vivo*, the high miR-378a-5p levels did not co-relate with changed ploidy in breast tumor cells. In the context of *in vitro* FISH data, it should be kept in mind that in these cells the elevated miRNA levels lasted for 3-4 days only while in the tumors the miRNA expression is anticipated to be high for a much longer time period. Importantly, depending on the chromosomes involved and the genetic background of the model system, aneuploidy can, besides cell death and/or cell cycle arrest, also provide selection advantage for the malignant cells (Thompson and Compton, 2008; Rutledge et al., 2016). Therefore, we assume that prolonged elevation in the expression of miR-378a-5p in tumors would lead to the generation of aneuploid cells that have different fates; the cells with high levels of aneuploidy are likely lost from the population while cells with low levels of numerical chromosome changes may survive and possess growth advantage. Moreover, *in vitro* miR-378a-5p overexpression did not result in increased percentage of monosomic or null cells. This suggests that these cells are eliminated rapidly from the population or are directed to senescence after the chromosome missegregation.

In breast cancer *in vivo*, we observe that high expression of miR-378a-5p and its host gene *PPARGC1B* co-relates with increased tumor grade and aggressiveness, which indicate that they both are expressed in concert. In our analysis, the basal-like breast tumor subtype exhibited significantly elevated expression of miR-378a-5p in comparison to Luminal A or B tumor subtypes. The basal-like breast tumor subtype, is negative for estrogen receptor (ER), Progesterone receptor (PR) and Human epidermal growth factor receptor 2 (Her2), and represents an aggressive disease with poor prognosis (Valentin et al., 2012).

Based on previous studies and our data, miR-378a-5p has emerged as a regulator of key cellular processes implicated in control of the hallmarks of cancer such as increased angiogenesis (Lee et al., 2007), switch from oxidative to glycolytic metabolism (Warburg effect) (Eichner et al., 2010) and aneuploidy (our results). All these factors can offer malignant cells selection and survival advantage, and enable the spread of the disease. In addition, the alteration of SAC signaling due to increase in the expression of miR-378a-5p and successive decrease in the AURKB levels can be a tumor promoting factor and/or confer resistance to paclitaxel that is widely used for the treatment of breast and ovarian cancers. Importantly, miR-378a-5p has also been identified as a circulating miRNA in the blood, which raises the opportunity that it could in future be used as a biomarker in cancer diagnostics (Liu et al., 2012). Specifically, based on our data, the miR-

378a-5p levels could in future assist in tumor grading/subtyping and in predicting the efficacy of MTA based chemotherapy.

6.2 Normal levels of miR-493-3p are needed for faithful chromosome segregation (II)

In our analysis miR-493-3p has emerged as a strong mitosis regulating miRNA, specifically due to its negative impact on the *MAD2* gene expression. It has been demonstrated previously that Mad2 is indispensable for faithful mitosis; deregulation of Mad2 leads to unfaithful chromosome segregation, aneuploidy and tumorigenesis in mice (Michel et al., 2001; Sotillo et al., 2007), and influences the recurrence-free survival in cancer patients (McGrogan et al., 2014). We found that miR-493-3p expression negatively co-relates with *MAD2* expression in cancer cell lines *in vitro* as well as in ovarian tumor samples *in vivo*. Our results indicate that miR-493-3p is one of the factors that fine-tunes the levels of Mad2 in a cell to support the maintenance of genomic balance and cells' response to clinically utilized MTAs.

The miR-493-3p is a negative regulator of Mad2. In cell culture, the ectopically introduced miR-493-3p led to the decrease of Mad2 (mRNA and protein), while inhibition of endogenous miR-493-3p by anti-miR-493-3p increased Mad2 protein levels. The results were similar across a panel of human cancer cell lines of diverse tissue origin, and thus we conclude that the miR-493-3p mediated regulation of Mad2 is universally conserved. Earlier, overexpression of miR-493-3p has been shown to suppress E2F1, a transcription factor for *MAD2* (Gu et al., 2014). We were able to confirm that miR493-3p indeed downregulated E2F1. Importantly, reduction of E2F1 by RNAi in human cell lines did not downregulate Mad2 mRNA and protein. Therefore, we conclude that E2F1 is not the only transcription factor for *MAD2* in these cells and it is unlikely that miR-493-3p would regulate Mad2 gene expression via E2F1 alone. Furthermore, we provide evidence that miR-493-3p targets mRNA of Mad2 directly and in a specific manner. First, the miR-493-3p suppresses the expression of a luciferase reporter plasmid that contains the *MAD2*-3'UTR sequence. Secondly, the cells transfected with TSB-MAD2 that competes with miR-493-3p for *MAD2*-3'UTR binding prevents the Mad2 downregulation even in the presence of excess miR-493-3p. Collectively, our data indicates that miR-493-3p directly targets a specific sequence within *MAD2*-3'UTR and suppresses *MAD2* gene expression post-transcriptionally.

