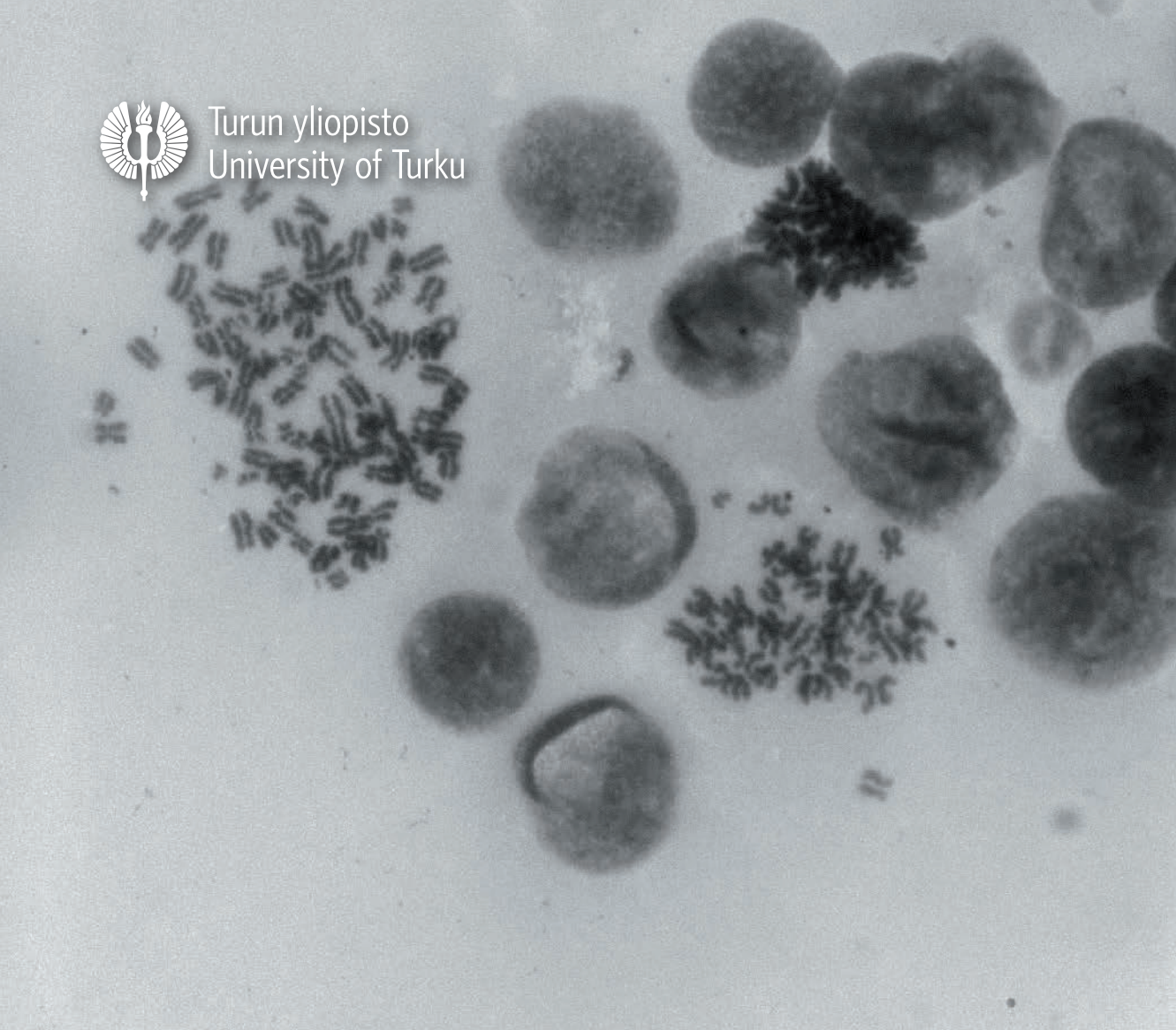

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To my family

ABSTRACT

Sofia Aakko (née Pruikkonen)
MicroRNA-mediated regulation of mitosis and taxane sensitivity in breast and ovarian cancer

University of Turku, Faculty of Medicine, Institute of Biomedicine, Physiology; Turku Doctoral Programme of Molecular Medicine (TuDMM); VTT Health, VTT Technical Research Centre of Finland; Turku Centre for Biotechnology, University of Turku and Åbo Akademi University

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Taxanes are microtubule targeting chemotherapeutics that block cell division (mitosis) and induce cell death. Taxanes are commonly used in the neoadjuvant chemotherapy for breast and ovarian cancer. However, the patient responses are hindered by the compounds' cytotoxicity and the commonly observed drug resistance. One route for an improved therapy outcome would be to determine the tumor cells' sensitivity to taxanes before starting the therapy. MicroRNAs (miRNAs) are central components of the post-transcriptional gene regulation. They are also potential biomarker molecules as their expression is known to differ between malignant and normal tissue. The aim of this thesis work was to identify mitosis-regulating miRNAs, characterize their cellular functions, and evaluate their potency in the prediction of tumor cells' taxane sensitivity. Two miRNAs, miR-493-3p and let-7b, were discovered to regulate the mitotic checkpoint and the sensitivity of cancer cells to a taxane (paclitaxel) *in vitro* by controlling the expression of *MAD2* and *AURKB,* respectively. Moreover, the expression of the miRNAs was found to be significantly altered in aggressive breast and ovarian tumors, which also correlated with reduced patient survival. A third mitosis-regulating miRNA, miR-193a-3p, was found to control cytokinesis via targeting the tumor suppressor *RASSF1A*. Excess of miR-193a-3p induced polyploidization and multipolarity in the next M-phase. Finally, a fourth miRNA, miR-203b-3p, was identified that sensitized breast and ovarian cancer cells to clinically relevant doses of paclitaxel through suppression of the anti-apoptotic protein Bcl-xL. Interestingly, this miRNA is a possible mediator of the previously reported c-Myc-induced sensitization to taxane therapy. Overall, the results presented here provide new information on the role of mitosis-regulating miRNAs in tumorigenesis and the drug sensitivity of breast and ovarian cancers. Profiling the expression of these miRNAs from tumors may advance cancer diagnostics and help to stratify the patients that would benefit most from taxane therapy.

Keywords: microRNA, mitosis, taxane, cancer

TIIVISTELMÄ

Sofia Aakko (os. Pruikkonen)
MikroRNA-välitteinen mitoosin ja taksaaniherkkyyden säätely rinta- ja munasarjasyövässä

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia; Molekyylilääketieteen tohtoriohjelma (TuDMM); VTT Health, Teknologian tutkimuskeskus VTT; Turun Biotekniikan keskus, Turun yliopisto ja Åbo Akademi

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Taksaanit ovat mikrotubulusten toimintaa estäviä syöpälääkkeitä, joiden sytotoksinen vaikutus perustuu solujaon (mitoosi) salpaamiseen. Taksaaneja käytetään yleisesti etenkin rinta- ja munasarjasyöpien liitännäisterapiassa. Taksaanien vakavat sivuvaikutukset potilaissa ja yleisesti havaittu lääkeresistenssi kuitenkin heikentävät terapian tehoa. Yksi mahdollinen keino parantaa hoidon tehoa olisi määrittää kasvaimen taksaaniherkkyys ennen terapian aloittamista. MikroRNA:t (miRNA) ovat tärkeitä geenien ilmentymisen säätelijöitä. Ne ovat myös lupaavia syövän biomarkkereita, koska niiden ilmentymisessä on havaittu merkittäviä eroja kasvainten ja normaalikudoksen välillä. Tässä väitöskirjassa on pyritty tunnistamaan mitoosia säätteleviä miRNA:ita, tutkimaan niiden toimintamekanismeja ja arvioimaan niiden käyttökelpoisuutta kasvainten taksaaniherkkyyden ennustamisessa. Kahden miRNA:n, miR-493-3p:n ja let-7b:n, todettiin säätelevän kohdegeeniensä *MAD2* ja *AURKB* kautta mitoottisen tarkastuspisteen toimintaa ja herkkyyttä taksaanille (paklitakseli) syöpäsoluviljelmissä. Molempien miRNA:iden ilmentymisen havaittiin muuttuneen aggressiivisemmissa rinta- ja munasarjasyöpäkasvaimissa sekä korreloivan potilaiden selviytymisennusteen kanssa. Kolmannelle mitoosia säätelevälle miRNA:lle, miR-193a-3p:lle, tunnistettiin uusi kohdegeeni, *RASSF1A*, jonka kautta miRNA säätelee sytokineesiä. Ylimäärä miR-193a-3p:tä aiheutti soluissa polyploidiaa sekä epänormaalien mitoottisten sukkuloiden määrän lisääntymisen. Neljännen tässä työssä tarkastellun miRNA:n, miR-203b-3p:n, havaittiin puolestaan alentavan solukuolemaa estävän *BCL2L1* geenin ilmentymistä ja siten herkistävän syöpäsolut paklitakselille. miR-203b-3p toimii mahdollisesti osana c-Myc–proteiinin ohjaamaa signalointireittiä, jonka on raportoitu kontrolloivan syöpäsolujen taksaanivastetta. Tämä väitöskirja tarjoaa uutta tietoa mitoosia säätelevien miRNA:iden roolista tuumorigeneesin ja taksaaniherkkyyden säätelyssä rinta- ja munasarjasyövissä. Näiden miRNA:iden tasojen määrittäminen tuumoreista voi tulevaisuudessa auttaa syövän diagnostiikassa sekä tunnistamaan potilaat, jotka hyötyvät eniten taksaaniterapiasta.

Avainsanat: mikroRNA, mitoosi, taksaani, syöpä

TABLE OF CONTENTS

ABBREVIATIONS

3’ untranslated region	3’UTR
4',6-Diamidino-2-Phenylindole	DAPI
Anaphase promoting complex/cyclosome	APC/C
antisense oligonucleotide	ASO
apoptosis-linked gene 2–interacting protein X	ALIX
B-Cell CLL/Lymphoma 2	Bcl-2
BCL2 Like 1	Bcl-xL
Budding uninhibited by benzimidazoles 1	Bub1
Budding uninhibited by benzimidazoles 3	Bub3
Budding uninhibited by benzimidazoles-related 1	BubR1
Cell division cycle 20	Cdc20
Cell division cycle 25 homolog B	Cdc25B
centromere protein A	CENP-A
centromere protein E	CENP-E
centrosomal protein 55	CEP55
chromosomal instability	CIN
chromosomal passenger complex	CPC
clear cell carcinoma	CCC
cleaved PARP	cPARP
cyclin-dependent kinase	Cdk
death in mitosis	DiM
deoxyribonucleic acid	DNA
Dickkopf WNT signaling pathway inhibitor 1	DKK1
Di George critical syndrome 8	DGCR8
Endosomal sorting complex required for transport III	ESCRT-III
Epidermal growth factor receptor	EGFR
Erb-B2 receptor tyrosine kinase 4	ErbB4
Family with sequence similarity 83 member D	FAM83D

fluorescence *in situ* hybridization	FISH
Frizzled class receptor 4	FZD4
guanosine-5'-triphosphate	GTP
high-grade serous carcinoma	HGSC
High mobility group AT-Hook 2	HMGA2
high-throughput screen	HTS
Insulin like growth factor 1 receptor	IGF1R
kinetochore-microtubule	kt-mt
Kinetochore scaffold 1	Knl1
Kirsten rat sarcoma viral oncogene homolog	KRas
locked nucleic acid	LNA
messenger RNA	mRNA
microRNA	miRNA
microtubule-binding protein cytoplasmic linker protein (CLIP)-associating protein	CLASP
microtubule organizing center	MTOC
microtubule targeting agent	MTA
Mitogen-activated protein kinase kinase 7	MKK7
Mitotic arrest deficient 1	Mad1
Mitotic arrest deficient 2	Mad2
Mitotic centromere-associated kinesin	MCAK
mitotic checkpoint complex	MCC
Multipolar spindle-1	Mps1
Myeloid cell leukemia 1	Mcl-1
nuclear envelope breakdown	NEBD
nuclear pore complex	NPC
nucleotide	nt
ovarian surface epithelium	OSE
pericentriolar matrix	PCM
polymerase chain reaction	PCR
Polo-like kinase	Plk

post-mitotic death	PMD
Protein phosphatase 1	PP1
Protein phosphatase 2	PP2A
Ras association domain family member 1	Rassf1
Ras homolog family member C	RhoC
RNA binding protein	RBP
RNA-induced silencing complex	RISC
Salt inducible kinase 2	SIK2
spindle assembly checkpoint	SAC
Syntaxin16	STX16
Tumor susceptibility 101	Tsg101

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numeral I-IV.

I Tambe M*, **Pruikkonen S*,** Mäki-Jouppila J, Chen P, Elgaaen BV, Straume AH, Huhtinen K, Cárpen O, Lønning PE, Davidson B, Hautaniemi S and Kallio MJ. Novel Mad2 targeting miR-493-3p controls mitotic fidelity and cancer cells' sensitivity to paclitaxel. Oncotarget. 2016. 7(11):12267-85.

II Mäki-Jouppila JHE, **Pruikkonen S**, Tambe MB, Aure MR, Halonen T, Salmela A-L, Laine L, Børresen-Dale A-L and Kallio MJ. MicroRNA let-7b regulates genomic balance by targeting Aurora B kinase. Mol Oncol. 2015. 9(6):1056-70.

III **Pruikkonen S** and Kallio MJ. Excess of a Rassf1-targeting microRNA, miR-193a-3p, perturbs cell division fidelity. Br J Cancer. 2017. 116(11):1451-1461.

IV **Aakko S,** Straume AH, Birkeland EE, Chen P, Qiao X, Lønning PE and Kallio MJ. c-Myc –regulated microRNAs, miR-203a-3p and miR-203b-3p, control Bcl-xL expression and paclitaxel sensitivity. *Manuscript*.

*equal contribution

1 INTRODUCTION

Gene expression is controlled at multiple levels: epigenetic, transcriptional, and post-transcriptional. Non-coding RNAs, and especially microRNAs (miRNAs), are central components of the post-transcriptional gene regulation. The importance of miRNA-mediated gene regulation is highlighted by the notions that the latest miRbase entry includes 2588 human miRNAs (miRbase release 21; (Kozomara and Griffiths-Jones, 2014) and that miRNAs may regulate more than 60 % of the human protein-coding genes (Friedman *et al*., 2009). Therefore, miRNAs participate in the control of virtually all important physiological processes such as development and proliferation. For these reasons, balanced miRNA expression is crucial to inhibiting pathological transformations (Chang and Mendell, 2007). In fact, the majority of human tumors exhibit altered levels of certain miRNAs (Calin *et al*., 2002; Lu *et al*., 2005).

Human tumors are also characterized by abnormal cell division (mitosis) and incorrect chromosomal content of cells (aneuploidy). Mitosis aims at equal distribution of genomic material to the daughter cells and thus is a carefully regulated process. One of the most important gatekeepers of mitotic fidelity is the spindle assembly checkpoint (SAC) that inhibits chromosome segregation until all chromosomes are correctly aligned on the division plane (Musacchio, 2015). A number of other proteins are required for example for bipolar mitotic spindle assembly and the establishment of correct microtubule-chromosome attachments (Cheeseman *et al*., 2006; Whitehead and Rattner, 1998). Cell division errors have been demonstrated to provoke aneuploidy (Ganem *et al*., 2009; Silkworth *et al*., 2009), but the role of aneuploidy in cancer is intriguing. Primarily, chromosome mis-segregation is considered to promote tumorigenesis by increasing the genomic heterogeneity of cells that offers a growth advantage (Levine *et al*., 2017; Sotillo *et al*., 2007). On the other hand, severe aneuploidy is detrimental to cell fitness (Sheltzer *et al*., 2017; Weaver *et al*., 2007) and induction of aneuploidy is also a therapeutic tool for killing tumor cells (Janssen *et al*., 2009; Zasadil *et al*., 2014). Indeed, mitosis is one of the oldest, although still developing, therapeutic areas in cancer treatment; conventional microtubule-targeting agents (MTA), such as docetaxel and paclitaxel, are still used as a part of the standard therapy in *e.g.* ovarian carcinoma (Vasey *et al*., 2004). Nevertheless, development of new cancer therapeutics with improved mitotic specificity has proven challenging (Boss *et al*., 2011; Goldberg *et al*., 2014; Yim, 2013).

Despite the tremendous increase of miRNA-research during the past decade, very little is known about miRNA-mediated control of mitosis and the role of this regulatory axis in cancer. miRNAs possess diagnostic and perhaps also therapeutic

value with regard to cancer, as their expression patterns can differentiate normal cells from cancerous cells; this can be utilized in tumor subtyping and grading (Haakensen *et al*., 2016; Lu *et al*., 2005). Considering the high frequency of mitotic defects and aneuploidy in cancer, cell division regulating miRNAs are potential biomarkers for cancer diagnostics. Moreover, profiling of the mitosis controlling miRNAs may also help to predict the clinical efficacy of MTAs that is dependent on the functional integrity of SAC (Furlong *et al*., 2012; Kasai *et al*., 2002).

This thesis work investigated the function of novel miRNAs controlling mitotic processes and genomic balance. Moreover, the role of these miRNAs in tumorigenesis and MTA response was studied in breast and ovarian carcinoma.

2 REVIEW OF THE LITERATURE

2.1 MicroRNAs (miRNAs)

2.1.1 miRNA biogenesis

MiRNAs have variable genomic origins, as they are transcribed from exonic regions or introns of coding and non-coding loci. Most miRNA genes have their own promoters, but miRNAs that reside in an intron of a protein-coding gene (host gene) are typically controlled by the host gene promoter (Ozsolak *et al*., 2008). Nearby miRNA genes may also form a polycistronic cluster, for example, the *MIR17~92* cluster, where the miRNAs are transcribed together to form one precursor miRNA molecule, which is then processed into several mature miRNAs. Clustering may enhance the efficacy of miRNA-mediated gene regulation, but it can also lead to upregulation of several oncogenic miRNAs due to the amplification of a single genomic locus (He *et al*., 2005). On the other hand, due to the evolution of miRNAs, paralogous miRNAs can be transcribed from distant genomic loci, such as members of the let-7 family (Lagos-Quintana *et al*., 2001). The transcription of miRNA genes is controlled by similar mechanisms as protein-coding genes, including the main transcription factors, c-Myc (Chang *et al*., 2008) and p53 (Tarasov *et al*., 2007), as well as epigenetic mechanisms (Brueckner *et al*., 2007; Lujambio *et al*., 2007).

Despite these variable genomic locations, miRNA genes are mainly transcribed by RNA polymerase II (Lee *et al*., 2004), and after transcription, the majority of miRNA transcripts enter a common processing machinery, which is illustrated in Figure 1 (reviewed in (Ameres and Zamore, 2013; Ha and Kim, 2014; Lin and Gregory, 2015)). The primary miRNA transcripts (pri-miRNA) are double-stranded, stem loop –structures with single-stranded overhangs at both ends. In the conventional miRNA biogenesis pathway, a complex called Microprocessor, formed by Drosha and Di George critical syndrome 8 (DGCR8) is responsible for pri-miRNA processing in the nucleus. The RNA-binding protein, DGCR8, enables the RNAse III endonuclease Drosha interaction with the pri-miRNA, and cleavage at the base of the pri-miRNA stem-loop (Denli *et al*., 2004; Gregory *et al*., 2004). The Microprocessor activity creates about a 35 nucleotide (nt) long hairpin structure called pre-miRNA. The pre-miRNA is transported into the cytoplasm by active, GTP-driven transport mediated by Exportin-5 (Yi *et al*., 2003). Then, a cytoplasmic RNase III endonuclease, Dicer, continues the processing of the miRNA precursor by cleaving the terminal loop and releasing a ~22 nt long double-

stranded RNA molecule (Bernstein *et al*., 2001; Grishok *et al*., 2001; Hutvágner *et al*., 2001). Dicer is an evolutionary conserved protein, the deletion of which is embryonically lethal in mice (Bernstein *et al*., 2003). Similarly to the Drosha endonuclease, the human Dicer associates with accessory RNA-binding proteins like the TAR RNA binding protein that enhances the pre-miRNA processing efficacy (Fukunaga *et al*., 2012; Haase *et al*., 2005).