We report that the gain or loss of miR-493-3p expression shows opposite cellular phenotypes. For example, the excess miR-493-3p leads to faster mitotic progres-

sion while inhibition of miR-493-3p results in mitotic delay. Previous studies have linked accelerated mitosis with premature APC/C activation mainly as a result of the loss of Mad2 function (Gorbsky et al., 1998). Furthermore, the erroneous APC/C activation causes abrupt degradation of its key mitotic substrates, namely Cyclin B1 and Securin, and induces premature separation of sister chromatids (Michel et al., 2004) which is also observed in the miR-493-3p overexpressing cells. These findings support the notion that excess of miR-493-3p in cells leads to downregulation of Mad2. In contrast, the high Mad2 levels are reported to cause increased mitotic duration mainly due to generation of hyperstabilized kinetochore-microtubule attachments (Kabeche and Compton, 2012). In our hands, suppression of miR-493-3p by anti-miRNA led to a similar cellular phenotype. Importantly, both these conditions (loss or gain of Mad2 function) induce error prone chromosome segregation and aneuploidy. Our data also shows that either the loss or gain of miR-493-3p induces mistakes in chromosome segregation, and thus underlines the physiological relevance of miRNA-mediated control of Mad2.

The SAC activity is lowered in cells with excess of miR-493-3p. In presence of taxol and nocodazole, the miR-493-3p overexpressing cells undergo forced mitotic exit within few hours after entering mitosis. This phenotype can result from the independent loss of function of several SAC proteins such as Mad2, Mad1, BubR1 and Bub1 (Meraldi et al., 2004; Michel et al., 2004; Meraldi et al., 2005). Our observation that in the miR-493-3p overexpressing prometaphase cells the Mad2 levels, but not the Bub1 levels, were significantly reduced supports the notion that the impact of miR-493-3p on SAC is mainly caused by suppression of Mad2. We also sought further proofs to highlight that *MAD2* downregulation is the primary reason for miR-493-3p induced SAC inactivation. Specifically, the cells co-transfected with TSB-MAD2 and miR-493-3p showed restoration of Mad2 levels back to normal that also resulted in increased SAC activity and normal sensitivity of cells to MTAs. However, it should also be noted that TSB-MAD2 can also interact with other miR-493-3p target mRNAs and may rescue their suppression; therefore we cannot fully exclude the possibility that other mitotic target genes of miR-493-3p exist, which together with downregulation of *MAD2* contribute to the cellular phenotype observed in the miR-493-3p transfected cells.

The retrospective analysis of ovarian tumor samples *in vivo* show inverse correlation between miR-493-3p and *MAD2* gene expression. In aggressive HGSC ovarian cancer, miR-493-3p was downregulated when compared to the ovarian surface epithelium and to a less aggressive clear cell carcinoma. In agreement, the *MAD2* levels were elevated in the aggressive HGSC cancer. Further data from assays with cultured OVCAR-8 and CAOV-3 cells, both of which are de-

rivatives of high-grade ovarian cancer, confirmed the inverse association between miR-493-3p and *MAD2* expressions. OVCAR-8 cells showed decreased miR-493-3p and elevated Mad2 levels, while CAOV-3 cells exhibited high miR-493-3p and low Mad2 levels. Although these cells are derived from tumors of similar grade, the observed differences in the gene expression profiles are not surprising as the cell lines originated from different individuals. This notion is in line with recent morphological, molecular biology and genetic data, indicating that ovarian cancer is very heterogeneous disease composed of different subtypes of tumors exhibiting dissimilar clinicopathologic features and tumorigenic behavior (reviewed in Blagden, 2015).

In cancer cells, the excess of endogenous or ectopically expressed miR-493-3p operates towards reducing the Mad2 below a threshold where the cell is unable to sustain normal SAC activity. In unperturbed conditions, the majority of cells in the CAOV-3 cell line, having high endogenous expression of miR-493-3p, were resistant to taxol, which is in contrast to the OVCAR-8 cell line having low expression of miR-493-3p and drug sensitive cells. In addition, the ectopic overexpression of miR-493-3p in both cell lines caused a similar cellular response; the frequency of mitotic slippage in the presence of MTAs was elevated as a consequence of lost Mad2 function.