Finally, the newly formed RNA duplexes are loaded onto AGO-protein(s) to form RNA-induced silencing complexes (RISC) and achieve gene silencing activity (Hammond *et al*., 2001). Since the mature miRNA in the RISC-complex needs to be single-stranded to enable target gene binding, the AGO-protein unwinds the miRNA duplex and the passenger strand is degraded, either actively by AGO2 or through RNA decay (Liu *et al*., 2004; Yoda *et al*., 2010). The remaining, active miRNA-strand (-3p or -5p) is referred to as the guide strand. Selection of the guide and the passenger strand is mainly dictated by thermodynamic stability; the strand with a relatively unstable 5' end is favored as the guide strand in most cases (Khvorova *et al*., 2003; Schwarz *et al*., 2003). However, the guide/passenger strand division is neither permanent nor is it universal. The ratio of -3p/-5p strand activity may vary in different tissues, but both strands can also be active simultaneously and even regulate the same genes and signaling pathways (Mataki *et al*., 2016; Ro *et al*., 2007; Yang *et al*., 2011).

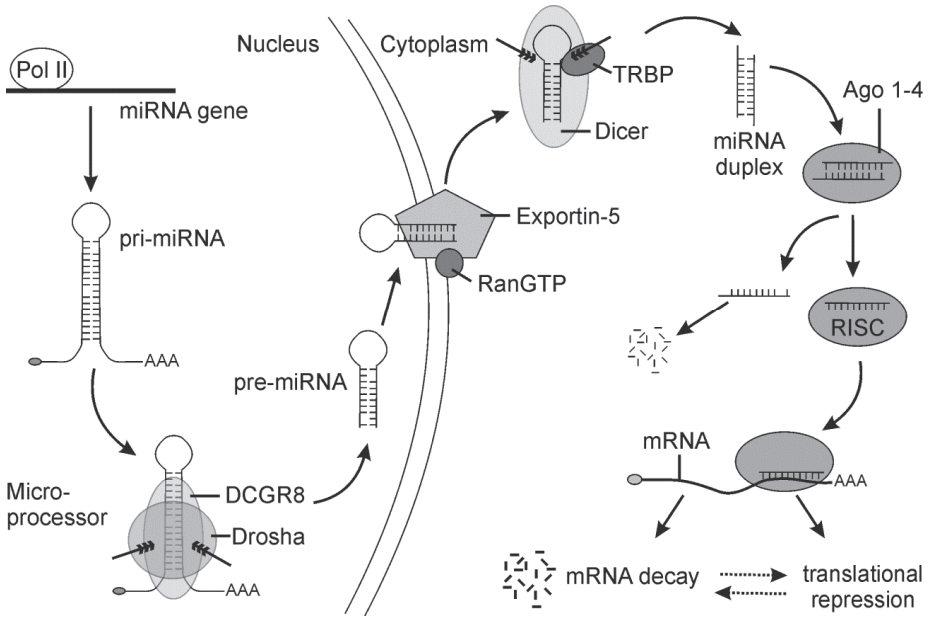

Figure 1. A schematic representation of the canonical miRNA biogenesis pathway and the mechanism of miRNA-mediated gene regulation. Modified from (Lin and Gregory, 2015).

The majority of miRNAs are processed through the canonical biogenesis pathway as described above, but vertebrates can also harness alternative processes to produce functional miRNAs. For example, some miRNAs may not be transcribed from the genome, but they are processed from other non-coding RNAs, such as the small nucleolar RNAs in a Dicer-dependent manner (Ender *et al*., 2008). The first non-canonical miRNAs biogenesis process was described for mirtrons, namely, miRNAs transcribed from intronic regions that are spliced to generate a pre-miRNA without Microprocessor activity (Okamura *et al*., 2007). Overall, the biological relevance of the non-canonical biogenesis processes is equivocal; according to current knowledge, about 1 % of the conserved miRNAs harness these alternative pathways (Ha and Kim, 2014).

2.1.2 Fundamentals of the miRNA function

The miRNAs regulate gene expression by binding to the target gene messenger-RNA (mRNA); the mature miRNA guides the RISC-complex to the target mRNA through sequence complementarity (Fig 1, reviewed in (Ameres and Zamore, 2013; Huntzinger and Izaurralde, 2011)). Most of the miRNA-binding sites are located in the 3'untranslated region (3'UTR) of the target mRNAs. The sequence complementarity over the nts 2-7 of miRNA 5' end, called the "seed", is enough for target gene recognition and suppression in mammals (Bartel, 2009). However, additional pairing around the miRNA nts 13-16 can enhance miRNA function (Grimson *et al*., 2007). Moreover, miRNAs have been detected to bind also to 5'UTR (Wang *et al*., 2016a) and coding sequence regions (Forman *et al*., 2008), but the 3'UTR sites are the most favorable for the RISC function (Bartel, 2009).

The miRNAs are generally regarded as negative regulators of gene expression, but the mechanism of action and the sequence of events for miRNA-mediated repression of gene expression are still under debate. In mammals, only the Ago2-protein has slicing activity (Liu *et al*., 2004), and mammalian miRNAs rarely possess the perfect complementary binding to the target that is needed for mRNA destruction (Yekta *et al*., 2004). Accordingly, translational inhibition alone was initially regarded to be the main mechanism of function for mammalian miRNAs. However, more recent studies have provided compelling evidence that in the majority of cases where the mammalian miRNA target protein is suppressed, the mRNA is also degraded (Baek *et al*., 2008; Guo *et al*., 2010). Whether the mRNA decay precedes or is due to translation inhibition and how these two processes are connected remains still evasive (Fig 1). Potentially, translation inhibition is the primary effect of mammalian miRNAs (Djuranovic *et al*., 2012). Nevertheless, deadenylation of the mRNA poly-A-tail has been shown to be the priming event in the

mRNA cleavage (Wu *et al*., 2006). For protein translation inhibition, multiple mechanisms have been proposed, including the inhibition of elongation and premature termination of translation (Huntzinger and Izaurralde, 2011), however, blockage at translation initiation has emerged as the most probable mechanism of action for the RISC complex (Djuranovic *et al*., 2012; Ricci *et al*., 2013).

Besides negative gene regulation, cases of gene expression activation by miRNAs have also been reported. However, activating miRNA functions are usually indirect, for example due to miRNA-mediated inhibition of a repressor element (Bruno *et al*., 2011), or appear only under special circumstances, such as during cell cycle arrest (Vasudevan *et al*., 2007).

A defining and interesting feature of miRNA-mediated gene regulation is the usually subtle effect on target gene expression (Baek *et al*., 2008; Bartel, 2009); hence miRNAs are regarded as fine-tuners of protein expression. On the other hand, each miRNA has hundreds of potential target genes, and one miRNA may have a powerful effect on a biological process in the case where it suppresses several genes in the same pathway or with a similar function (Bartel, 2009). Moreover, as more than half of the mammalian protein coding genes are potentially regulated by miRNAs (Friedman *et al*., 2009), it is conceivable that miRNAs regulate important physiological processes, especially development, differentiation, cell death, and metabolism (reviewed in (Chang and Mendell, 2007; Kloosterman and Plasterk, 2006)). However, not much is known, for example, about miRNA-mediated regulation of cell cycle and cell division. Most of the reports to date concern the altered expression of cell cycle regulating miRNAs in cancer, allowing uncontrolled cell proliferation and abnormal cell division (discussed further in Chapter 2.6.3.5). Moreover, even less is known about the cell cycle dependent regulation of miRNA expression (reviewed in (Bueno and Malumbres, 2011)).

2.2 Cell cycle – phases and regulation

The fundamental function of the cell cycle is to replicate the genomic content of the cell, coded into deoxyribonucleic acid, (DNA) and pass it equally to the two daughter cells. These two phases are called the S phase (DNA synthesis) and the M phase (mitosis), respectively. Between replication and division, the cell goes through two gap phases, G1 and G2, to allow time for the synthetization of proteins needed in the S- and M-phase. Together the G1, S, and G2 phases form an interphase (Fig 2). The duration of cell cycle depends on the cell type and environmental conditions, but the cell cycle of a typical cultured human cell is approximately 24 h, of which the interphase consumes 23 h and the M phase 1 h.

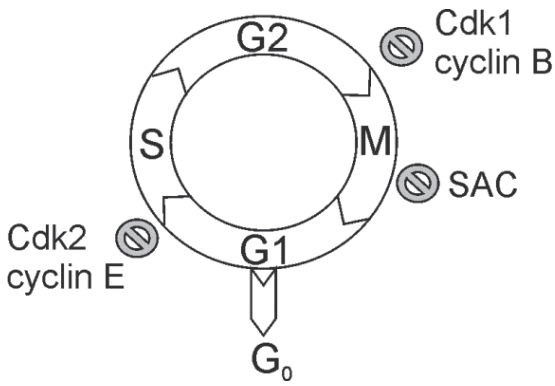

Figure 2. Illustration of cell cycle phases, major checkpoints, and principal regulators.

The cell cycle is carefully controlled by checkpoint switches that monitor both ongoing cell cycle processes and environmental conditions. The major biochemical controllers of cell cycle are the cyclin-dependent kinases (Cdk) and their counterpart cyclins. The levels of specific cyclins fluctuate according to the cell cycle phase and at their expression peak, they activate the corresponding Cdk and induce functional activation of the target proteins. Some of the cyclin-Cdk complexes are needed for progression through the G1 or S phase, but some also serve as checkpoint switches that trigger the next cycle phase (Fig 2). At the end of G1, the cell has to either start the next cycle or exit. This decision is dependent on extracellular conditions; if these are favorable, the cell proceeds through the replication checkpoint and enters a new S phase. If the environmental circumstances do not favor growth, the cell can go to a resting state called G zero (G0) and may even remain there until the end of its lifespan. In vertebrates, the S phase is biochemically triggered by the accumulation of cyclin E and consequent Cdk2 activation (Ohtsubo *et al*., 1995). After DNA replication, the cell enters the G2 phase, where the integrity of the newly synthesized DNA and the environmental conditions are checked before mitotic entry (the G2/M checkpoint). The G2/M transition is mainly controlled by the cyclin B-mediated activation of Cdk1, but also dephosphorylation of Cdk1 by cell division cycle 25 C (Cdc25C) is needed (Hoffmann *et al*., 1993). The third major cell cycle checkpoint, SAC, guards the chromosome segregation at the metaphase-to-anaphase transition, and its function is not based on Cdk activation, but rather the proteolytic degradation of regulatory proteins. SAC components and their function are discussed in more detail in Section 2.4.1.

2.2.1 The M-phase

The M-phase can be further divided into nuclear division (mitosis) and cytoplasmic division (cytokinesis). In this thesis, mitosis is defined as six sequential phases, also including the cytoplasmic division as illustrated in Figure 3. Mitosis starts in prophase with condensation of the replicated chromosomes. Simultaneously, the mitotic spindle begins (described in more detail in Chapter 2.3) to form in the cytoplasm where the two centrosomes nucleate the spindle microtubules. Nuclear envelope breakdown (NEBD) depicts the start of prometaphase and allows the spindle microtubules to access the chromosomes and arrange them to the division plane. In metaphase, all the chromosomes of the cell are attached to both spindle poles from their centromeric protein platforms, kinetochores, and are aligned at the metaphase plate. This attachment satisfies the SAC and allows anaphase onset, where the cohesion between sister chromatids is removed, and the chromatids are synchronously separated and pulled in opposite directions. Shortening of the kinetochore-bound microtubules is responsible for the initial separation of the sister chromatids in anaphase A. Then the chromatids are pulled farther away from each other in anaphase B, as motor proteins move the spindle poles apart, and the spindle elongates. The nuclear division is finalized in telophase, when the nuclear envelope starts to reform around the two sets of decondensed chromosomes, and the cytoplasmic division is also initiated. Finally, an actin-myosin contractile ring furrows the cytoplasm into two parts (cytokinesis). The daughter cells remain connected by a thin cytoplasmic bridge, called a midbody, until the abscission machinery finally pinches the two cells apart. After final separation, the daughter cells can enter a new cycle of duplication and division.

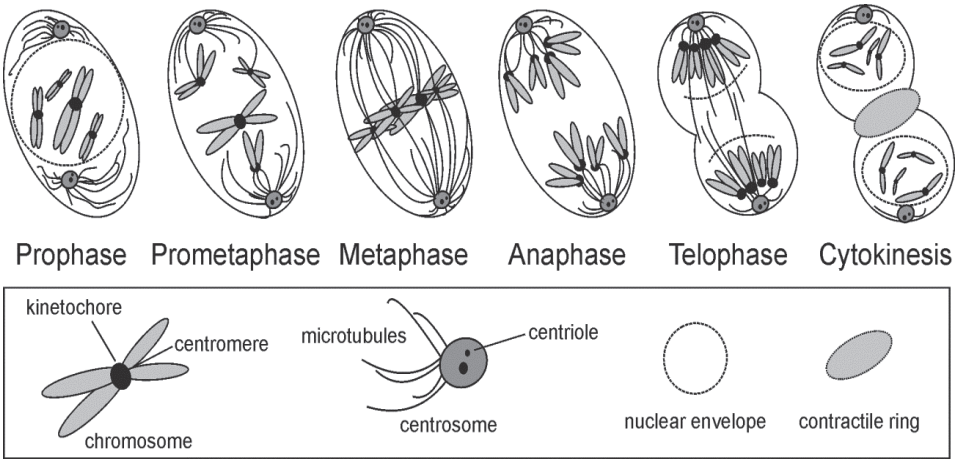

Figure 3. The phases of mitosis and the main structures involved in mitotic division.

2.3 The mitotic spindle controls and powers chromosome segregation

2.3.1 Centromere and kinetochore

The sister chromatid arms separate as the DNA condenses in prophase, and the chromatid pair is held together only from the middle via the centromeric regions. The centromeric heterochromatin serves as a platform for the multiprotein structure called kinetochore that connects the chromosomes to the spindle (Fig 3). Centromeres are characterized by a specific histone H3 variant, centromere protein A (CENP-A) (Sullivan *et al*., 1994; Warburton *et al*., 1997), which together with the chromosomal passenger complex (CPC, discussed in more detail in Chapter 2.4.1.3) forms the innermost level of the trilayered kinetochore (reviewed in (Cheeseman and Desai, 2008; Nagpal and Fukagawa, 2016)). CENP-A also associates with the constitutive centromere-associated network, which contains 16 centromere proteins and forms the middle layer of the kinetochore (Weir *et al*., 2016). The main component of the outer kinetochore is the evolutionary conserved KNM network, which consists of the Kinetochore scaffold 1 (Knl1), the Mis12 complex, and the Ndc80 complex (Cheeseman *et al*., 2006). The outmost layer is responsible for microtubule binding and thus defines the kinetochore activity, as discussed in detail in Section 2.3.3.

2.3.2 Microtubules

Microtubules are composed of heterodimeric tubulin subunits (α-tubulin + β-tubulin). The heterodimers join head-to-tail to form protofilaments, which are then bundled laterally to form a hollow cylinder. Microtubules are polar and dynamic structures; the more stable minus ends are buried in the microtubule organizing centres (MTOC, centrosome) with the more dynamic plus ends pointing away. The plus ends can bind kinetochores, stay free (interpolar microtubules) or interact with the cell cortex (astral microtubules) (see Fig 3). The plus ends change constantly between growth and shrinkage states, depending on whether the exposed β-tubulin is bound to the guanosine-5'-triphosphate (GTP) or –diphosphate, respectively. This phenomenon, known as dynamic instability, is one of the powering factors in chromosome movement during anaphase (Alushin *et al*., 2014; McIntosh *et al*., 2010; Mitchison and Kirschner, 1984).

2.3.3 Kinetochore-microtubule attachments

The current "search-and-capture" model for the formation of proper kinetochore-microtubule (kt-mt) attachments was introduced thirty years ago to explain mitotic spindle assembly. The model is based on dynamic microtubules "searching" for kinetochores and "capture" leading to microtubule stabilization and stable kt-mt attachment (Kirschner and Mitchison, 1986). Few refinements have been added to the model to explain the relatively high swiftness of this stochastic process. For example, kinetochore-driven microtubule nucleation and microtubule-associated motor proteins help, but are not essential, in spindle assembly (Maiato *et al*., 2004; Walczak *et al*., 1998). When a microtubule meets a kinetochore, the KNM network components, the Ndc80-complex and Kln1, both bind the microtubule. The KMN network facilitates the interaction, and the binding affinity is greatest when the network is complete (Cheeseman *et al*., 2006). Actually, human kinetochores form first a lateral connection to the microtubule, which is then converted to a stable end-on attachment. For example, centromere protein E (CENP-E) and mitotic centromere-associated kinesin (MCAK) mediate the lateral to end-on conversion (Shrestha and Draviam, 2013). An important regulator of kt-mt attachments is Aurora B, as Ndc80 phosphorylation by Aurora B decreases microtubule binding affinity, which is essential for the destabilization of incorrect attachments (Cheeseman *et al*., 2006). Indeed, many incorrect attachments form during the stochastic process of kinetochore capture by the microtubules, including syntelic and merotelic attachments wherein both sister kinetochores are attached to the same pole, or one kinetochore is attached to both poles, respectively. The SAC (discussed in Chapter 2.4.1) monitors the correctness of the kt-mt attachments, which prevents chromosome segregation until all chromosomes have established amphitelic, *i.e*. bipolar, attachments.

Besides forming proper attachments to microtubules, kinetochores also need to produce or convert the forces needed for chromosome segregation. Some kinetochore localized proteins, such as the mammalian microtubule-binding protein cytoplasmic linker protein (CLIP)-associating proteins (CLASP), can promote chromosome segregation via direct control of spindle microtubule dynamics (Pereira *et al*., 2006). Moreover, the intrinsic dynamic instability of microtubules can be harnessed for chromosome segregation if stable kt-mt attachments are simultaneously maintained. Attachment plasticity is created by a binding mode of numerous weak interactions, where several Ndc80 molecules in a kinetochore bind one microtubule (Dong *et al*., 2007; Joglekar *et al*., 2006). In yeast, the Dam1 protein connects the dynamic instability of microtubules to chromosome movement in anaphase. Dam1 forms a ring around the kinetochore-bound microtubule and couples the cargo (chromosome) to the depolymerizing microtubule. A vertebrate homolog of Dam1 was long searched for,

but now the Spindle And Kinetochore Associated Complex Subunit 1 (Ska1) has emerged as a potential candidate for this function (Schmidt *et al*., 2012; Welburn *et al*., 2009).

2.3.4 Centrosomes

Two centrosomes form the poles of the bipolar mitotic spindle; they nucleate and organize the microtubules and thus serve as MTOCs. Centrosomes are formed by a perpendicularly oriented centriole pair surrounded by a pericentriolar matrix (PCM), including hundreds of proteins (Fig 3). An important PCM component is γ-tubulin ring complex that is responsible for microtubule nucleation (Zheng *et al*., 1995). Both daughter cells inherit two centrioles in cell division, which then nucleate a daughter centriole in the S-phase. The two centriole pairs are disengaged upon mitotic entry, after which PCM expansion and centrosome maturation begins (reviewed in (Conduit *et al*., 2015)). This centriole cycle is linked to cell cycle progression and tightly controlled by, for example, Polo-like kinases (Habedanck *et al*., 2005; Haren *et al*., 2009). Interestingly, centrosomes are not essential for spindle assembly in vertebrates, but they do greatly enhance chromosome segregation fidelity (Khodjakov *et al*., 2000; Sir *et al*., 2013). However, in untransformed human cells centrosome loss has been suggested to eventually trigger a p53 dependent cell cycle arrest (Lambrus *et al*., 2015; Wong *et al*., 2015).

2.3.5 Motor proteins

Motor proteins couple adenosine triphosphate hydrolysis to conformational change and cargo movement; they are important for mitotic spindle assembly and chromosome segregation. Microtubule-bound motor proteins usually exhibit directional specificity: kinesins move towards the plus end and dyneins to the minus end. The most vital mitotic motor protein is kinesin-5 (also known as Eg5 and KIF11), which contains two motor domains that enable the binding of anti-parallel microtubules. Kinesin-5 mediated microtubule sliding in opposite directions pushes the poles apart, which is needed for bipolar spindle assembly, but may also contribute to chromosome segregation in anaphase B (Kapitein *et al*., 2005; Whitehead and Rattner, 1998). In the absence of kinesin-5, nuclear envelope-associated dyneins can pull the spindle poles apart via astral microtubules (Raaijmakers *et al*., 2012). Another class of kinesins, chromokinesins that are bound to chromosome arms, pushes the mitotic chromosomes away from the poles by walking toward the microtubule plus end (polar ejection force), thereby

contributing to both chromosome alignment and condensation (Antonio *et al*., 2000; Mazumdar *et al*., 2004).

2.4 Biochemical control of mitosis

2.4.1 Spindle Assembly Checkpoint (SAC)

2.4.1.1 Mitotic checkpoint complex (MCC) mediates SAC signaling

The evolutionary conserved mitotic checkpoint, SAC, inhibits premature sister chromatid separation in prometaphase by monitoring the kt-mt attachments (reviewed in (Foley and Kapoor, 2013; Musacchio, 2015)). MCC is the main effector of SAC signaling and is illustrated in Figure 4. The human MCC consists of the following proteins: mitotic arrest deficient 2 (Mad2), budding uninhibited by benzimidazole-related 1 (BubR1; *S. cerevisiae* Mad3), budding uninhibited by benzimidazole 3 (Bub3), and cell division cycle 20 (Cdc20); a co-activator of the Anaphase Promoting Complex/Cyclosome (APC/C) ubiquitin ligase (Sudakin *et al*., 2001). In late prophase, MCC binds to and inhibits the activity of APC/C-Cdc20, which is responsible for directing the anaphase inhibitor proteins securin and cyclin B1 for proteasome-mediated destruction (Alfieri *et al*., 2016; Hein and Nilsson, 2014; Izawa and Pines, 2014; Yamaguchi *et al*., 2016). Upon kinetochore attachment to microtubule(s) in metaphase, the SAC is turned off and active APC/C-Cdc20 degrades securin and cyclin B1. Securin degradation allows separase to cleave cohesin between sister chromatids, and cyclin B1 elimination promotes anaphase entry via Cdk1 inactivation as shown in Figure 4 (Foley and Kapoor, 2013; Musacchio, 2015).

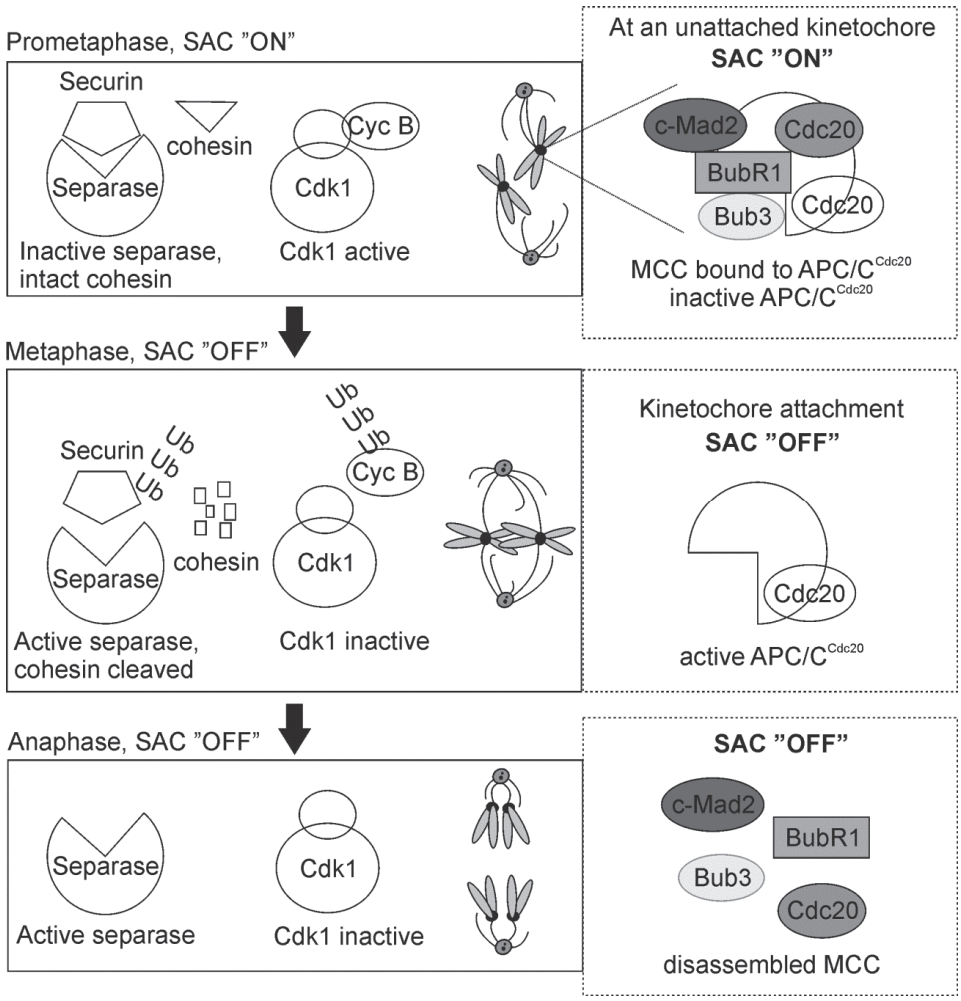

Figure 4. A schematic model of the SAC function during mitotic progression from prometaphase to anaphase. The boxes on the right depict the function/status of the MCC and its target, APC/C, in each phase. See text for more discussion. Modified from (Foley and Kapoor, 2013; Musacchio, 2015).

In the MCC, BubR1 is the eventual APC/C-Cdc20 inhibitor, but Mad2 is indispensable for the initial complex formation, which is depicted in Figure 5 (Alfieri *et al*., 2016; Han *et al*., 2013; Lara-Gonzalez *et al*., 2011; Yamaguchi *et al*., 2016). Briefly, Mad2 forms a heterodimer with mitotic arrest deficient 1 (Mad1) at the unattached kinetochores, which enables the conversion of cytosolic inactive open-Mad2 (o-Mad2) to active closed Mad2 (c-Mad2) (De Antoni *et al*., 2005). The active c-Mad2 binds to Cdc20 and primes it for BubR1 binding, which then restrains the ability APC/C-Cdc20 to bind its substrates (Fig 5) and arrests the cell in (pseudo)metaphase (Alfieri *et al*., 2016; Han *et al*., 2013; Yamaguchi *et al*.,

2016). The Mad2-template model allows the robust activation of the checkpoint. Besides Mad1, for example, Aurora B, multipolar spindle-1 (Mps1), and Knl1 contribute to MCC recruitment to unattached kinetochores (Foley and Kapoor, 2013; Musacchio, 2015).

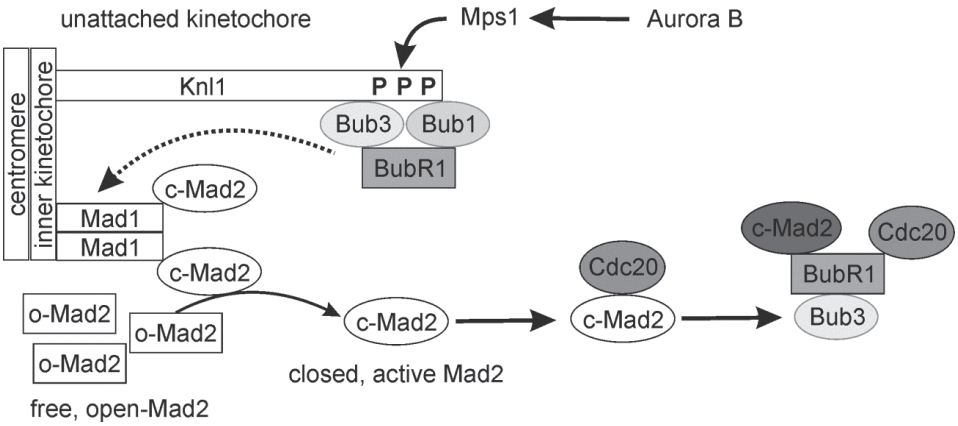

Figure 5. The Mad2 template model for MCC recruitment and assembly at the unattached kinetochores. See text for more discussion. Modified from (Foley and Kapoor, 2013; Musacchio, 2015).

2.4.1.2 Mad2

Mad2 is closely associated with this thesis effort and it is described below in more detail as an example of a core SAC protein.

Kinetochore localized Mad2 is essential for MCC formation and the generation of robust SAC signal. However, Mad2 also has a kinetochore-independent function: it controls mitotic timing, together with cytosolic BubR1, possibly via inhibiting the APC/C during early mitosis (Gorbsky *et al*., 1998; Meraldi *et al*., 2004). In addition to the kinetochore-bound and cytosolic pools, Mad2 also localizes to the nuclear pore complexes (NPC) in the nuclear membrane of interphase cells. Mad1-Mad2 complexes at the NPCs drive the interphase MCC assembly, allowing rapid MCC formation and APC/C inhibition at unattached kinetochores in prometaphase. (Rodriguez-Bravo *et al*., 2014; Sudakin *et al*., 2001)

Accordingly, in cell culture and mouse models, partial or complete loss of Mad2 causes a premature exit from mitosis, accompanied by the uncoordinated separation of sister chromatids and the induction of aneuploidy (Michel *et al*., 2004, 2001). In response to the severe chromosome segregation defects, depletion of *MAD2L1* (hereafter *MAD2*) triggers cell death and senescence in cultured human

cells (Lentini *et al*., 2012; Michel *et al*., 2004; Yun *et al*., 2009), and *Mad2* null mice die at embryonic age E6.5 (Dobles *et al*., 2000). On the other hand, Mad2 overexpressing cells also frequently exhibit lagging chromosomes and chromatin bridges. The chromosome mis-segregation is not applicable to the SAC defect, but rather to the hyperstabilization of kt-mt attachments and a lack of Aurora B-mediated error correction (Kabeche and Compton, 2012; Sotillo *et al*., 2007).

As balanced Mad2 expression is indispensable for the fidelity of chromosome segregation and the maintenance of genomic stability, it needs be tightly controlled. E2F transcription factors have been demonstrated to bind and promote the expression of *MAD2*, but the activity of E2Fs is normally restricted by retinoblastoma protein until the S phase (Hernando *et al*., 2004; Schvartzman *et al*., 2011). c-Myc (Menssen *et al*., 2007) and BRCA1 (Wang *et al*., 2004) can also activate *MAD2* transcription via binding to the gene promoter. Furthermore, *MAD2* transcription is controlled by the β-TRCP-REST –axis: degradation of the repressor-element-1-silencing transcription factor (REST) by the β-TRCP ubiquitin ligase licenses *MAD2* expression in the G2 cell cycle phase (Guardavaccaro *et al*., 2008). Post-transcriptional Mad2 regulation involves the miRNAs miR-433 and miR-28-5p, which suppress Mad2 expression via direct binding to the *MAD2* mRNA (Furlong *et al*., 2012; Hell *et al*., 2014). Moreover, an association with the NPC protein Trp stabilizes the Mad2 protein possibly via proteolysis inhibition (Schweizer *et al*., 2013).

2.4.1.3 Aurora B kinase and the Chromosomal Passenger Complex (CPC)

While the MCC stops mitotic progression in the presence of erroneous kt-mt attachments, the evolutionary conserved CPC is crucial for correcting the abnormal attachments. CPC consists of Aurora B, INCENP, surviving, and borealin and is named for its dynamic localization during mitosis: CPC resides at the inner centromere in prometaphase and metaphase, at the spindle midzone in anaphase, and at the midbody in telophase. Aurora B is the catalytic component of the complex, while INCEP, survivin, and borealin are responsible for proper targeting and activation of the complex (Cooke *et al*., 1987; Jeyaprakash *et al*., 2007; Kaitna *et al*., 2000). During chromosome alignment, Aurora B kinase phosphorylates the outer kinetochore protein Ndc80, weakening its microtubule binding affinity (Cheeseman *et al*., 2006) and allowing the uncoupling of faulty attachments. Accordingly, the chemical inhibition of Aurora B increases the rate of syntelic attachments, when the error correction is flawed (Hauf *et al*., 2003). Aurora B may also directly promote destabilization of the improperly attached microtubules by regulating the microtubule depolymerizing activity of the MCAK kinesin (Lan *et al*.,

2004). Moreover, phosphorylation of the Ska complex by Aurora B disturbs the complex's interaction with the KNM network, thereby inhibiting the formation of stable end-on kt-mt attachments (Chan *et al*., 2012).

In addition to controlling kt-mt attachment stability, CPC and especially Aurora B promote recruitment of MCC to unattached kinetochores via the upstream kinase Mps1 (see Fig 5, (Ditchfield *et al*., 2003; Saurin *et al*., 2011). Indeed, Aurora B activity is needed to arrest cells in mitosis in response to MTAs (Ditchfield *et al*., 2003; Hauf *et al*., 2003). In line with its function in the SAC, the centromeric levels of Aurora B are higher at unattached kinetochores (Salimian *et al*., 2011) where the kinase is also spatially nearer to its outer kinetochore substrates due to the non-stretched state of the unattached kinetochore (Liu *et al*., 2009).

2.4.1.4 SAC activation and inactivation

Early studies ultimately linked SAC activity to unattached kinetochores: one unattached kinetochore is enough to arrest a cell in mitosis (Rieder *et al*., 1995), and as discussed above, MCC proteins localize dynamically to the unattached kinetochores (Li and Benezra, 1996). However, whether the checkpoint signaling is mediated by microtubule attachment to kinetochore, stretch at kinetochore created by bilateral attachment, or both, has remained obscure (Foley and Kapoor, 2013). Recently, by expressing a non-phosphorylatable variant of Ndc80, thereby allowing stable kt-mt attachments without bi-orientation, two groups have independently demonstrated that stable end-on kt-mt attachments are enough to satisfy the SAC and allow chromosome segregation. Thus, according to the current view, SAC satisfaction is neither dependent on intra-kinetochore tension nor the architectural changes in the kinetochore, which are induced by microtubule binding (Etemad *et al*., 2015; Tauchman *et al*., 2015).

As anaphase ensues rapidly after the last chromosome is correctly attached and moved to the spindle equator, the SAC needs to be promptly silenced. To allow swift checkpoint silencing, MCC is continuously assembled and disassembled (Uzunova *et al*., 2012). In addition, several mechanisms have been demonstrated to stimulate the complex dismantlement or contribute to the removal of MCC proteins from the kinetochores. For example, the microtubule-associated motor protein dynein can transfer SAC proteins from the kinetochores to the centrosomes along microtubules (Howell *et al*., 2001), and a Mad2-antagonising protein, $p31^{comet}$, can promote MCC disassembly (Miniowitz-Shemtov *et al*., 2015; Westhorpe *et al*., 2011). Moreover, when the APC/C bound MCC obtains a certain conformation, the MCC component Cdc20 is ubiquitinylated by APC/C that advances MCC dismantling (Alfieri *et al*., 2016; Yamaguchi *et al*., 2016). Further,

changes in the spatial kinetochore organization upon microtubule binding may promote removal of the SAC proteins; although that is not essential for checkpoint inactivation *per se* (Aravamudhan *et al*., 2015; Tauchman *et al*., 2015). In addition to the physical removal of SAC proteins from the kinetochores, the checkpoint-associated protein phosphorylation also needs to be reversed by, for example, protein-phosphatase 1 (PP1) and 2 (PP2A) (Nijenhuis *et al*., 2014).

2.4.2 Cytokinesis and abscission machinery

Cytokinesis is the cytoplasmic division that follows chromosome segregation (see Fig 3). Cytokinesis is driven by an actin-myosin- based contractile ring that forms in the spindle midzone where the anti-parallel microtubules overlap with each other (central spindle). Upon contraction of the ring, a cleavage furrow forms, and the equatorial regions of the plasma membrane are pulled toward the midzone. When the cell is almost pinched in two, the daughter cells still remain connected for a while through the bundle of anti-parallel microtubules, now called midbody. Finally abscission cleaves this connection, thereby liberating two identical daughter cells (reviewed in (Fededa and Gerlich, 2012)).

The declining Cdk1 activity in anaphase allows the recruitment of protein regulator of cytokinesis 1, centralspindlin complex, and CPC to the spindle midzone, all of which are essential for stabilization of the anti-parallel microtubules and formation of the central spindle (Hümmer and Mayer, 2009; Landino and Ohi, 2016; Mishima *et al*., 2004; Zhu *et al*., 2006). The connection to Cdk1 inactivation also ensures that cytokinesis is not initiated before chromosome segregation (Mishima *et al*., 2004). The central spindle controls accumulation and activation of RhoA GTPase in the equatorial cell cortex (Yüce *et al*., 2005). RhoA activation then triggers the formation of the contractile ring, but centralspindlin and CPC also promote the ring assembly (Fededa and Gerlich, 2012; Lewellyn *et al*., 2011). How exactly the contractile ring exerts the forces needed for cleavage furrow formation remains unclear.

The contractile ring cleaves the cytoplasm nearly in two, and the remaining microtubule bridge is dissolved by abscission. In mammals the bridge (midbody) accumulates more than a hundred proteins; most of which are related to vesicle trafficking and recycling endosomes, such as Rab11, the Exocyst complex, and SNARE proteins (Gromley *et al*., 2005; Skop *et al*., 2004). These proteins mediate the narrowing of the intracellular bridge (Schiel *et al*., 2012), but the final abscission takes place on both sides of the midbody and is driven by the endosomal sorting complex required for transport (ESCRT)-III (Guizetti *et al*., 2011). The ESCRT-III localizes to the abscission sites through a complex signaling cascade

that includes centrosomal protein 55 (CEP55), apoptosis-linked gene 2–interacting protein X (ALIX) , and tumor susceptibility gene -101 (Tsg101) (Fededa and Gerlich, 2012). The preceding bridge narrowing is needed for correct ESCRT-III localization (Schiel *et al*., 2012), and these two processes also share upstream regulatory factors, such as Syntaxin16 (STX16) (Neto *et al*., 2013). To avoid chromosome damage, abscission is inhibited until all chromatin is cleared away from the cleavage site, also known as the NoCut checkpoint. The NoCut signal is mediated by Aurora B, which activates an inhibitory ESCRT-III subunit to delay abscission (Carlton *et al*., 2012; Mendoza *et al*., 2009).

2.4.3 Rassf1

The dominant isoform of Ras Association Domain Family Member 1 (*RASSF1*), Rassf1a, has several mitotic functions: it binds and stabilizes microtubules (Liu *et al*., 2003; van der Weyden *et al*., 2005), regulates cytokinesis (Guo *et al*., 2007; Song *et al*., 2009a; Tommasi *et al*., 2011), and potentially inhibits APC/C activity in early prometaphase (Liu *et al*., 2007; Song *et al*., 2004). Accordingly, Rassf1a localization in mitosis changes from microtubules and centrosomes to midzone in telophase (Guo *et al*., 2007; Song *et al*., 2004), where it recruits abscission machinery components. Rassf1a transition to midzone is initiated by Aurora B phosphorylation (Song *et al*., 2009a). Rassf1a is also phosphorylated by Aurora A kinase in mitosis, which regulates the microtubule binding capacity of Rassf1a (Rong *et al*., 2007). Depletion, suppression, or mislocalization of Rassf1a causes a myriad of mitotic defects: multipolar spindles, anaphase lagging chromosomes, declined centrosome integrity, and cytokinesis failure. However, the effect of Rassf1a on mitotic timing has remained controversial (Dallol *et al*., 2007; Guo *et al*., 2007; Song *et al*., 2004, 2005; Tommasi *et al*., 2011). Despite the mitotic defects, *Rassf1a* suppression alone is not enough to generate aneuploidy *in vivo*, but concomitant genomic lesions, such as loss of p53, are also required (Tommasi *et al*., 2011; van der Weyden *et al*., 2005).