Interestingly, the *in vivo* analysis of ovarian cancer (Oslo and TCGA cohort) and breast cancer patient samples (Bergen cohort) indicated that patients with high miR-493-3p expression undergo reduced disease specific survival, post-chemotherapy. One likely contributing factor is the tumor cells' resistance towards chemotherapy drugs. We hypothesize that the patients with high miR-493-3p levels would be less sensitive to MTAs such as paclitaxel, which is used as current standard of care chemotherapy, in combination with platinum, for the treatment of advanced ovarian, breast and non-small cell lung cancers after cytoreductive surgery (McGuire et al., 1989; Sledge et al., 1994; Bonomi et al., 1997). Analysis of the data, indeed, indicated that stage III breast cancer patients whose tumors expressed high levels of miR-493-3p showed a significantly poorer response to the paclitaxel therapy when compared to epirubicin treatment arm. Besides the impact on SAC signaling, cancer cells with excess miR-493-3p were observed to undergo senescence, which may provide another mean to escape the drug treatment, also *in vivo* (Gordon and Nelson, 2012). miR-493-3p causes cellular senescence most likely as a result of its negative impact on its target genes *MAD2* (our data), *MKK7* (Sakai et al., 2014) and *E2F1* (Gu et al., 2014) that are all implicated in the induction of senescence. In conclusion, our data links intimately the altered expression of miR-493-3p and *MAD2* to cancer cells' sensitivity to MTAs and provides the first evidence of the diagnostic value of the miRNA in the selection of the first line chemotherapy.

Our study is the first to explore the role of miR-493-3p in control of mitosis and Mad2 expression. However, it should be kept in mind that miRNA-mediated regulation of Mad2 has been studied previously with positive findings; miR-433 and miR-28-5p are other validated miRNAs to target the mRNA of Mad2. Interestingly, miR-493-3p and miR-433 reside in the same miRNA cluster at Chr14q32, which is found deregulated in melanoma, ovarian cancer, and GISTs (Zehavi et al., 2012; Zhang et al., 2008; Haller et al., 2010). In the future, analyzing the expression patterns of Mad2 regulating miRNAs from patient samples can help to determine the tumor subtype or predict the efficacy of clinically used MTAs.

6.3 Dusp3 maintains spindle bipolarity by preventing aberrant Erk1/2 activation in M phase (III)

We identified a previously unknown function of Dusp3 in the maintenance of spindle bipolarity during mitosis. Our study is the first to demonstrate significance of Dusp3 in mitotic cells and it provides an explanation for the earlier observation of high Dusp3 expression during G2/M phase (Rahmouni et al., 2006). We show that in HeLa cells, the reduced levels of Dusp3 leads to formation of multipolar mitotic spindles in an Erk1 or Erk2 activity-dependent manner. The *in silico* analysis indicates that the Dusp3 siRNAs' used in our study are specific for the phosphatase as they did not show marked sequence similarity with other Dusp family members (sequence similarity was <57%). Based on these results we propose that within the Dusp family of phosphatases no other member possesses functional redundancy with Dusp3 in the process of maintaining spindle bipolarity. However, we cannot rule out the possibility that Dusp3 could influence the function of a protein(s) other than Erk1/2 that is directly involved in preserving normal spindle architecture at M phase.

In line with previous reports, we observe that reduced levels of Dusp3 leads to increased phosphorylation of Erk1/2 and Jnk, and generates a delay in cell cycle progression (Rahmouni et al., 2006; Hao and ElShamy, 2007; Wagner et al., 2013). In addition, we report that levels of c-Jun and phospho-c-Jun are also elevated in these cells. Interestingly, the Jnk activation was detected even before the serum stimulus, which indicates that the Dusp3 silenced cells might be experiencing cellular stress. Earlier studies have highlighted that loss of Dusp3 leads to induction of cellular senescence and upregulation of $p21^{cip1}$ (Rahmouni et al., 2006), both of which are indicators of cellular stress (Ben-Porath and Weinberg, 2004). Importantly, the aberrant high activity of Erk1/2 can cause cell cycle arrest at early G2 phase, delay cells' entry into M phase and induce multipolar spindles (Shinohara et al., 2006; Cui et al., 2010; Saavedra et al., 1999). In support of these findings, we show that in cell culture constitutively active Erk1/2

increases the frequency of cells with more than two spindle poles, while in cells treated with Erk1/2 inhibitors the architecture of mitotic spindle was normal, which is consistent with a previous report (Shinohara et al., 2006). Therefore based on our results we propose that increased Dusp3 expression during G2/M phase functions to keep the Erk1/2 activity in check to facilitate normal mitotic progression.