Rassf1a is a known tumor suppressor protein that is lost in different types of cancers, mostly due to hypermethylation of the gene promoter (reviewed in (Donninger *et al*., 2007)). Besides epigenetic silencing, genetic lesions, such as allelic deletions (Chen *et al*., 1994; Hogg *et al*., 2002; Ito *et al*., 2005; Wistuba *et al*., 1997) and inactivating mutations (Kashuba *et al*., 2009), also contribute to Rassf1a loss in cancer. As a proof of the tumor suppressor concept, *Rassf1a* knockout mice are more susceptible to tumor development; both spontaneously and in response to carcinogen induction (Tommasi *et al*., 2005, 2011; van der Weyden *et al*., 2005). Rassf1a has been suggested to restrict cell proliferation and malignant

growth via the inhibition of cyclin D1 accumulation and G1/S transition (Shivakumar *et al*., 2002) although the final impact of *RASSF1A* depletion on cell growth may depend on the other genetic alterations that are present (Whitehurst *et al*., 2008). Even though Rassf1a also promotes apoptosis (Baksh *et al*., 2005; Matallanas *et al*., 2007), inhibition of cell cycle progression is essential for its growth suppressive function. Notably, the Rassf1a microtubule binding domain seems to be responsible for the cell cycle obstruction, independent of cyclin D1 regulation (Donninger *et al*., 2014).

2.5 Mitotic defects and cancer – implications in tumorigenesis and therapy

2.5.1 SAC defects

Defects in the mitotic checkpoint allow the segregation of misaligned chromosomes, which may promote chromosomal instability (CIN), aneuploidy, and tumorigenesis. Several animal and cell models' expressing abnormal levels of individual SAC proteins have been created to test this hypothesis. Studies on these models have validated the concept and demonstrated how important it is for cells and organisms to maintain normal levels of SAC proteins.

For example, complete loss of Mad2 triggers cell death in cultured human cancer cells and is embryonically lethal in mice, because of severe chromosome mis-segregation (Dobles *et al*., 2000; Michel *et al*., 2004). To the contrary, *Mad2* haploinsufficiency causes moderate aneuploidy that promotes tumorigenesis (Michel *et al*., 2001). Interestingly, overexpression of Mad2 also causes aneuploidy, which was found to drive cancer formation and tumor relapse in mice (Rowald *et al*., 2016; Schvartzman *et al*., 2011; Sotillo *et al*., 2007, 2010). Similar observations have been derived from other SAC mouse models, *e.g.*, mice that exhibited either partial depletion of *BubR1* (Rao *et al*., 2005) or *Cenp-E* (Weaver *et al*., 2007), excess of *Ndc80* (Diaz-Rodriguez *et al*., 2008), or mutated *Mps1* (Foijer *et al*., 2014).

Importantly, the altered expression of checkpoint components has been demonstrated in different types of human tumors (Furlong *et al*., 2012; Grabsch *et al*., 2003; Hernando *et al*., 2004; Liang *et al*., 2014; Meng *et al*., 2015). Moreover, overexpression of Ndc80 (Meng *et al*., 2015) and both overexpression and lowered levels of Mad2 have been linked to poor patient prognosis (Furlong *et al*., 2012; Hernando *et al*., 2004). However, mutations in checkpoint genes, including *MAD2*, *BUB1*, *BUBR1*, and *BUB3* are rare (Gemma *et al*., 2001; Hernando *et al*., 2001;

Ruddy *et al*., 2008; Shichiri *et al*., 2002), suggesting that transcriptional and post-transcriptional mechanisms are mainly responsible for checkpoint protein alterations in tumors. The relevance of altered SAC protein levels and a consequent increase in chromosomal instability from the perspective of malignant cell growth are discussed in more detail in Chapter 2.5.3.

2.5.2 Centrosome defects and multipolarity

An abnormal centrosome number was linked to a high rate of whole chromosome mis-segregation, also known as CIN, in the early 20th century by Theodor Boveri. Since, corroborating evidence has emerged, *i.e.*, centrosome defects correlate with CIN in human tumors (Denu *et al*., 2016; Lingle *et al*., 2002; Pihan *et al*., 2003) and proteins' governing the centrosome cycle, such as Aurora A and Polo-like kinase 4 (Plk4), are overexpressed in many cancerous lesions and correlate with poor patient prognosis (Landen *et al*., 2007; Marina and Saavedra, 2014).

Still, whether extra centrosomes are a cause or a consequence of tumorigenesis has remained under debate. Cells with excess centrosomes usually arrest in the cell cycle or die (Ganem *et al*., 2014; Holland *et al*., 2012). However, most human cells can cluster the extra centrosomes into two MTOCs and assemble a (pseudo)bipolar spindle. A kinesin motor protein, HSET, is the major driver of centrosome clustering (Chavali *et al*., 2016; Kwon *et al*., 2008), but also functional SAC, spindle tension, and CPC contribute to the coalescence (Leber *et al*., 2010). Despite the capability of a cell with clustered extra centrosomes to divide in a bipolar manner, the presence of extra foci often causes the generation of merotelic attachments that can induce chromosome segregation errors. This ambiguous process provides a mechanistic explanation for how centrosome defects may promote CIN and cancer formation (Ganem *et al*., 2009; Silkworth *et al*., 2009). Indeed, recent evidence from *Plk4* overexpressing mice demonstrates a causal relationship between extra centrosomes, aneuploidy, and tumorigenesis, even in the presence of an intact p53 (Levine *et al*., 2017; Serçin *et al*., 2015).

As centrosomes are duplicated in each cell cycle, defects in this process are an apparent reason for an abnormal centrosome number. As mentioned above, overexpression or abnormal activity of Plk4 leads to overduplication of the centrosomes (Habedanck *et al*., 2005; Holland *et al*., 2012; Levine *et al*., 2017; Serçin *et al*., 2015). Moreover, a G2 arrest can induce a Polo-like kinase 1 (Plk1)-mediated re-duplication of centriole (Loncarek *et al*., 2010). At the end of mitosis, both daughter cells should inherit one centrosome, and thus, tetraploidy-inducing

conditions (cytokinesis failure, mitotic slippage, cell fusion, endoreduplication) can also lead to centrosome amplification. Another reason for formation of extra poles is the fragmentation of the PCM; for example, CLASP proteins and ninein are needed to protect the centrosome from forces exerted by misaligned chromosomes that can cause PCM scattering (Logarinho *et al*., 2012).

2.5.3 Aneuploidy – a double-edged sword

Whereas CIN refers to the rate of whole chromosome mis-segregation, numerical aneuploidy is defined as a state of an abnormal chromosome number that deviates from the modal and is not a multiple of the haploid chromosome set (polyploidy). Apart from meiotic non-disjunction and genomic amplification of individual chromosomes, aneuploidy is typically caused by whole chromosome mis-segregation in mitosis. As illustrated in Figure 6, discussed above, in Chapter 2.4.1.2, and 2.4.1.3, defects in the mitotic checkpoint (A), faulty correction of erroneous kt-mt attachments (D), and an abnormal centrosome number (C) are prevalent causes of aneuploidy. Also, problems with sister chromatid cohesion may promote mis-segregation (B) (see Fig 6).

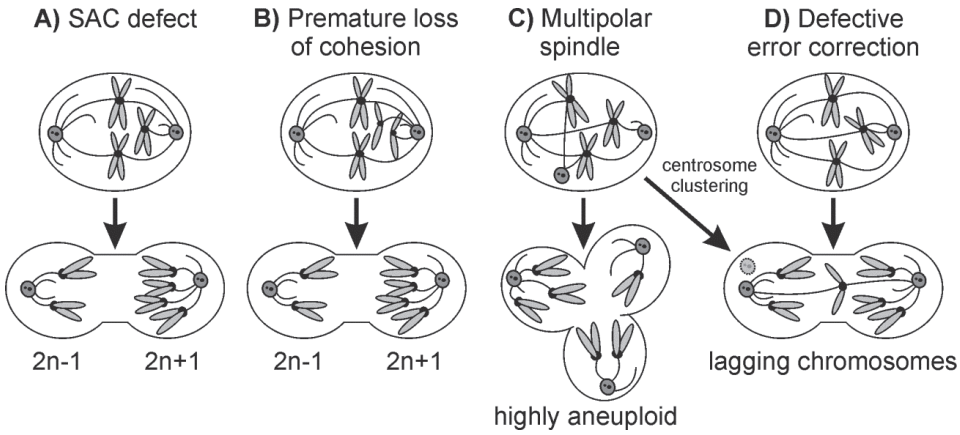

Figure 6. Mitotic errors that can promote aneuploidy. Multipolar spindles can be shifted to (pseudo)bipolar via centrosome clustering, but this process often still results in lagging chromatids. Modified from (Holland and Cleveland, 2009).

Although aneuploidy is a hallmark of cancer, the causal relationship of aneuploidy and cancer has remained unclear. Aneuploid mouse models, exhibiting *e.g.*, an altered expression of mitotic proteins, have displayed variable effects on tumorigenesis: suppression of tumor growth (Zasadil *et al*., 2016), no promotion of spontaneous or carcinogen-induced tumorigenesis (Ricke *et al*., 2012), and modest

(Michel *et al*., 2001) or prominent (Levine *et al*., 2017; Serçin *et al*., 2015; Sotillo *et al*., 2007) induction of tumorigenesis. Cleveland and colleagues have demonstrated in cultured human cancer cells (Kops *et al*., 2004) and with a mouse model (Weaver *et al*., 2007) that the degree of genomic imbalance may determine whether cell proliferation is enhanced or hindered in response to aneuploidy. For example, *Cenp-E* heterozygous mice exhibit aneuploidy and develop tumors spontaneously. However, under conditions of pre-existing low level aneuploidy, *Cenp-E* depletion has a tumor suppressive effect likely due to the massive genomic instability that is induced. These findings have led to a hypothesis according to which mild aneuploidy can offer a growth advantage and promote cancer cell growth, whereas a high rate of aneuploidy is intolerable even for cancer cells (Weaver *et al*., 2007).

How does aneuploidy then affect cell growth both negatively and positively? In an aneuploid mouse model, the size of the extra chromosomes correlated with the severity of the proliferation defect (Williams *et al*., 2008). This suggests that the extra chromosomes and their genes disrupt the transcriptome and proteome homeostasis and cause a stress that the cell has to cope with. Indeed, recent studies have demonstrated that marked changes exist in the stress-related metabolism and autophagy signaling pathways in aneuploid human cells, concomitantly with a proliferation defect (Ohashi *et al*., 2015; Santaguida and Amon, 2015; Stingele *et al*., 2012). On the other hand, aneuploidy further promotes chromosome mis-segregation, at least partially due to replication stress created by the extra chromosomes (Passerini *et al*., 2016; Sheltzer *et al*., 2011). These additional genomic and karyotypic alterations may promote cell transformation and provide evolutionary adaptability along with a growth advantage for the cancerous cells (Sheltzer *et al*., 2017).

The dual role of aneuploidy in terms of tumorigenesis and the high incidence of aneuploidy in human neoplasms provide a therapeutic opportunity for cancer treatment. Different approaches have proven that elevating the rate of chromosome mis-segregation (CIN) from modest to high, is an efficient mean to halt cancer cell proliferation and tumor growth (Janssen *et al*., 2009; Kops *et al*., 2004; Silk *et al*., 2013; Weaver *et al*., 2007; Zasadil *et al*., 2016). For example, in human cells or mice, simultaneous reduction of two proteins that are important for chromosome alignment and SAC function (*e.g.* Mps1, BubR1, CENP-E, Mad2), increases chromosome mis-segregation rate and cell death, and suppresses tumorigenesis when compared to models that are heterozygous for one of the proteins (Janssen et al., 2009; Silk et al., 2013). Similar results were obtained with a mouse model where initial CIN and tumorigenesis is induced by adenomatous polyposis coli (*Apc*) mutation; partial *Cenp-E* depletion in these mice inhibited tumor progression by increasing CIN and cell death (Zasadil et al., 2016). However, the risks of increasing

CIN by a particular therapy in cancer patients need to be carefully considered to avoid development of secondary tumors (Zasadil *et al*., 2016). Interestingly, low and clinically relevant concentrations of paclitaxel that induce chromosome segregation defects kill cancer cells more effectively when the cells already harbor numerical chromosome changes (aneuploidy) (Janssen *et al*., 2009). Thus, exploring the genomic status of the tumors may help to stratify those patients that would have the best benefit-to-risk ratio for genomic instability -inducing cancer therapeutics.

2.6 Mitosis, miRNAs and cancer – therapeutic and diagnostic opportunities

2.6.1 Mitosis as a therapeutic target

Drugs that target the cell cycle or cell division processes comprise a large group of the cancer therapeutics. MTAs are classical anti-mitotic drugs that have been used in the clinics for decades. Clinical MTAs can be roughly divided according to their mechanism of action into microtubule stabilizing (taxanes, epothilones) and depolymerizing (vinca alkaloids) agents. The vinca alkaloids are mainly used to treat hematological cancers, whereas taxanes and epothilones are part of the standard treatments for breast, ovarian, and prostate cancers as well as non-small-cell lung cancer and Kaposi's sarcoma (reviewed in (Jordan and Wilson, 2004)). When applied to cultured human cancer cells, these drugs induce a SAC-mediated, prolonged mitotic arrest followed by cell death (Jordan *et al*., 1991, 1996; Yvon *et al*., 1999). Based on cell culture studies, the induction of mitotic arrest is considered to be the mechanism of action also *in vivo*. However, already 20 years ago few studies suggested that the efficacy of MTAs, especially at low concentrations, might at least partially depend on their ability to increase aneuploidy (Ikui *et al*., 2005; Paoletti *et al*., 1997). This alternative hypothesis gained supportive evidence a few years ago, as Weaver and colleagues demonstrated that the intratumoral concentrations of paclitaxel in breast cancer patients do not cause mitotic arrest, but multipolar spindles and chromosome mis-segregation, the latter being essential for drug efficacy (Zasadil *et al*., 2014). Also, changes in the balance of pro- and anti-apoptosis proteins during MTA-induced prolonged mitosis have been suggested to play a role in drug-mediated cell death (Minn *et al*., 1996; Shi *et al*., 2008). Further studies in different cancer types are still needed to better elucidate the *in vivo* mechanism of action of MTAs.

Unfortunately, MTAs perturb microtubule functions in non-malignant cells as well, which causes severe side effects for the patients, such as neuropathy and neutropenia (Gidding *et al*., 1999; Markman, 2003). This issue has stimulated the development of mitosis specific therapeutics that would have better selectivity for cancer cells. Part of this concept was based on the findings from SAC-activating and mitotic arrest causing MTAs. For example, the first Plk1 inhibitor, BI2536, induced a mitotic arrest followed by cell death in cultured cancer cells and inhibited tumor growth in a xenograft model (Steegmaier *et al*., 2007). Also, inhibitors of kinesin motor proteins, such as Cenp-E and Eg5, caused mitotic arrest by preventing the detachment of kinesin from microtubules, resulting in chromosome alignment problems and a centrosome separation defect, respectively (Lad *et al*., 2008; Wood *et al*., 2010).

Another rationale in the development of the next generation anti-mitotic drugs has been the concept of increasing the frequency of aneuploidy beyond the toleration of cancer cells. A plethora of compounds have been developed to target the mitotic Aurora kinases, A and B. Inhibition of Aurora A induces aneuploidy, as the cells divide after transient mitotic arrest despite the presence of spindle defects (Hoar *et al*., 2007; Kaestner *et al*., 2009). In addition to erroneous chromosome segregation, the inhibition of Aurora B also abolishes cytokinesis resulting in severe polyploidization (Ditchfield *et al*., 2003; Hauf *et al*., 2003; Kaestner *et al*., 2009). Other interesting drug targets with similar expected outcome include, for example, Ndc80 (Wu *et al*., 2008) and Mps1 kinase (Schmidt *et al*., 2005; Tardif *et al*., 2011). Inhibition of mitotic exit is a third rationale applied to the development of new anti-mitotic drugs. Depletion or inhibition of Cdc20 induces a robust mitotic arrest followed by cell death, as mitotic exit is completely inhibited (Huang *et al*., 2009; Manchado *et al*., 2010; Zeng *et al*., 2010). This strategy also has efficiently restrained tumor formation in a mouse model (Manchado *et al*., 2010).

2.6.1.1 Anti-mitotic cancer therapeutics in the clinics

Unfortunately, most of the next generation anti-mitotic drugs tested in clinical trials thus far have shown modest potency and persisting adverse effects, mainly myelosuppression (Boss *et al*., 2011; Goldberg *et al*., 2014; Yim, 2013). To the contrary, the traditional MTAs have proven reasonably effective in clinical use: for example patients with metastatic breast cancer that received paclitaxel as first-line chemotherapy demonstrated response rates ranging from 25 % to over 50 % (Holmes *et al*., 1991; Nabholtz *et al*., 1996), and relapsed childhood leukemia cases have had a 57 % remission rate in response to the vinca alkaloids

vincristine and vindesine (Anderson *et al*., 1981). Thus, MTAs are still today used as a part of the standard (neo)adjuvant chemotherapy for triple-negative breast cancer (Frasci *et al*., 2009) and epithelial ovarian carcinoma (McGuire *et al*., 1996; Vasey *et al*., 2004), for example. However, many traditional MTAs are substrates for the drug efflux pump proteins, such as the P-glycoprotein, which can confer resistance to therapy in the clinics (Jordan and Wilson, 2004). A new type of MTAs that are not affected by the efflux pumps, epothilones, have been developed to conquer this resistance mechanism (Rothermel *et al*., 2003). Also, expression of specific tubulin isotypes, such as βIII-tubulin, can confer a resistance to taxanes (Verdier-Pinard *et al*., 2003). More information is needed about the mechanism of action of MTAs in the clinics, as it is the key to battling the resistance problems and enhancing the efficacy of MTA therapy.

2.6.1.2 Competition between cell death and slippage promoting pathways dictates the response to anti-mitotic cancer therapeutics

The efficacy of anti-mitotic drugs is essentially determined by the fate of the treated cells. A prolonged mitotic arrest can result in either death from the arrest, abnormal division leading to aneuploidy, or exit from mitosis without division, also known as slippage. The latter alternative constitutes a potential hazard, as the slipped cells, with 4n genomic content, can continue cycling through endoreduplication (see Fig 7A). Preferably, these cells either undergo post-mitotic death or cell cycle arrest (senescence) (Brito and Rieder, 2009; Gascoigne and Taylor, 2008). Interestingly, a recent study has shown that the response of individual cells from an established cell line to anti-mitotic drugs varies notably. For example, a HeLa cell population exhibited 7 different fates in response to paclitaxel treatment (Gascoigne and Taylor, 2008).