Furthermore, the multipolar phenotype caused by Dusp3 depletion was rescued by chemical inhibition of Erk1/2 but not Jnk activity. Treatment of Dusp3 silenced cells with Erk1/2 inhibitor FR180204 or Mek inhibitor PD98059, or overexpression of WT-Dusp3 protein in the cells led to decreased Erk1/2 activity that was found to prevent the formation of multipolar spindles. Moreover, we demonstrate that induction of multipolarity by Dusp3 RNAi required the presence of either Erk1 or Erk2; if both kinases were silenced together no spindle aberrations were observed in the Dusp3 depleted cells. Collectively, our results suggest that in order to maintain bipolar spindle architecture Dusp3 inactivates both Erk1 and Erk2. However, the exact nature and timing of this regulation in terms of the cell cycle stage remains to be elucidated.

The multipolarity can arise as a consequence of aberrant centrosome number and/or loss of centrosome integrity. The causes for generation of multiple centrosomes include centriole overduplication, failure of cytokinesis, cell fusion and *de novo* centriole assembly (Maiato and Logarinho, 2014). On the other hand loss of centrosome integrity is characterized by centriole disengagement and/or PCM fragmentation during mitosis (Maiato and Logarinho, 2014). At this moment, we lack experimental evidence to precisely explain the molecular mechanism by which the extra spindle poles arise in the cells with diminished levels of Dusp3 and/or hyperactive Erk1/2. However, we can exclude the involvement of cytokinesis failure and cell fusion as these were not observed to occur in the Dusp3 silenced cells.

The cell cycle arrest observed after loss of Dusp3 may present one mechanism by which the centrosome number increases in these cells. Earlier studies have shown that the cell cycle arrest at G2/M phase coupled with activated Cdk2:Cyclin A/E provides the cells with a capability to reduplicate the centrosomes (Dodson et al., 2004; Matsumoto et al., 1999; Meraldi et al., 1999). The success of this abnormal process depends on the status of $p21^{cip1}$, which counteracts centrosome reduplication (Matsumoto et al., 1999; Meraldi et al., 1999). Interestingly, the cells with reduced Dusp3 exhibit both G2 cell cycle arrest and increased levels of $p21^{cip1}$ (Rahmouni et al., 2006). The Dusp3 silenced cells exhibiting G2 arrest can potentially initiate the centrosome reduplication cycle, but in order to complete this process and to produce extra centrosomes, these cells should find a way to keep the activity of $p21^{cip1}$ low and Cdk2 higher. Whether or not this route contributes

to spindle multipolarity in Dusp3 silenced cells remains to be elucidated. Another possible mechanism explaining multipolarity in the Dusp3 silenced cells involves the activation of Erk1/2 that can lead to overproduction of AURKA, which has been previously linked to induction of mitotic aberrations including generation of multipolar spindles (Zhou et al., 1998; Meraldi et al., 2002; Lentini et al., 2007). The AURKA transcription factors E4TF1, EST1, EST2 and GABP (Tanaka et al., 2002) are regulated by Erk1/2 activity (Furukawa et al., 2006; Foulds et al., 2004; Flory et al., 1996). Based on this we hypothesize that depletion of Dusp3 results in AURKA upregulation via increased activity of Erk1/2 that in turn contributes to the formation of extra spindle poles. At this time we, however, cannot point out the precise mechanisms for the induction of multipolarity after loss of Dusp3. One simple way to gain more insights would be examination of the centriole number in the aberrant spindle poles. For example, the aberrant centriole disengagement usually produces extra spindle poles with one centriole instead of the normal two. Moreover, the PCM fragmentation can lead to formation of spindle poles devoid of centrioles while the centrosome amplification displays extra spindle poles each with two centrioles (Maiato and Logarinho, 2014). Future studies are needed to resolve this matter.