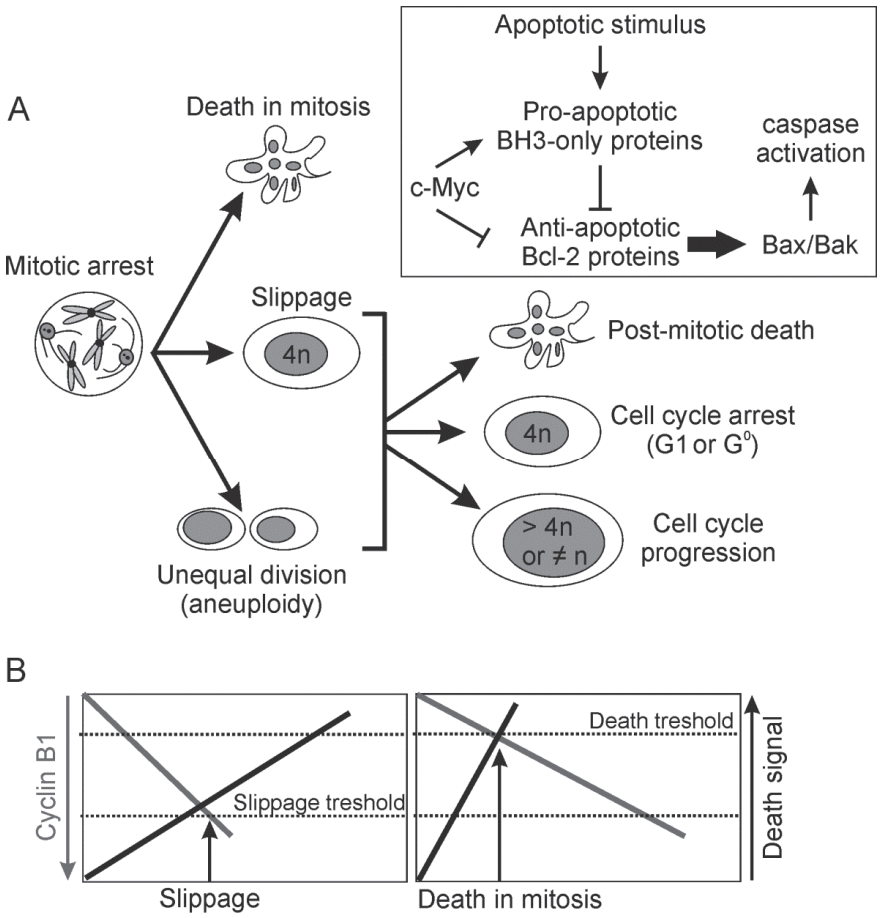

Figure 7. (A). Possible cell fates in response to drug-induced mitotic arrest and a simplified illustration of the intrinsic apoptosis pathway, which mediates mitotic and post-mitotic death, presented in a separate box. (B). The competing networks model. Panel A modified from (Gascoigne and Taylor, 2009; Topham *et al*., 2015; Youle and Strasser, 2008) and panel B from (Gascoigne and Taylor, 2008).

Gasgoine and Taylor have proposed a model of two independent, but competing, networks to explain the cell fate determination during prolonged mitotic arrest, presented in Figure 7B. This model is based on the notion that even in the presence of active SAC, cyclin B1 is gradually degraded by the proteasome, and a certain threshold exists to trigger an exit from mitosis (Brito and Rieder, 2006). Simultaneously, cell death signaling pathways are activated. Accordingly, the fate of the cells depends on whether the cyclin B1 levels fall below the exit threshold or do the death promoting signals first exceed a level that triggers apoptosis. Intra- and interline variation in cell fates may be explained by genomic diversity that modulates the thresholds and individual pathway activities (Gascoigne and Taylor, 2008; Kueh *et al*., 2016).

Owing to extensive research, the mitotic exit network is well established. Much less is known about the cell death signaling during prolonged mitosis. The caspase-mediated intrinsic apoptosis pathway is the best candidate for mitotic cell death mediation (Allan and Clarke, 2007; Brito and Rieder, 2009; Gascoigne and Taylor, 2008). As depicted in the box in Figure 7A, upon apoptotic stimuli, the pro-apoptotic BH3-only proteins, such as Bid, Bim, Puma and Noxa, neutralize the anti-apoptotic Bcl-2 family members, for example, B-Cell CLL/Lymphoma 2 (Bcl-2), BCL2 Like 1 (Bcl-xL), and Myeloid Cell Leukemia 1 (Mcl-1). Inactivation of these anti-apoptotic factors allows the pro-apoptotic Bak and Bax to create pores in the mitochondrial membrane and activate the caspases (reviewed in (Youle and Strasser, 2008)). Interestingly, promotion of the death pathway using BH3-mimetics, which inhibit the anti-apoptotic Bcl-2 family proteins, can increase the frequency of cell death upon treatment with anti-mitotic agents. For example, the Bcl-2 family inhibitors, navitoclax (ABT-263) and ABT-737, enhance the efficacy of the taxane and kinesin-5 inhibitor treatments in different preclinical cancer models (Oakes *et al*., 2012; Shi *et al*., 2011; Tan *et al*., 2011; Wali *et al*., 2017; Wang *et al*., 2015). In addition, the oncogene c-Myc was recently found to be a major regulator of the intrinsic apoptosis pathway (Fig 7A box) and thus govern not only mitotic cell fate, but also post-mitotic death signaling (Topham *et al*., 2015). Furthermore, Cdk1 has been implicated as a modulator of cell fate since it phosphorylates Bcl-2 proteins and caspases; on the other hand it is itself regulated by cyclin B1, thereby possibly connecting the two competing pathways (Allan and Clarke, 2007; Terrano *et al*., 2010). Recently, miRNAs were also suggested playing a role in MTA-resistance via regulating SAC proteins and the components of the intrinsic apoptosis pathway, for example, or through their other cancer-related target genes (reviewed in (Cui *et al*., 2013)).

2.6.2 Genomic and transcriptional alterations cause abnormal miRNA expression in cancer

The first evidence for the involvement of miRNAs in cancer signaling came from Calin and colleagues who demonstrated that the genomic locus harboring *MIR15A* and *MIR16-1* genes was deleted in more than half of the chronic lymphocytic leukemia cases (Calin *et al*., 2002). Loss of the *MIR15/16* locus can drive leukemia formation in mice, probably due to an altered expression of cell cycle regulating proteins (Klein *et al*., 2010). Later, another group showed that miRNA expression profiles vary between healthy and malignant tissues in different types of human cancers (Lu *et al*., 2005).

Following this first report on loss of miR-15/16 in cancer due to genomic deletion, many human miRNA genes were found to be located in fragile sites of the genome and thus are prone for deletions, amplifications and translocations (Calin *et al*., 2004). Genomic alterations are one reason for changed miRNA expression in cancer, but changes in the epigenetic status of a miRNA gene locus may also contribute (Brueckner *et al*., 2007; Lujambio *et al*., 2008). Moreover, many miRNA transcription factors, such as p53 and c-Myc, are dysregulated in cancer and may drive abnormal miRNA expression (Chang *et al*., 2008; Tarasov *et al*., 2007). Mutations in protein coding genes are frequent in cancer, and single-nucleotide polymorphisms in the miRNA binding sites can inhibit miRNA-mediated gene regulation completely (Chin *et al*., 2008). However, mutations in the mature miRNA sequences in tumors do appear to be rare (Saunders *et al*., 2007).

Individual miRNAs can be dysregulated in tumors due to the these described reasons, but a global downregulation of miRNA expression in neoplasms has also been reported (Lu *et al*., 2005). A possible explanation for widespread loss of miRNA expression is dysregulation of the miRNA biogenesis machinery. For example, the nuclease Dicer can be either upregulated or downregulated depending on the cancer type (Lin and Gregory, 2015), and *DICER1* mutations can predispose to cancer (Slade *et al*., 2011). Also, RNA binding proteins (RBP) control the biogenesis, stability, and activity of miRNAs, and cancer cells may either circumvent this control or exhibit altered levels of RBPs (Miles *et al*., 2012).

2.6.3 Tumor suppressor and oncogenic miRNAs

miRNAs regulate essentially all central processes related to cancer ("hallmarks of cancer"), including proliferation, cell death, motility, and angiogenesis. For example, the tumor suppressors, miR-15/miR-16 (Cimmino *et al*., 2005) and let-7 family (Johnson *et al*., 2005), promote cell death and suppress cell proliferation, respectively. Some miRNAs regulate only one aspect of tumorigenesis, such as the exclusively metastasis promoting miR-10b (Ma *et al*., 2010), while other miRNAs, such as let-7, are involved in both primary tumorigenesis and cancer spreading (Johnson *et al*., 2005; Zhao *et al*., 2014). Importantly, individual miRNAs are usually classified as tumor suppressors or oncomiRNAs although the emerging evidence suggests that a miRNA may have both tumor suppressive and oncogenic functions, and the net effect of the miRNA may vary between different cancer types and the stages of tumorigenesis (reviewed in (Svoronos *et al*., 2016)).

Examples of how the aberrant expression of cell cycle and mitosis regulating miRNAs can contribute to tumorigenesis are described below. Further, the known

cancer-related functions of miR-493-3p, let-7b-5p, miR-193a-3p and miR-203b-3p, studied in this thesis, are also discussed below.

2.6.3.1 miR-493-3p and cancer

The human *MIR493* gene resides in the long arm of chr14, more precisely at the chr14q32.2 locus (miRbase (Kozomara and Griffiths-Jones, 2014). The *MIR493* gene is part of a miRNA gene cluster that consists of ten genes. Furthermore, near this cluster is located another, larger miRNA cluster of about 40 miRNA genes. Both of these clusters belong to the imprinted *DLK1-DIO3* (*Dlk1-Gtl2* in mice) domain, which is conserved among mammals (Seitz *et al*., 2004). Changes in the copy number and the epigenetic status of the domain and consequent abnormal expression of the miRNA genes it harbors, have been reported in different types of cancers, as for example epithelial ovarian (Zhang *et al*., 2008a), melanoma (Zehavi *et al*., 2012) and acute promyelocytic leukemia (Manodoro *et al*., 2014).

The majority of the reported direct target genes of miR-493-3p are involved in the regulation of cancer cell metastasis. Suppression of Mitogen-Activated Protein Kinase Kinase 7 (MKK7) (Sakai *et al*., 2014), Insulin Like Growth Factor 1 Receptor (IGF1R) (Okamoto *et al*., 2012), Ras Homolog Family Member C (RhoC), and Frizzled Class Receptor 4 (FZD4) (Ueno *et al*., 2012) by miR-493-3p decreases the invasion and metastatic settlement of colon and bladder cancer cells. Conversely, according to a more recent report by Jia and colleagues, increased miR-493-3p levels in gastric cancer cells and tumors can promote cell invasion and proliferation as well as induce chemoresistance by downregulating Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) (Jia *et al*., 2016). Previously miR-493-3p was shown to inhibit lung cancer cell invasion and cell growth *in vitro* and *in vivo* via targeting *E2F1* (Gu *et al*., 2014). The capacity of miR-493-3p to suppress angiogenesis in rat brain endothelial cells (Li *et al*., 2016) is also relevant for tumorigenesis.

2.6.3.2 let-7b-5p and cancer

The evolutionary conserved let-7 miRNA family includes 10 mature miRNAs in humans (let-7a, b, c, d, e, f, g, i, miR-98 and miR-202) and the encoding genes are scattered to 9 chromosomes. The *LET7B* gene is in cluster with the *LET7A3* gene in chromosome 21 (miRbase, (Kozomara and Griffiths-Jones, 2014). Due to the various genomic locations, the expression of *LET7* genes is regulated by different mechanisms. However, the oncogene c-Myc has been shown to repress the

expression of several *LET7* family members, including *LET7B* (Bueno *et al*., 2011; Chang *et al*., 2008; Sampson *et al*., 2007). The levels of mature let-7 miRNAs are controlled also post-transcriptionally, by the RNA binding protein, Lin28, that blocks the processing of *LET7* pri-miRNAs (Viswanathan *et al*., 2008).

The let-7 miRNA was initially identified in *C. elegans* as a controller of developmental timing (Reinhart *et al*., 2000). Respectively in humans, let-7 miRNAs promote cell differentiation, inhibit proliferation and are regarded as suppressors of malignant cell growth (Johnson *et al*., 2007; Yu *et al*., 2007). Suppression of several let-7 target genes, such as Ras, High Mobility Group AT-Hook 2 (HMGA2), various cyclins and Cdks (Johnson *et al*., 2007, 2005; Schultz *et al*., 2008; Yu *et al*., 2007) and c-Myc (Sampson *et al*., 2007) contribute to the miRNA-mediated inhibition of tumorigenesis. For example, tumors of the skin and lung (Johnson *et al*., 2007; Schultz *et al*., 2008) have lower let-7b levels, compared to corresponding normal tissue, and low let-7b expression is also connected to the poor survival of ovarian carcinoma patients (Nam *et al*., 2008). The effect of let-7 miRNAs on cell death with cancer drug treatment is more complex: let-7a has been reported to inhibit caspase-3 mediated apoptosis (Tsang and Kwok, 2008); however, on the other hand, let-7c promotes cell death by suppressing the anti-apoptosis protein Bcl-xL (Shimizu *et al*., 2010).

2.6.3.3 miR-193a-3p and cancer

Human miR-193a belongs to the same family as miR-193b, albeit their coding genes reside in different chromosomes, namely, in the long arm of chromosome 17 and in the short arm of chromosome 16, respectively. The *MIR193A* gene is not clustered with other miRNAs, as the nearest ones are more than 10 kilobases away, but the *MIR193B* is genomically clustered with *MIR365A* (miRbase (Kozomara and Griffiths-Jones, 2014).

The *MIR193A* gene promoter has been reported to be hypermethylated in lung (Heller *et al*., 2012; Seviour *et al*., 2016), breast (Pronina *et al*., 2017), and oral cancers (Kozaki *et al*., 2008), which leads to reduced miRNA expression in tumors. Accordingly, miR-193a-3p has tumor suppressive functions in different types of cancers, as miR-193a-3p suppresses cell proliferation by regulating important cell cycle proteins, such as Kirsten Rat Sarcoma Viral Oncogene Homolog (KRas; (Seviour *et al*., 2016) and components of the Epidermal Growth Factor Receptor (EGFR)-network (Uhlmann *et al*., 2012). The suppression of KRas expression by miR-193a-3p also diminishes the metastatic capability of tumor cells (Seviour *et al*., 2016). However, other target genes of this miRNA, as for example Erb-B2 Receptor Tyrosine Kinase 4 (ErbB4) (Yu *et al*., 2015) and RAB27B (Pu

et al., 2016), may also mediate the suppression of cell invasion. The tumor suppressor function of miR-193a-3p is further enhanced by the promotion of apoptosis due to suppression of the anti-apoptosis protein Mcl-1 (Kwon *et al*., 2013), and miR-193a-3p may also have an impact on chemoresistance in bladder cancer cells (Li *et al*., 2015; Lv *et al*., 2015).

2.6.3.4 miR-203b-3p and cancer

Human *MIR203B* is located in the chromosome 14 long arm (14q32.33) in a cluster with *MIR203A* (miRbase) (Kozomara and Griffiths-Jones, 2014). Virtually all reports on miR-203 are about miR-203a, as it is more widely conserved than miR-203b. The *MIR203* gene is embedded in a CpG island and loss of miR-203 due to promoter hypermethylation has been reported in a variety of tumors and cancer cell lines, including hematological malignancies and cancers of the lung, breast, cervix, and gastrointestinal tract origin (Botezatu *et al*., 2010; Bueno *et al*., 2008; Kozaki *et al*., 2008; Wang *et al*., 2014; Zhang *et al*., 2011). EGFR/MEK/ERK and Hedgehog pathways also suppress miR-203, and hyperactivity of these pathways in cancer may contribute to declined miR-203 levels (Sonkoly *et al*., 2012; Zhou *et al*., 2014a).

As the miR-203 levels are low in cancer, several tumor suppressive functions have been reported for it; miR-203 suppresses cell proliferation and promotes apoptosis by regulating the anti-apoptosis proteins Bcl-w (Bo *et al*., 2011) and survivin (Bian *et al*., 2012). Moreover, miR-203 suppresses the metastatic capability of cancer cells through several target genes in both cultured cancer cells and mouse metastasis models (Saini *et al*., 2011; Viticchiè *et al*., 2011; Zhang *et al*., 2011). In skin cancer, miR-203 is considered to be a master regulator of tumorigenic processes, as it targets the oncogene *MYC* (Lohcharoenkal *et al*., 2016). To the contrary, in another report, inhibition of miR-203 was shown to suppress c-Myc levels and the growth of breast cancer cells (Muhammad *et al*., 2016).

By suppressing the anti-apoptotic Bcl-w and survivin, miR-203 also enhances sensitivity to cisplatin and paclitaxel treatment in bladder and lung cancers, respectively (Wang *et al*., 2014; Zhang *et al*., 2015). However, the results on the miR-203 effects on drug sensitivity are variable. For example, in colon cancer miR-203 has been described as sensitizing resistant cells to paclitaxel by suppressing salt-inducible kinase 2 (SIK2) (Liu *et al*., 2016) and Akt (Li *et al*., 2011), while inducing resistance to platinum treatment by suppressing ATM (Zhou *et al*., 2014b).

2.6.3.5 Mitosis controlling miRNAs and cancer

Errors in mitosis are evidently involved in tumorigenesis, and thus, the role of mitosis-regulating miRNAs in cancer has attained emerging interest. miR-433, miR-28-5p have been demonstrated to regulate the important SAC protein, Mad2, and another SAC component, Mad1, is a target for miR-125b. Overexpression of these miRNAs induces chromosome segregation errors and aneuploidy in human cancer cells (Bhattacharjya *et al*., 2013; Furlong *et al*., 2012; Hell *et al*., 2014). Suppression of nucleoporin Nup124 by the ectopic expression of miR-133b has generated similar mitotic defects in cultured human cancer cells (Bhattacharjya *et al*., 2015). Three research groups have independently reported that overexpression of *PLK1*-targeting miRNAs (miR-100, miR-210, miR-509-3-5p) induces a G2/M arrest accompanied by mitotic spindle and chromosomal abnormalities (Li *et al*., 2014; Shi *et al*., 2009; Wang *et al*., 2016b). In addition to Plk1, miR-210 regulates a number of other mitotic proteins, including Bub1, cell division cycle 25 homolog B (Cdc25B), family with sequence similarity 83 member D (FAM83D), and cyclin F, perturbing mitotic progression and chromosome segregation when overexpressed (He *et al*., 2013). Regarding the end of M-phase, miR-1290 is overexpressed in colon cancer tissue and can promote tumorigenesis by delaying cytokinesis and inducing multinucleation by suppressing Kif13b (Wu *et al*., 2013).

2.6.4 miRNA therapies

Cell growth related miRNA functions and the misexpression of miRNAs in cancer can offer new therapeutic opportunities for cancer treatment, as re-introduction of lost tumor suppressor miRNAs or inhibition of oncogenic miRNAs can limit malignant cell proliferation and/or block metastasis(reviewed in (Li and Rana, 2014; Rupaimoole and Slack, 2017)). A schematic example of miRNA therapies in cancer treatment is presented in Figure 8.

Small molecule compounds, such as the hypomethylating agent 5-azacytidine, may provide the means to restore the expression of miRNAs on a larger scale. However, the biological consequences are probably very context dependent (Bandres *et al*., 2009; Lujambio *et al*., 2007). More specific restoration of individual miRNAs could be achieved with double-stranded miRNA mimicking oligonucleotides or viral miRNA expression vectors (Chen *et al*., 2011). Serious safety issues are associated with the clinical use of the viral vectors, and thus the miRNA mimics possess more therapeutic potential. Also the mimic oligonucleotides require an *in vivo* delivery system, such as liposomal nanoparticles that can be coated with tumor-specific antibodies to improve specificity (Tivnan *et al*., 2012; Trang

et al., 2011). A liposome-embedded miR-34 mimic for treatment of liver cancer entered clinical trials a few years ago, but the study was soon halted due to severe immunological side effects (Agostini and Knight, 2014).

Different kinds of miRNA inhibitors, antimiRNAs, have been studied in preclinical models for the inhibition of undesired miRNA function (Rupaimoole and Slack, 2017). AntimiRNAs are single-stranded synthetic antisense oligonucleotides (ASO), which bind to a specific miRNA and inhibit its activity. ASOs are differentially modified to improve their stability and efficacy. Locked nucleic acid (LNA) modification, which locks the sugar structure into an exact conformation, has proven to be most effective in cultured cell models and animals (Elmen *et al*., 2007; Lennox and Behlke, 2010). Similar to the miRNA mimics, the ASOs also require a vehicle system for efficient *in vivo* delivery. Currently, two differentially modified miR-122 antisense oligonucleotides are in phase II clinical trials for the treatment of hepatitis C virus infection (Janssen *et al*., 2013; Rupaimoole and Slack, 2017). miRNA sponges are competitive inhibitors of miRNA targets, as they carry several binding sites for the miRNA and effectively sequester the miRNA away from its targets. Because the miRNA recognition sites in the sponge correspond to the miRNA seed, sponges can simultaneously bind and inhibit all members of a miRNA family (Ebert *et al*., 2007).

Many challenges still remain in the development of miRNA therapeutics for the clinics. The capability of miRNAs to target several genes may be beneficial for therapy efficacy, but this feature also increases the risk of unwanted effects. In addition, the similar seed sequences of miRNA family members introduce a specificity challenge for the miRNA inhibitors (Obad *et al*., 2011). Targeted delivery helps minimize the risk of off-target effects, and modulating the pri-miRNA instead of the mature form could provide specificity over other miRNA family members, as the pri-miRNA sequences differ more (Li and Rana, 2014). Importantly, potential liver toxicity (Stanton *et al*., 2012) and the abnormal activation of the immune system (Hildebrandt-Eriksen *et al*., 2012; Hornung *et al*., 2005), triggered by the oligonucleotides, their chemical modifications, or delivery systems, need to be both carefully studied during the preclinical development of any miRNA therapeutics.

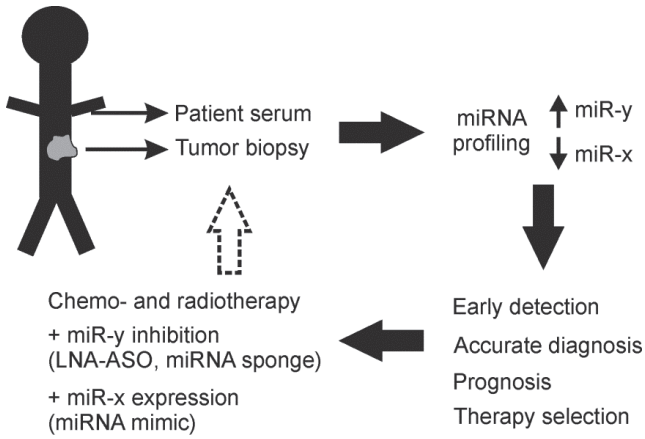

Figure 8. miRNAs as diagnostic and therapeutic tools in cancer treatment. Modified from (Lujambio and Lowe, 2012).

2.6.5 miRNAs as diagnostic tools

As the development of miRNA-based therapeutics to bedside is expected to still take a relatively long time, perhaps a more contemporary option is to utilize miRNAs as diagnostic tools, for cancer and other diseases (see Fig 8). Several notions support the biomarker potential of miRNAs. First, miRNA expression profiles have been demonstrated to distinguish tumor tissue from normal tissue and be even more accurate in the classification of tumor subtypes than gene expression profiles are (Lu *et al*., 2005). Breast cancer is an example of a very heterogeneous disease, wherein miRNA profiling has demonstrated its utility in determination of tumor subtype and invasive capacity (Haakensen *et al*., 2016; de Rinaldis *et al*., 2013). Also, the tissue of origin for metastatic colonies can often be verified using miRNA profiling (Rosenfeld *et al*., 2008). Secondly, miRNAs are more stable than mRNAs and thus can be reliably quantified even from formalin-fixed, paraffin-embedded tissue samples (Liu and Xu, 2011). Thirdly, miRNAs are also present and measurable in several body fluids, offering an exciting new opportunity for non-invasive diagnostics. Most importantly, the circulating miRNAs seem to be excreted from the primary tumor tissue, and in liquid biopsies their expression profiles differ markedly for cancer patients and healthy individuals. Moreover, the circulating miRNAs are very stable despite the high RNase activity in the blood (Lawrie *et al*., 2008; Mitchell *et al*., 2008; Weber *et al*., 2010). This extreme stability is achieved through miRNA packaging into exosomes, which can then be taken up by a recipient cell, further suggesting a functional aspect for miRNA delivery via circulation (Kosaka *et al*., 2010).

2.6.6 miRNAs and cancer therapy resistance

A major obstruction in cancer treatment is both intrinsic and acquired therapy resistance. Most anti-cancer therapies target signaling pathways that are essential for cell survival, for example, proliferation, apoptosis, cell cycle, and division. As the abnormal expression of miRNAs perturbs the function of these essential processes (Chang and Mendell, 2007; Kloosterman and Plasterk, 2006), it is not surprising that recent reports suggest that miRNAs are also involved in cancer therapy resistance (Cui *et al*., 2013; Svoronos *et al*., 2016). Hence, an interesting possibility for miRNA therapeutics is their use in combination with conventional chemotherapeutic agents and radiotherapy, as illustrated in Figure 8. Importantly, miRNA profiling can be used to predict drug response (see Fig 8). Reports on miRNA-mediated regulation of chemotherapy response are emerging, and examples of miRNA-mediated regulation of MTA response, the subject of this thesis effort, are discussed below.

MTAs, such as paclitaxel, docetaxel and vinblastine, are still widely used as anti-cancer therapeutics although they are strong cytotoxic agents with both drug resistance and side effect issues (see Chapter 2.6.1). Recent miRNA research has indicated that specific miRNAs may alter MTA function by interfering with the expression of SAC proteins, β-tubulin isotypes, cell death modulators, or drug efflux pump proteins (reviewed in (Cui *et al*., 2013)). Furlong and colleagues reported that *MAD2*-targeting miR-433 induced paclitaxel resistance in ovarian cancer cells through abrogation of SAC and high expression of miRNA associated with poor survival of ovarian cancer patients (Furlong *et al*., 2012). Also, low miR-200c levels were associated with poor survival among ovarian carcinoma patients because miR-200c promotes MTA sensitivity by suppressing the expression of type III β-tubulin (Cochrane *et al*., 2010; Leskela *et al*., 2010). Several other miRNAs, such as let-7a, miR-203a, and miR-34a, have been reported to modify the MTA response in cultured human cancer cells via changed expression of anti- and pro-apoptosis factors (Kojima *et al*., 2010; Liu *et al*., 2016; Tsang and Kwok, 2008). Lastly, let-7g sensitized ovarian cancer cells to paclitaxel and vinblastine via indirect suppression of the drug efflux pump protein multidrug resistance 1.

The accumulated evidence suggests that altered expression of miRNAs plays an important role in tumorigenesis and cancer therapy response. To enable the use of miRNAs as diagnostic and/or therapeutic tools in future cancer treatment, more studies using preclinical models and human cancer cohort datasets are needed to identify the potential biomarker miRNAs and elucidate their mechanisms of action.

3 AIMS OF THE STUDY

This thesis aims at the identification and characterization of novel mitosis-regulating miRNAs, and study of their role in tumorigenic processes and drug sensitivity. The results are expected to provide new insights on the use of miRNAs in cancer diagnostics in the future.

The specific goals of this thesis thus were:

1. Characterize the effects of altered miR-493-3p and let-7b-5p expression on mitosis, genomic balance, and cancer cell taxane response as well as identify the mitotic target gene(s) of the miRNAs.

2. Identify novel miRNA(s) that regulate the tumor suppressor protein, Rassf1a, and study their role in the cell division processes and maintenance of genomic stability.

3. Identify potent miRNAs that target the anti-apoptosis protein, Bcl-xL, and analyze their impact on cancer cell survival and taxane response.

4. Analyze the potential association of the studied miRNAs with the parameters of tumorigenesis and patient survival data in breast and ovarian cancer cohorts.

4 MATERIALS AND METHODS

4.1 Cell lines (I-IV)

Human cancer cell lines used in this thesis work are listed in Table 1.

Table 1. Immortalized human cancer cell lines used in this study.

Cell line	Description	Used in
CaOV-3	ovarian adenocarcinoma	I, IV
HCT116	colorectal carcinoma	I, II, III
HeLa	cervical adenocarcinoma	I, II, III
H2B-GFP HeLa	HeLa cells stably expressing H2B-GFP	I, II
H2B-GFP/tubulin-mCherry HeLa	HeLa cells stably expressing H2B-GFP and mCherry-tubulin	III
MCF-7	breast adenocarcinoma	I, IV
MDA-MB-231	breast adenocarcinoma	II
MDA-MB-231 SA	breast adenocarcinoma	II, IV
OVCAR-8	ovarian adenocarcinoma	I, III, IV

4.2 Transient transfections (I-IV)

Pre-miR™ miRNA precursors (I, II; Ambion, Thermo Fisher Scientific, Carlsbad, CA, USA), miRIDIAN microRNA mimics (III, IV; GE Dharmacon, Lafayette, CO, USA), Anti-miR™ miRNA inhibitors (I, III; Ambion), miRCURY LNA ™ microRNA Target Site Blockers (I, II; Exiqon, Copenhagen, Denmark) and short interfering RNAs (I-IV) were transiently transfected into cells using HiPerFect (I-IV; Qiagen, Valencia, CA, USA) or SiLentFect (II; Bio-Rad, Hercules, CA, USA) transfection reagent. For co-transfecting oligonucleotides with plasmid constructs, either Lipofectamine 2000 or 3000 (I-IV; Invitrogen, Thermo Fisher Scientific) was used. Most analyses were performed 24-72 h after transfection, except senescence was assayed 4 days after transfection.

4.3 Methodology to study the mitotic phenotype (I-IV)

4.3.1 Live-cell imaging (I-IV)

To monitor the long-term proliferation of living cells, Incucyte live-cell imaging devices (Essen BioSciences, Hertfordshire, UK) were used. Operetta high-content imaging system (PerkinElmer, Waltham, MA, USA) and Axiovert 200M inverted microscope (Zeiss GmbH, Oberkochen, Germany) with an environment chamber were used for high resolution imaging of shorter durations, *e.g.* to visualize M-phase progression in detail (Table 2). When needed, the mitotic population was enriched with cell cycle synchronization using the double-thymidine block method. In brief, subconfluent (50%) cell population was treated with 2mM thymidine for 19 h, washed and let recover for 8-9 h. A second thymidine treatment was applied at least for overnight, before releasing the cells. Transient transfections were done either before the first thymidine treatment or between the blocks. Alternatively, MTAs were used for accumulating cells in mitosis to analyse their behavior and fate under various experimental conditions.

Table 2. Imaging equipment.

Equipment	Camera	Program	Application	Used in
Incucyte HD and FLR		Incucyte 2010	Automated live-cell imaging (phase-contrast and fluorescence)	I-IV
Operetta high-content imaging system		Harmony®	Automated imaging of live and fixed cells (phase-contrast and fluorescence)	I, III
Zeiss Axiovert 200M inverted microscope	AxioCam MRm camera	Zeiss Axiovision	Live-cell imaging (fluorescence)	II
ScanR imaging system		ScanR software	Automated fluorescent imaging of fixed cells	I, II
Zeiss Axiovert 200M inverted microscope	Zeiss AxioCam MRc (colour) ccd camera	Zeiss Axiovision	Phase-contrast imaging of fixed cells	I
Zeiss Axiovert 200M inverted microscope	Hamamatsu Orca ER	Metamorph v6.2r6	Fluorescent imaging of fixed cells (z-stacks)	I, II, III

4.3.2 Microscopy of fixed cell specimens (I-III)

The architecture of the mitotic spindle and possible presence of multilobed nuclei, polyploid cells, lagging chromosomes, chromatin bridges or other mitotic anomalies, were assessed from fixed cell using the Operetta or ScanR imaging systems (Olympus, Tokyo, Japan) and the Zeiss Axiovert 200M microscope with Metamorph software (Table 2), all equipped with phase-contrast and fluorescence optics. For fluorescence imaging of cells that did not stably express tagged proteins, labeling with specific antibodies (Table 4) and DNA staining with 4',6-Diamidino-2-Phenylindole (DAPI) were applied. Briefly, cells were grown on coverslips and fixed with 2-4% paraformaldehyde in PHEM (60nM PIPES, 25mM HEPES, 10mM EGTA, 4mM $MgSO_4$) containing 0.5% Triton-X-100. For tubulin immunostaining, the fix was supplemented with 0.2% glutaraldehyde. Some antibodies required a short pre-extraction with PHEM/0.5% Triton-X-100. Unspecific antibody binding was blocked with 20% boiled normal goat serum in MBST buffer (10mM MOPS, 150mM NaCl, 0.05% Tween 20). Both primary and secondary antibodies were diluted in 5% blocking solution and incubated for 1h. After DAPI-staining (1:10 000 in H_2O) coverslips were mounted on microscopy slides with Vectashield (Vector laboratories).

4.4 Methods for studying genomic balance (I-III)

4.4.1 Fluorescence in situ hybridization (FISH) (I-III)

Changes in the cells' chromosome number were assessed with FISH, using probes for two or three different chromosomes. Trypsinized HCT116 cells were first swelled with 75mM KCl at +37°C for 15 min and then fixed with ice-cold methanol-acetic acid (3:1). The fixed cells were dropped on a glass slide, air-dried and then denaturated and hybridized with the FISH probe (Abbott Inc., Chicago, IL, USA). Hybridization and the following washings were conducted according to the manufacturer's instructions. Finally, DNA was stained with DAPI. Images for analysis were acquired with the ScanR Imaging system (I-II) or the Axiovert 200M microscope and Metamorph software (III) (Table 2).

4.4.2 Chromosome spreads (I, III)

To detect structural chromosomal abnormalities and premature sister chromatid separation, chromosome spreads were prepared from mitotic cells of a cycling

population. Samples were prepared similar to FISH assay and then stained with Giemsa's Azure Eosin Methylene Blue Solution (Merck, Darmstadt, Germany) after dropping the cells on the slide. The samples were studied with the Axiovert 200M microscope with Metamorph software (Table 2).

4.5 Gene and miRNA expression analysis methods (I-IV)

4.5.1 Quantitative miRNA and gene expression PCR (I-IV)

RNA was extracted from cells using either miRVana™ miRNA isolation kit (Ambion) or RNeasy RNA extraction kit (Qiagen). To measure mature miRNA levels, Taqman microRNA Reverse Trancription Kit (Applied Biosystems, Thermo Fisher Scientific) and miRNA specific Taqman MicroRNA assays (Applied Biosystems) were used. RNU6B was used for normalization. cDNA for mRNA quantification was prepared with iScript cDNA synthesis kit (Bio-Rad). Primers and probes (Roche, Basel, Switzerland) used for Taqman-based quantitative PCR are listed in Table 3. PCR was run with 7900HT Fast Real-Time PCR System (Applied Biosystems) at the Finnish Microarray and Sequencing Centre (Turku Centre for Biotechnology) or with CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad), and results analyzed with the corresponding manufacturer's software and comparative Ct method.

Table 3. Primers and probes used in quantitative PCR.

Gene	Strand	Sequence	Probe	Used in
AURKB	sense antisense	ATTGCTGACTTCGGCTGGT GTCCAGGGTGCCACACAT	#69	II
E2F1	sense antisense	TCCAAGAACCACATCCAGTG CTGGGTCAACCCCTCAAG	#5	I
GAPDH	sense antisense	AGCCACATCGCTCAGACAC or ACGACCAAATCCGTTGACTC GCCCAATACGACCAAATCC or CTCTGCTCCTCCTGTTCGAC	#60	I, II, III
MAD2L1	sense antisense	CGCGTGCTTTTGTTTGTGT GCTGTTGATGCCGAATGAGA	#32	I
RASSF1A	sense antisense	GCTCGTCTGCCTGGACTG CTCCACAGGCTCGTCCAC	#23	III
STX16	sense antisense	CAGCTGTTAGCCGAGCAAGT CATCAGCAAGCTCGTCCAG	#19	III

4.5.2 Illumina microarray (II)

To study gene expression changes induced by excess miR-493-3p, H2B-GFP cells were transfected with either miR-control or miR-493-3p using Dharmafect (GE Dharmacon). Samples were collected 24h after transfection and total RNA was extracted with miRVana™ miRNA isolation kit. miRNA overexpression in the samples was confirmed with quantitative PCR and RNA quality was checked with Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA). Illumina microarray from three biological replicates was run and the results analyzed at the Finnish Microarray and Sequencing Centre (Turku Centre for Biotechnology).

4.5.3 Immunoblotting (I-IV)

To quantify the expression of proteins at population scale, immunoblotting with specific antibodies was performed. Cells were first lysed with APC-buffer (20mM Tris-HCl, 100mM KCl, 50mM sucrose, 1mM $MgCl_2$, 0.1mM $CaCl_2$, 0.5% Triton-X-100) or with RIPA-buffer (50mM Tris-HCl, 150mM NaCl, 0.5% DOC, 0.1% SDS, 1% NP-40) supplemented with protease- and phosphatase inhibitors (Roche). Protein samples were run on 4-20% gradient SDS-PAGE gels (Bio-Rad) and then transferred to nitrocellulose membrane. Blocking was performed either with 5% milk, 5% BSA or Odyssey blocking buffer (LI-COR) and the membrane was then incubated with a primary antibody (Table 4) diluted in blocking solution or TBS-T (0.1% Tween 20), usually for overnight at +4°C. Secondary antibodies were used at 1:5000 concentration and 1 h incubation at room temperature. Either the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA) or chemiluminescence and the ImageQuant LAS 4000 scanner (Fujifilm, GE Healthcare, Chicago, IL, USA) or conventional film was used for protein detection. For quantification protein signals were normalized to GAPDH expression in each sample.

4.5.4 Immunofluorescence (I-III)

To study protein localization and to quantify their amounts at specific cellular sites in different phases of mitosis, immunostaining (chapter 4.3.2) and fluorescence imaging of fixed cells using the Zeiss Axiovert 200M inverted microscope and the Metamorph software (Table 2) were performed. Signal intensities and size of the stained areas were measured from maximum projections prepared from the acquired image z-stacks using the Metamorph software.

Table 4. Primary antibodies and their dilutions used for immunoblotting (WB) and immunofluorescence (IF).

Antigen	Species	Supplier, catalog number	Dilution	Used in
Aurora B	rabbit	Abcam, ab2254	WB, 1:800	II
AIM-1	mouse	BD Biosciences, 611083	WB and IF, 1:1000	II
Bcl-xL	rabbit	Cell Signaling, 2764	WB, 1:1000	IV
Bub1	rabbit	Abcam, ab9000	IF, 1:150	I
CEP55	mouse	Abnova, H00055165-A01	IF, 1:200	III
CETN3	mouse	Abnova, H00001070-M01	IF, 1:500	III
cleaved caspase-3	rabbit	Cell Signaling, 9664	WB, 1:1000	IV
cleaved PARP	mouse	Cell Signaling, 9546	WB, 1:1000	I, IV
c-Myc	rabbit	Abcam, ab32072	WB, 1:1000	IV
CREST (human autoimmune sera)	human	Antibodies Incorporated	IF, 1:200	I, II
cyclin B1	mouse	BD Biosciences, 554178	WB, 1:500	I
E2F1	mouse	Santa Cruz, sc-251	WB, 1:500	I
GAPDH	mouse	Advanced Immuno-Chemical Inc. or HyTest Ltd., mAb 6C5	WB,1:30000-50000	I-IV
Mad2	mouse	Abcam, ab10691	WB, 1:500, 1:1000	I
Mad2	mouse	Santa Cruz, sc-65492	IF, 1:75	I
pericentrin	rabbit	Abcam, ab4448	IF, 1:500	II, III
phospho-Cenp-A Ser7	rabbit	Upstate, 05-792	IF, 1:1000	II
Rassf1a	mouse	Abcam, ab23950 or Acris antibodies, SM6017	WB, 1:500	III
securin	mouse	Abcam, ab3305	WB, 1:250	I
Syntaxin16	rabbit	Atlas antibodies, HPA041019	WB, 1:750, IF, 1:250	III
α-tubulin	mouse	Abcam, ab7291	IF, 1:200	II
α-tubulin	rat	Abcam, ab6160	IF, 1:500	III

4.6 Luciferase reporter assay (I-IV)

Possible miRNA binding to the target gene mRNA was studied with luciferase reporter assay. In brief, cells were co-transfected with miRNA mimic and a firefly luciferase reporter construct containing the gene 3'UTR (or whole mRNA sequence). A Renilla luciferase construct was also included for normalization. 24 or

48h after transfection the luminescence signal produced by the plasmid was measured with Dual-Glo Luciferase Assay system (Promega, WI, USA) and EnSight™ or EnVision™ plate reader (PerkinElmer). The primers used for cloning the Aurora B luciferase construct are listed in Table 5. For Aurora B and Rassf1a, the miRNA binding was confirmed by performing the luciferase reporter assay with a plasmid where the miRNA binding site was mutated using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) and the primers described in Table 6.