The data on Dusp3 in cancer is very limited, and further studies are needed before any conclusions can be made regarding the possible role of Dusp3 in tumorigenesis. The expression of Dusp3 appears to vary between cancers. For example, in breast cancer and NSCLC, Dusp3 expression is decreased when compared to non-malignant control tissues (Hao et al., 2007; Wang et al., 2011) while in prostate and cervix cancer the phosphatase levels are upregulated (Arnoldussen et al., 2008; Henkens et al., 2008). Importantly, the occurrence of multipolar cells within a tumor is a quite normal phenomenon. In addition, the multipolar mitotic cells challenge the maintenance of genomic balance due to the induction of merotelic attachments that increases the risk of producing lagging chromosomes and chromosome bridges during anaphase. The resulting low level aneuploidy usually favors the cancer cell growth (Ganem et al., 2009; Silkworth et al., 2009). We present mitosis-specific functions of Dusp3 and reveal that decreased levels of the phosphatase lead to the formation of mitotic spindles with extra poles, which is known to generate aneuploidy, a hallmark of cancer (Ganem et al., 2009; Silkworth et al., 2009). Conversely, the overexpression of Dusp3 did not appear to have any adverse effects on spindle architecture or SAC signaling, and therefore excess of the phosphatase may not cause genomic instability. Future research is anticipated to lead to the identification of new mitotic substrates of Dusp3 and thereby shed more light on the roles of the phosphatase in control of cell cycle and cell growth in healthy and pathological growth conditions.

7 SUMMARY AND CONCLUSIONS

Error free chromosome segregation is an absolute requirement for the preservation of genomic stability. During mitosis, SAC functions as a key signaling pathway to provide time for completion of all prerequisites needed for faultless chromosome segregation. Defective SAC signaling increases the risk of chromosome missegregation that can lead to aneuploidy, and further to stimulation of tumorigenesis. Despite many aspects of SAC functions have been well characterized at the molecular level, the mechanisms regulating activity and gene expression of SAC proteins has remained poorly understood. A number of SAC genes have been found to be deregulated in tumors and this can provide cancer cells growth advantage via at least two separate routes; by allowing cell proliferation in spite of aneuploidy and by providing the cells with an ability to evade antimitotic drug effects. Our work has led to the identification and functional characterization of new factors that fine-tune the expression of SAC genes and work as molecular on/off switches in mitotic processes.

The summary and conclusions of the research presented in this thesis include:

I. We identified miR-378a-5p as a novel regulator of mitosis signaling and gate-keeper of genomic balance. Altered expression of miR-378a-5p may contribute to breast cancer tumorigenesis and affect tumor cells' sensitivity to MTA therapy. Excess of miR-378a-5p in cultured cancer cells abrogated SAC signaling, which led to a number mitotic anomalies including multipolarity and aneuploidy, failure of cytokinesis and consequent polyploidization of the cell population, and development of resistance against MTAs. miR-378a-5p was found to control the activity and protein levels of Aurora B kinase , which provides a plausible explanation for the mitotic mistakes observed in the miRNA overexpressing cells. Suppression of Aurora B by miR-378a-5p occurred indirectly via the RTK-Mapk pathway; cells with an excess of miR-378a-5p exhibited increased RTK signaling that led to hyperactivation of Mapk-Erk1/2, which in turn negatively affected the Aurora B function. *In vivo*, high levels of miR-378a-5p were found to associate with increased grade and aggressive form of breast cancer.

II. Our data established miR-493-3p as a key SAC regulating miRNA and an important determinant of MTA sensitivity *in vitro* and *in vivo*. The balanced expression of miR-493-3p was required for maintenance of genomic equilibrium and normal MTA response; cancer cells with altered expression of miR-493-3p exhibited pleiotropic mitotic defects including impaired mitotic timing, premature separation of sister chromatids, induction of aneuploidy and override of MTA imposed M phase block. miR-493-3p was found to target directly the 3'UTR of *MAD2L1* mRNA and suppress the gene expression. The introduction of anti-miR-493-3p into cells led to a decrease in the levels of endogenous miR-493-3p, which caused an upregulation of Mad2 protein levels resulting in lagging chromosomes and chromosome bridges that are reported phenotypes of Mad2 overexpression. In ovarian and breast cancer patients, high levels of miR-493-3p were found to correlate with aggressive cancer and lower patient survival. Especially compelling was the finding that breast cancer patients whose tumors exhibited high levels of the miR-493-3p showed poor survival when treated with paclitaxel chemotherapy in comparison to epirubicin treatments.

III. We found that elevated expression of phosphatase Dusp3 is needed to keep the Erk1/2 activity in check to avoid the formation of multipolar spindles during mitosis. In cells with reduced Dusp3 levels the activity of both Erk1/2 and Jnk was increased. However, the formation of the extra spindle poles was found to be dependent only on the activity of Erk1/2 while Jnk had no role in the process. The overexpression of RNAi resistant WT-Dusp3 restored the spindle bipolarity in the Dusp3 silenced cells without causing any noticeable mitotic defects. The data suggests that the main mitotic function of Dusp3 is to maintain spindle bipolarity and thereby support genomic balance.

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