Table 5. Primers used in cloning the Aurora B luciferase reporter constructs.

Gene	Strand	Sequence	Used in
AURKB 3'UTR	sense antisense	ATCGACTAGTTGATGGTCCCTGTCATTCACT ATCGACGCGTTGAGTACAAAAAGCTTCAGCC	II
AURKB	sense antisense	ATCGACTAGTGGAGAGTAGCAGTGCCTTGGA ATCGACGCGTTGAGTACAAAAAGCTTCAGCC	II

Table 6. Primers used for site-directed mutagenesis.

Gene	Strand	Sequence	Used in
AURKB	sense antisense	GGATCCCTAACTGTTCCCTTATCTGTTTTCGC ATTCCTCCTTTGTTTATAAAGGCTGAAG CTTCAGCCTTTATTAAACAAAGGAGGAATGC GAAAACAGATAAGGGAACAGTTAGGGATCC	II
RASSF1A	sense antisense	GCCGTGTGAGTGTGACAGGTTACGTGGGGC CTGTGGAATGAG CTCATTCCACAGGCCCCACGTAACCTGTCAC ACTCACACGGC	III

4.7 Proliferation, cell cycle and cell fate analysis (I-IV)

To study cell cycle distribution, cells stained with propidium iodide were analyzed with flow cytometry using BD FACSCalibur equipped with CellQuest Pro software (BD Biosciences, San Jose, CA, USA) (I, II, III). Cell death was measured also by microscope-based dead cell indexing from fixed specimens (III) and immunoblotting for cell death markers (I, IV). Cell viability was measured by CellTiter-Glo® Luminescent Cell Viability Assay (Promega) and EnSight™ Multimode Plate Reader, according to manufacturer's instructions (IV). Senescence was detected using β-galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA) and Axiovert 200M inverted microscope with color camera (Table 2). Senescence induction was scored as stained area normalized to cell number.

4.8 Clinical data analyses (I-IV)

Retrospective analyses of human tumor samples and related patient information were conducted using six different breast and ovarian cancer cohort datasets (Table 7). The miRNA and mRNA expression acquisition and analysis methods, and patient characteristics are described in more detail in the corresponding references.

Table 7. Patient cohorts used in this study. In cohort A, miRNA profiles were available from 25 patient in both epi- and tx-arms for analyses in I and III. For analyses in IV, miRNA and mRNA profiles were available from 85 patients in epi arm and 105 patient in tx-arm.

Cohort /dataset	miRNA analysis	Gene expression analysis	References	Used in
Cohort A ***Bergen*** 223 breast carcinoma tumors (randomized in epi and tx treatment arms)	50bp single end sequencing	Illumina HT-12 microarray	(Chrisanthar *et al*., 2011)	I, III, IV
Cohort B ***MICMA*** 101 primary breast carcinoma tumors	Agilent b 8_15k, Human miRNA Microarray Kit (V2), design ID 019118	Agilent 4x44K one-color oligo array	(Enerly *et al*., 2011; Naume *et al*., 2001)	II, III
Cohort C ***TCGA breast 1*** 395 primary breast cancer tumors	Illumina miRNA sequencing	Agilent custom 244K whole genome microarrays	(Koboldt *et al*., 2012)	II
Cohort D ***TCGA breast II*** 1172 primary breast cancer samples	Illumina miRNA sequencing		(Koboldt *et al*., 2012)	IV
Cohort E ***Oslo*** 12 HGSC, 9 CCC, 9 OSE	Affymetrix miRNA 2.0 Arrays	Affymetrix Human Genome U133 Plus 2.0	(Elgaaen *et al*., 2010, 2012; Vilming Elgaaen *et al*., 2014)	I
Cohort F ***TCGA ovary*** 572 ovarian carcinoma tumor and 8 normal ovarian tissue samples	Agilent 8x15K miRNA-specific arrays	Affymetrix Human Genome U133 Plus 2.0	(Bell *et al*., 2011; Ganzfried *et al*., 2013)	I, III

4.9 Statistical analyses (I-IV)

Two-tailed paired Student's t-test was used for comparing two groups and one-way ANOVA with Tukey's HSD as a post-hoc test for comparing more than two groups. Respectively, the non-parametric tests Mann-Whitney U and Kruskal-Wallis were used to study the null-hypothesis when the analyzed data was not normally distributed. For statistical analysis of FISH results, either Chi-Square test (II) or one-way ANOVA (I) was applied. K-means clustering method (k=2) was used for separating the patient groups according to miRNA or gene expression for survival analysis. The difference between Kaplan-Meier survival curves was studied using the log-rank test.

5 RESULTS

5.1 The miR-493-3p dictates mitotic checkpoint function and cancer cells sensitivity to paclitaxel

5.1.1 miR-493-3p expression governs SAC function

Human miR-493-3p was one of the hit anti-mitotic miRNAs in our high-throughput screen (HTS, Fig 9). In the screen, miR-493-3p overexpressing human cancer cells manifested a premature exit from MTA-induced mitotic arrest, characterized by multilobed nuclei (I, Fig 1C), thereby suggesting that SAC signaling is perturbed (Winsel *et al*., 2014).

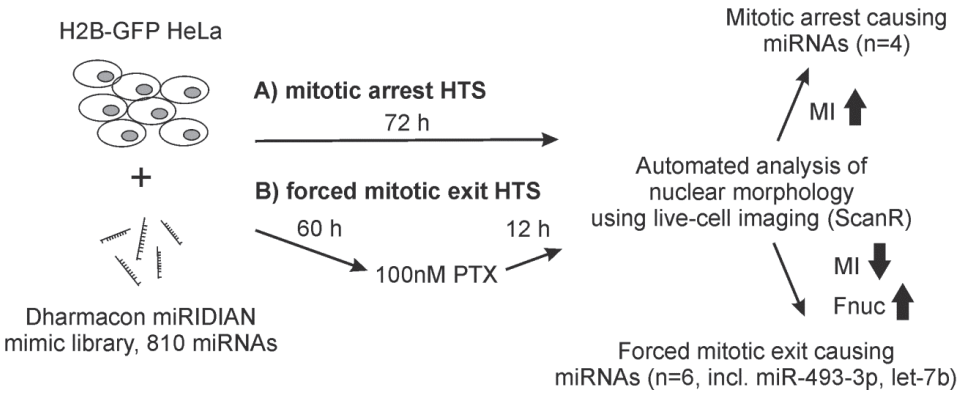

Figure 9. A schematic representation of the HTS for anti-mitotic miRNAs that would cause mitotic arrest (A) or induce a forced exit from paclitaxel-induced mitotic block (B). miR-493-3p (I) and let-7b (II) were hits from the latter (B). PTX=paclitaxel, MI=mitotic index, Fnuc=fragmented nuclei.

The HTS phenotype was validated in paclitaxel and/or nocodazole-treated HeLa, OVCAR-8 and CaOV-3 cells utilizing live-cell imaging (I, Fig 1D-E, 7B). For example, in HeLa cells, the frequency of mitotic slippage increased from the intrinsic 10 % to almost 50% in cell population with a thousands fold overexpression of miR-493-3p (I, Fig 1B, D). The majority of the cells exited the M-phase after a transient arrest of a few hours. In paclitaxel-treated ovarian carcinoma cell lines, the cells' endogenous miR-493-3p expression correlated with the intrinsic rate and timing of mitotic slippage. Almost 80% of the CaOV-3 cells, having very high miR-439-3p expression, exhibited a forced exit from taxol block on average in less than 5 h; whereas in the OVCAR-8 cell population, having markedly lower miR-

493-3p levels, 38% of the cells underwent a forced exit from taxol arrest after an average delay of over 8 h (I, Fig 6A, 7A).

In unperturbed HeLa cells, override of SAC through miR-493-3p overexpression accelerated mitotic progression, measured as a NEBD-to-anaphase duration, by an average of 13.5 min (I, Fig 1E). Conversely, suppressing endogenous miR-493-3p levels to about 60% of the normal, with an anti-miRNA, prolonged mitosis on average by almost 20 min (I, Fig 4A, D).

5.1.2 miR-493-3p regulates SAC by a direct targeting of Mad2

The notable override of SAC led us to consider key SAC proteins as potential target genes of miR-493-3p. In a microarray gene expression analysis from miR-493-3p overexpressing cells, the MCC component *MAD2* was the most potently suppressed mitotic gene (I, Table S1). Moreover, the *MAD2* 3'UTR contains a possible binding site for miR-493-3p (I, Fig 2A). Finally, the miR-493-3p binding to the *MAD2* 3'UTR was confirmed experimentally with a luciferase reporter assay (I, Fig 2B). In line with the direct targeting of *MAD2*, excess of miR-493-3p efficiently suppressed *MAD2* mRNA and protein expression in a panel of five human cancer cell lines with different tissues of origin: HeLa (cervix), HCT116 (colon), MCF7 (breast), OVCAR-8 (ovary), and CaOV-3 (ovary) (I, Fig 2C-D, S1, 6D). Importantly, Mad2 expression was greatly diminished at the kinetochores of prometaphase cells in miR-493-3p overexpressing cell population when compared to the control (I, Fig 2E).

To establish that the observed SAC defect is due to Mad2 suppression, we used a competing oligonucleotide that binds to the same site in the *MAD2* 3'UTR as the miRNA (Mad2 target site blocker, TSB-MAD2). Co-transfection of the TSB-MAD2 with miR-493-3p restored Mad2 protein levels to 70% of normal and also inhibited the forced mitotic exit almost completely (I, Fig 2G-H, S3). Manipulation of the endogenous miR-493-3p–Mad2 –axis with a miR-493-3p inhibitor demonstrated more than two-fold upregulation of Mad2 protein at the population scale and even greater accumulation of Mad2 at the unattached kinetochores (I, Fig 4A-C). During our work, miR-493-3p was reported to directly target *E2F1* (Gu *et al*., 2014), which is also a putative driver of *MAD2* transcription (Hernando *et al*., 2004), raising the possibility that miR-493-3p may also regulate Mad2 levels indirectly via E2F1. Although miR-493-3p also suppressed *E2F1* expression in our hands, silencing *E2F1* using RNAi had no effect on *MAD2* mRNA or protein levels in our model (I, Fig S2), suggesting that E2F1 is not involved in the miR-493-3p – Mad2 regulatory axis.

5.1.3 *Altered miR-493-3p expression perturbs chromosome segregation and induces aneuploidy in vitro*

In addition to checkpoint deficiency, altered Mad2 levels have been reported to lead to chromosome segregation defects and aneuploidy (Dobles *et al*., 2000; Kabeche and Compton, 2012; Michel *et al*., 2001; Sotillo *et al*., 2007). Indeed, we observed a premature securin degradation and a 22% and 15% increase in the frequency of prematurely separated sister chromatids in miR-493-3p overexpressing HeLa and HCT116 cells, respectively (I, Fig 3A-B). On the other hand, increased Mad2 levels in anti-miR-493-3p transfected cells caused an increase in lagging chromosomes and chromatin bridges, both of which are indications of abnormal chromosome segregation (I, Fig 4E). As a consequence of chromosome separation problems, excess miR-493-3p generated aneuploidy in the chromosomally stable HCT116 cells. The percentage of aneuploid cells, detected with FISH, significantly increased for both studied chromosomes (12 and 21) in miR-493-3p overexpressing cell population, compared to the control (I, Fig 3C). Most of the aneuploid cells were trisomic, but monosomies were also frequently observed (I, Table S2). Importantly, the aneuploidy seemed to be a direct consequence of miR-493-3p-mediated Mad2 suppression, as the TSB-MAD2 rescued the rate of aneuploidy nearly to the level observed in the scrambled control miRNA transfected cells (I, Fig 3C).

5.1.4 *miR-493-3p-induced mitotic defects lead to senescence and cell death*

As genomically imbalanced cells may be eliminated from the cell population through cell cycle exit, and *MAD2* depletion has been linked to senescence induction (Lentini *et al*., 2012; Prencipe *et al*., 2009), we checked using β-galactosidase staining whether miR-493-3p overexpressing cells exhibit senescence. Indeed, there was 3.5-4 times more β-galactosidase staining in miR-493-3p overexpressing cell populations compared to the control cells (I, Fig 3D HeLa, Fig S4 MCF-7). Also the senescence appeared to be a Mad2-specific phenotype, as co-transfection of the TSB-MAD2 reversed the senescence completely in HeLa cells (I, Fig 3D).

In addition to senescence induction, excess of miR-493-3p also slightly increased cell death frequency. Indeed, 72h after miRNA transfection a flow cytometry analysis of propidium iodide stained cells indicated an increase of sub-G1 population in miR-493-3p transfected cells compared to miR-control (10.7 +/- 2.6% vs. 4.7 +/- 1.2%, p=0.04; Fig 8A), and immunoblotting showed increased expression of cleaved PARP (cPARP) protein compared to the control miRNA (5.3 +/- 2.4 –fold, p=0.04; Fig 8B).

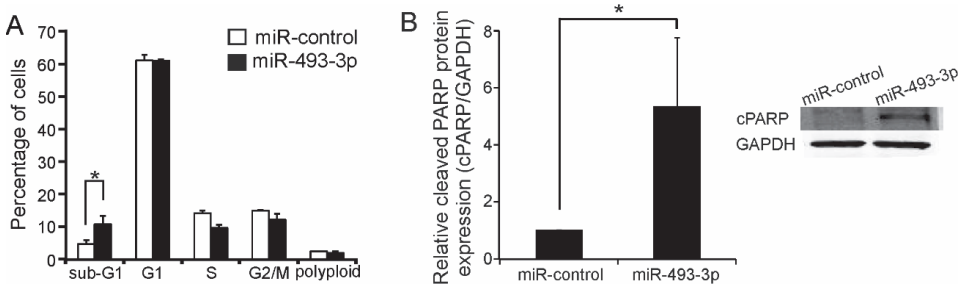

Figure 10. A. Cell cycle analysis from propidum iodide stained HeLa cells transfected with miR-control or miR-493-3p. B. Pooled quantification and a representative immunoblot of cPARP expression in miR-control and miR-493-3p transfected HeLa cells. All data come from 3 independent experiments, shown as mean +/- s.d.

5.1.5 High miR-493-3p is associated with poor survival of ovarian and breast cancer patients

To seek evidence for biological relevance of the miR-493-3p – Mad2 –axis *in vivo*, we performed retrospective analyses of two ovarian carcinoma cohorts. In the Oslo cohort (E), miR-493-3p showed a clear expression pattern; the miRNA expression was lower in the high-grade serous tumors (HGSC) compared to clear cell carcinomas (CCC) and normal ovarian epithelium samples (OSE). *MAD2* mRNA expression displayed a reverse pattern with a higher expression level in HGSC compared to OSE (I, Fig 5A). In the larger TCGA cohort (F), the *MAD2* expression scheme repeated, but there were no significant differences in the miR-493-3p levels between the different grades of tumors (I, Fig 5B).

Ovarian carcinoma patients are routinely treated with a combination of taxane and platinum (McGuire *et al*., 1996; Vasey *et al*., 2004). Interestingly, high miR-493-3p expression was found to have a significant association with poor survival of HGSC patients in the TCGA dataset (I, Fig 7C). To strengthen our findings on ovarian carcinoma, we performed a similar analysis in a breast cancer cohort (A, Bergen). In the breast cancer dataset, high miR-493-3p expression also correlated significantly with poor disease-specific survival. Importantly, when these patients were classified according to their randomly assigned therapy regimen, the miR-493-3p correlation to survival manifested only in the paclitaxel-treated patients, not in the epirubicin therapy group (I, Fig 7D-F).

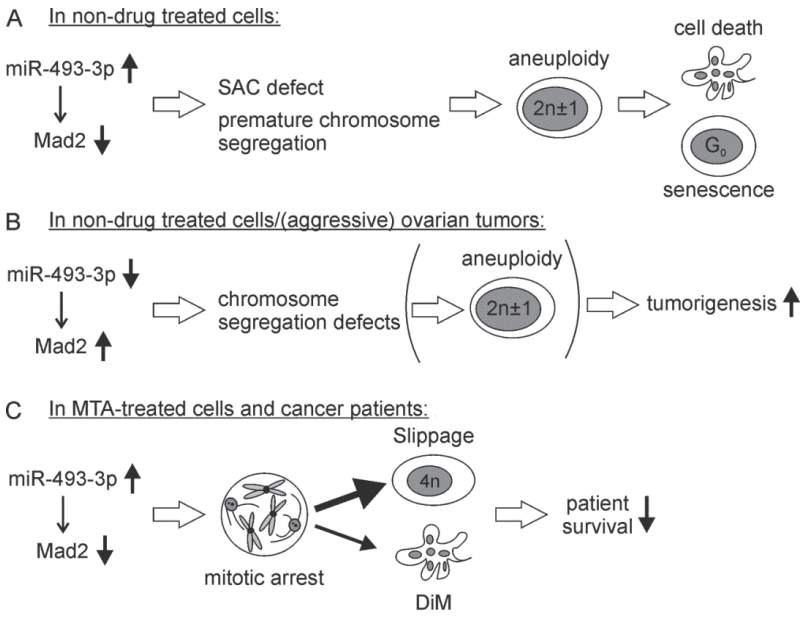

Figure 11. A graphic summary of how altered miR-493-3p levels affect (A-B) the genomic balance of cultured human cancer cells, tumorigenesis *in vivo*, and (C) MTA-response in cultured cells and in breast and ovarian cancer patients. DiM=death in mitosis, MTA=microtubule targeting agent, and the SAC=spindle assembly checkpoint.

5.2 Tumor suppressor miRNA, let-7b-5p, regulates Aurora B expression and genomic balance

5.2.1 Excess of let-7b-5p abrogates the mitotic checkpoint and genomic balance in cultured human cancer cells

Overexpressed miRNA let-7b-5p (from here on referred to as let-7b) was observed to override a MTA-induced mitotic block in our previously performed HTS. Confirmatory results were obtained from live-cell imaging assays of nocodazole and taxol- treated HeLa cells with excess let-7b: almost half of the population underwent a forced mitotic exit without cytokinesis and the mitotic index started to decline after 10h of drug treatment (II, Fig 1A-C). Flow cytometric cell cycle analysis demonstrated a significant increase of 4N (G2/M) and polyploid cells in the let-7b overexpressing population compared to the miR-control (II, Fig 1D). Over half of the let-7b overexpressing cells also exhibited a multipolar mitotic spindle in an analysis of fixed cells stained with pericentrin and α-tubulin specific antibodies (II,

Fig 5). Moreover, the genomic balance of the let-7b overexpressing cells was disrupted as indicated by an increase of different aneuploid cell populations in FISH assays, performed with probes for three different chromosomes (II, Fig 4C).

Under drug-free culture conditions, excess of let-7b almost tripled the average duration of mitosis, as it was 1.3h in to control population and 3.7h in the let-7b overexpressing cell population. This increase probably rose from a third of the let-7b overexpressing population that stayed in mitosis for significantly longer and died from that arrest (II, Fig 1B).

5.2.2 Let-7b disturbs mitotic signaling through suppressing Aurora B kinase

Aurora B kinase is a potential mitotic target gene of let-7b for several reasons. First, let-7b has a predicted binding site in the *AURKB* 3'UTR. Secondly, a previous study has suggested based on microarray data that *AURKB* is one of the genes that responds to the modulation of let-7b levels (Johnson *et al*., 2007). Third, reduction of Aurora B levels could explain the escape from MTA-induced mitotic block in the let-7b overexpressing cells (Ditchfield *et al*., 2003; Hauf *et al*., 2003; Kallio *et al*., 2002; Marxer *et al*., 2014). Indeed, overexpression of let-7b led to diminished *AURKB* mRNA and protein expression in all four tested human cancer cell lines, as well as reduced phosphorylation of CENP-A at unattached kinetochores, which is an indication of decreased Aurora B kinase activity (II, Fig 2, 4A, S2A-C). Let-7b also exhibited a significant negative Pearson correlation with *AURKB* mRNA expression in two breast cancer cohort datasets (B: -0.41 and C: -0.37; II, Fig 7G and S4), suggesting that let-7b may also control *AURKB* expression *in vivo*. As further evidence of Aurora B inhibition, let-7b also potentiated the function of the chemical Aurora B inhibitor, Barasertib, (Yang *et al*., 2007) when the drug effect was measured as a frequency of polyploidy induction (II, Fig 6). Moreover, a luciferase reporter assay indicated that let-7b binds to its predicted site in the *AURKB* mRNA 3'UTR (II, Fig 3A-B). Binding to this site seems to mediate the suppression of Aurora B since co-transfection of a competing by target site blocker oligonucleotide, which binds to the same site in the *AURKB* 3'UTR as let-7b, alleviated the let-7b-mediated Aurora B protein suppression completely (II, Fig 3C).

5.2.3 *Low let-7b expression is associated with clinicopathological markers of breast cancer aggressiveness and poor patient prognosis*

The let-7 family of miRNAs are regarded as tumor suppressor miRNAs because they target several oncogenes, suppress proliferation and invasion, and their expression is often lower in tumors compared to their levels in normal tissues (Johnson *et al*., 2007, 2005; Schultz *et al*., 2008; Zhao *et al*., 2014). Accordingly, analysis of the MICMA breast cancer cohort data (B) indicated that let-7b expression was lower in tumors of a higher grade or aggressive subtype (HER2, basal-like), as well as in tumors with positive HER2 status, negative ER status, and mutated p53. Lower let-7b level also correlated significantly with poor relapse-free survival of the patients (II, Fig 7). Importantly, of all the 20 different mature let-7 family miRNAs, the expression of let-7b-5p differed the most between different grade tumors (II, Fig S5). Moreover, the observed let-7b expression pattern in different molecular tumor subtypes and the miRNA correlation with tumor HER2 and ER status were confirmed in a larger breast cancer cohort dataset (C, II, Fig S4).

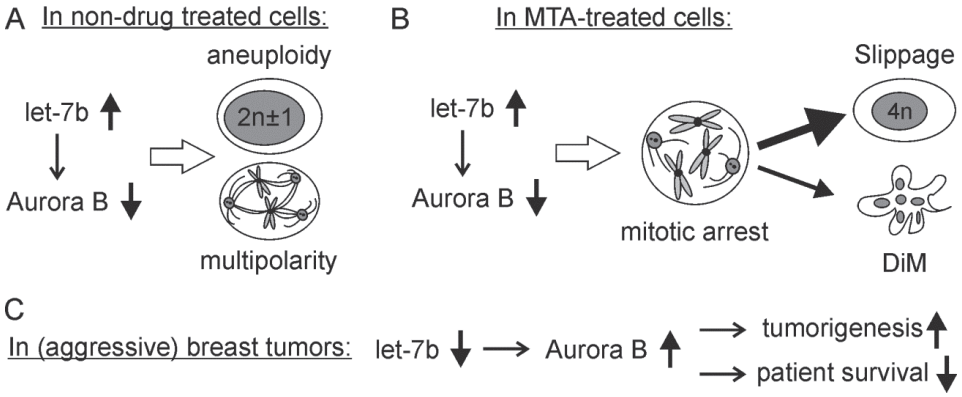

Figure 12. A graphic representation of how excess let-7b affects (A) mitosis and genomic balance in non-drug treated cells and (B) cell fate upon MTA treatment. The thickness of the arrows represents the relative frequency of the fate in B. (C) The role of the let-7b/Aurora B –axis in breast cancer *in vivo*. DiM=death in mitosis, MTA=microtubule targeting agent.

5.3 miR-193a-3p controls mitosis by targeting the tumor suppressor Rassf1a

5.3.1 miR-193a-3p is a direct regulator of Rassf1a expression

Rassf1a is a tumor suppressor protein that has been implicated in M-phase control: it has been reported to control cytokinesis and mitotic timing, perhaps via the inhibition of APC/C activity in early prometaphase (Guo *et al*., 2007; Song *et al*., 2004, 2009a). As the miRNA-mediated control of Rassf1a has remained largely unexplored, we performed a screen for *RASSF1A* targeting miRNAs that would cause mitotic anomalies when abnormally expressed. For this screen, we selected both miRNAs (n=55) that have a predicted binding site in the *RASSF1A* 3'UTR and miRNAs (n=19) that showed a significant negative correlation (< -0.22) with *RASSF1A* mRNA expression in a breast cancer cohort data (B, MICMA). By assessing the miRNAs ability to induce mitotic errors related to the earlier reports on Rassf1a suppression phenotypes (multipolarity, polyploidy, lagging chromosomes) and/or to lower the *RASSF1A* mRNA and protein expression, we identified 8 potential hit miRNAs (III, Fig 1). One of these, miR-181a-5p, was excluded from the study, as it was already reported to target *RASSF1A* at the beginning of our project(Bräuer-Hartmann *et al*., 2015; Meng *et al*., 2012), which provided validation of our screening strategy.

The primary aim of the study was to identify miRNAs that regulate Rassf1a expression directly. When the binding of the hit miRNAs to the *RASSF1A* 3'UTR was experimentally tested with luciferase reporter assay, only miR-193a-3p was found to directly target *RASSF1A* 3'UTR (III, Fig 2A). We further confirmed that miR-193a-3p significantly suppressed *RASSF1A* mRNA and protein expression in different human cancer cell lines (HeLa, HCT116, OVCAR-8) by 30-50 % (III, Fig 2C-D). Importantly, inhibition of the endogenous miR-193a-3p with a miRNA inhibitor (anti-miR-193a-3p) resulted in 1.5 –fold upregulation of Rassf1a protein (III, Fig 2E), thereby suggesting that miR-193a-3p is a biologically relevant regulator of Rassf1a expression.

5.3.2 Excess of miR-193a-3p impairs cytokinesis

To get more detailed information on the miR-193a-3p induced mitotic defects, we performed live-cell imaging assays in synchronized HeLa and OVCAR-8 cells. In both cell lines, a significant proportion of the cells overexpressing miR-193a-3p failed to undergo proper cytokinesis (on average 18.7 and 8.0%, respectively). This

defect resulted in an increased frequency of polyploid, multinuclear interphase cells. Also the duration of mitosis (NEBD-to-anaphase) was slightly longer (8 min) in cell population with excess miR-193a-3p, but the M-phase duration did not correlate with the cytokinesis failure (III, Fig 3).

Rassf1a regulates cytokinesis by engaging the SNARE protein STX16 to the spindle midzone in telophase. STX16 then recruits essential proteins of the cytokinesis executing machinery to the midbody. (Neto *et al*., 2013; Song *et al*., 2009a) We confirmed deregulation of the Rassf1a-STX16 pathway by miR-193a-3p; STX16 was repressed and mislocalized in cells that overexpressed the miRNA. In the interphase cells, the normally compact STX16 staining at Golgi/endosomal compartment was dispersed to a two times larger area, and STX16 levels were significantly diminished at the midbodies of the telophase cells. We also observed a slight, but variable, downregulation in the midbody signal of CEP55 that is recruited by STX16. At the population scale, STX16 protein levels were suppressed by 40% in cells with an excess of miR-193a-3p (III, Fig 4).

5.3.3 miR-193a-3p overexpression induces multipolar mitotic spindles, mitotic arrest and cell death

The cells that fail in cytokinesis typically exhibit extra centrosomes that can cause problems in spindle arrangement and chromosome alignment in the subsequent M-phase. On average, 47% of the mitotic cells in miR-193a-3p overexpressing population exhibited a multipolar spindle (III, Fig 5A). In line with the observed polyploidization, in almost half of the multipolar cells, all poles were centrin-3 positive. However, the partial lack of centrioles in the majority of multipolars suggests that excess miR-193a-3p also induced *de novo* centrosome defects, such as PCM fragmentation (III, Fig 5B).

Multipolar mitotic spindles, despite their possibly transient nature, can increase genomic instability through the induction of lagging chromosomes (Ganem *et al*., 2009; Silkworth *et al*., 2009). However, no gross aneuploidy was detected with miR-193a-3p overexpression in FISH assays, nor did the chromosomes exhibit any major structural changes in metaphase spreads.

In line with the observed mitotic anomalies and the longer duration of mitosis, the mitotic index increased in miR-193a-3p overexpressing cell populations, suggesting the presence of a transient mitotic delay. In addition to an accumulation into the M-phase, excess of miR-193a-3p induced a marked increase in cell death frequency as assessed by microscopy and flow cytometry (III, Fig 5C and data not shown).

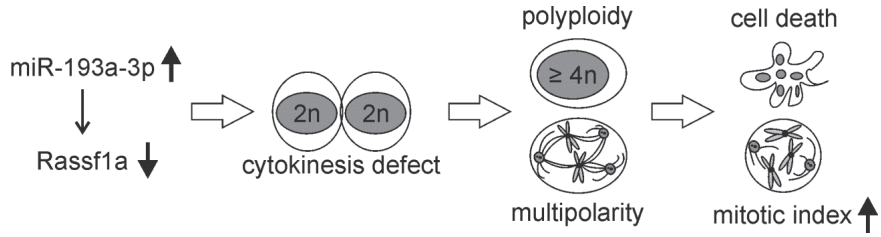

Figure 13. A summary of miR-193a-3p's Rassf1a-dependent mitotic effects and their cellular consequences.

5.4 c-Myc mediates taxane sensitivity through upregulation of Bcl-xL regulating miRNAs, miR-203a-3p and miR-203b-3p

5.4.1 miR-203b-3p promotes the sensitivity of cancer cells and patients to taxane treatment

The anti-apoptotic Bcl-2 family member, Bcl-xL, is a component of the intrinsic apoptosis pathway and an important regulator of taxane-mediated cell death and cancer cell drug sensitivity (Bennett *et al*., 2016; Topham *et al*., 2015; Wong *et al*., 2012). As not much is known about the regulation of Bcl-xL expression, we aimed to study the miRNA-mediated control Bcl-xL and thus, taxane sensitivity. To this end, we selected four potential *BCL2L1* targeting miRNAs based on their predicted binding to *BCL2L1*, negative correlation with *BCL2L1* and positive correlation with cancer cells' taxane response (NCI cell line data panel and/or literature), and correlation with patient survival in at least one of the studied cancer cohorts (A, D, and F). We then tested the effect of these four miRNAs on taxane sensitivity in breast and ovarian cancer cell lines. In live-cell imaging experiments, only overexpression of miR-203b-3p proved to significantly enhance cell death from 10nM paclitaxel in all four tested cell lines (MDA-MB-231 SA, MCF-7, OVCAR-8, CaOV-3; IV, Fig 1). The frequency of death in mitosis (DiM) was increased in all cell populations that overexpressed miR-203b-3p, but post-mitotic death (PMD) was increased by the miRNA only in the breast cancer cell lines. For example, in MDA-MB-231 SA cells, both DiM and PMD were equally increased, raising the total frequency of death from the 25.4% in the control population to 54.7% in the miR-203b-3p overexpressing cells. Furthermore, the onset of both DiM and PMD was accelerated by excess miR-203b-3p in MDA-MB-231 SA cells (IV, Fig 1).

Corroborating the live-cell imaging assay results, the cell proliferation measurement in MDA-MB-231 SA and CaOV-3 cells after the 48h paclitaxel treatment

indicated on average a 34% and 23% decrease in proliferation, respectively (IV, Fig 2B). At the same timepoint, the expression of cell death marker proteins, cPARP and cleaved caspase 3, were also increased in the miR-203b-3p overexpressing cell populations when compared to the controls (IV, Fig 2A).

As taxanes are used as part of neoadjuvant chemotherapy in breast cancer treatment (Frasci *et al*., 2009; Henderson *et al*., 2003) and the miR-203b-3p-mediated sensitization to paclitaxel *in vitro* was strongest in the breast cancer cell lines, we analyzed miR-203b-3p expression in relation to the therapy response and patient survival in two breast cancer cohort datasets (A and D). In the TCGA II cohort (D), high miR-203b expression significantly correlated with better patient survival (IV, Fig 2C), but no similar pattern was observed in the Bergen cohort (A; IV, data not shown). However, we did notice a slightly higher miR-203b-3p expression in the patient groups that exhibited a complete or partial response to paclitaxel therapy, when compared to the patients with poorer therapy response (IV, Fig 2D-E). Moreover, the observation by Topham and colleagues of high *MYC* expression in breast cancer patients that respond well to therapy was confirmed in the Bergen cohort. Higher *MYC* expression clearly correlated with the better survival of breast cancer patients, but interestingly only in the taxane-treated group and not in the epirubicin therapy group (IV, Fig 4E-F, S2B).

5.4.2 miR-203b-3p directly regulates Bcl-xL expression

To verify Bcl-xL suppression by miR-203b-3p that most likely explains the miRNA-induced sensitization to taxanes, we studied miR-203b-3p binding to the *BCL2L1* mRNA 3'UTR and the miRNAs capability to suppress Bcl-xL levels. Luciferase reporter assay confirmed that miR-203b-3p truly binds to the *BCL2L1* 3UTR (IV, Fig 4A), enabling it to regulate the gene's expression. Further still, miR-203b-3p suppressed Bcl-xL protein expression in all human cancer cell lines that were studied (MDA-MB-231 SA, MCF-7, CaOV-3) (IV, Fig 4B). Moreover, miR-203b-3p demonstrated a significant negative correlation with *BCL2L1* mRNA expression in the Bergen breast cancer cohort dataset (A, -0.15, p=0.04; IV, Fig 3C).

5.4.3 c-Myc potentially controls the expression of Bcl-xL regulating miRNAs, miR-203a-3p and miR-203b-3p

Analysis of the Bergen breast cancer cohort data (A) also indicated a positive correlation between *MYC* and miR-203b-3p (0.15) in the taxane therapy group, but not in the epirubicin therapy arm (-0.06; IV, Fig 3E). Interestingly, *MIR203B* is

genomically clustered with *MIR203A*, the mature miRNA product of which has been previously reported to indirectly suppress Bcl-xL expression (Li *et al*., 2011). In the cohort dataset, miR-203a-3p exhibited a similar positive correlation with *MYC* in the taxane-treated patients only (0.17; IV, Fig 3F) as miR-203b-3p, and the expression of these two miRNAs also correlated strongly (0.69, $p<0.001$; IV, Fig 3D). Based on these results and the notion that c-Myc is known to regulate the expression of several miRNAs (Bueno *et al*., 2011; Chang *et al*., 2008; Sampson *et al*., 2007) and has potential binding sites upstream of the miR-203 genes, we hypothesized that c-Myc acts as a transcription factor for both miRNAs. Silencing of c-Myc by RNAi in CaOV-3 cells (< 10% of the endogenous c-Myc protein left) suppressed the miR-203a-3p and miR-203b-3p expression by an average of 39% and 65%, respectively. Interestingly, the repression of c-Myc affected miRNA expression only when cells were treated overnight with 10nM paclitaxel before harvesting (IV, Fig 4D and S2A).

The potential c-Myc induced upregulation of the Bcl-xL suppressing miRNAs, miR-203b-3p and miR-203a-3p, is a possible mechanism for the c-Myc-mediated Bcl-xL repression, which has been reported before, but the mechanism has remained unclear (Eischen *et al*., 2001; Topham *et al*., 2015). A significant negative correlation between *MYC* and *BCL2L1* was found also in the Bergen breast cancer cohort (A, -0.26, $p<0.001$; IV, Table S1) and *BCL2L1* expression was significantly lower in tumors with high *MYC* when compared to the cases with low *MYC* (IV, Fig 3A). Similar to the correlations between *MYC* and miR-203a/b, the *MYC*/*BCL2L1* correlation was stronger in the taxane-treated patient group (-0.46, $p<0.001$) and was not present at all in the epirubicin therapy arm (-0.03; IV, Fig 3B).

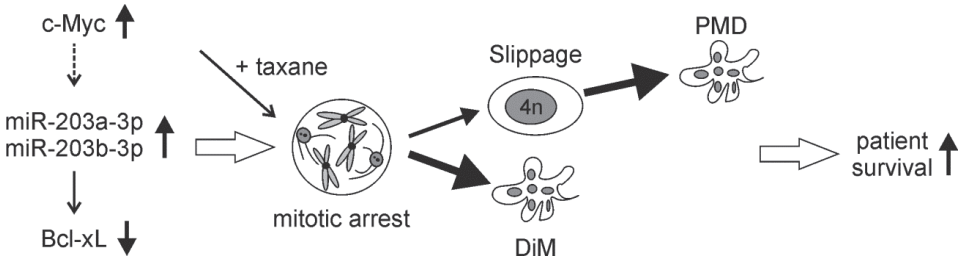

Figure 14. A summary of how the c-Myc-miR-203a/b-Bcl-xL –axis may modulate taxane sensitivity *in vitro* and *in vivo*. The thickness of the arrows represents the relative frequency of the fate in taxane-treated miR-203 overexpressing cells/tumors. DiM=death in mitosis, PMD=post-mitotic death.

6 DISCUSSION

6.1 Identification and target validation of mitosis-regulating miRNAs (I-IV)

The discovery of mitosis-regulating miRNAs in this thesis work was based on two different screening approaches: a microscopy-based functional screen and a target gene-driven *in silico* screen. Publications I and II were based on hits from the anti-mitotic phenotype screen, which was performed using a human miRNA mimic library (Winsel *et al*., 2014). As the number of known miRNAs has increased rapidly over the past few years, we later utilized a time and money saving target gene-based screening approach for identification of Rassf1a and Bcl-xL regulating miRNAs (III, IV). Both screening approaches were proven successful in this thesis work: at least one hit miRNA, whose overexpression caused an anticipated phenotype by suppressing the expected target gene, was discovered in each project. However, the anti-mitotic phenotype screen yielded more hit miRNAs compared to the target gene –driven screens. It should be also noted that the *in silico* –filtering of the candidate miRNAs in the latter screens is also slightly prone for human influence.

Identification and validation of the hit miRNA target genes is a challenging step in the phenotype-based screening approach. We tackled this question by using miRNA target gene prediction softwares (I, II) and global mRNA expression analysis (I). Different target prediction tools were also applied in the *in silico* screens for the selection of potential *RASSF1A*- and *BCL2L1*-targeting miRNAs, together with other databases (*e.g.* NCI cell line database) and a literature survey (III, IV). The binding prediction softwares are helpful but the results should be interpreted with caution as their algorithms may *e.g.* overly emphasize perfect sequence complementarity in miRNA-mRNA interactions (Ritchie and Rasko, 2014). On the other hand, the genome-wide gene expression analyses may be useful for recognizing the biologically relevant miRNA targets, but they do not include those rare targets that are repressed only at the protein level. All of the miRNAs studied in this thesis were experimentally validated to bind directly to the target mRNAs' 3'UTRs and suppress their expression (I-IV).

miRNA-induced changes in target gene expression are usually subtle and miRNAs are regarded as fine-tuners of gene expression (Baek *et al*., 2008; Bartel, 2009). In this thesis work, a variable degree of target gene repression (15-70%) by the different miRNAs was observed in various *in vitro* models. The intensity of target gene suppression may depend on *e.g.* the abundance of other miRNA target genes and the amount of Argonaute proteins in the cell (Janas *et al*., 2012; Seitz, 2009).

Importantly, SAC is very dependent on protein stoichiometry and sensitive to even a 20% reduction in a checkpoint protein quantity (Heinrich *et al*., 2013), making mitosis vulnerable for altered miRNA levels.

miRNA and target gene expressions were also retrospectively studied *in vivo* using breast and ovarian carcinoma cohorts. Supporting the findings observed in cell models, miR-493-3p and *MAD2* showed opposite expression patterns in ovarian carcinoma (I, Fig 5) and a negative correlation was detected between let-7b and *AURKB* in two independent breast cancer datasets (II, Fig 7G and S4D). Similarly, miR-203b-3p had a significant negative correlation with *BCL2L1* in the Bergen breast cancer cohort (IV, Fig 3C). In contrast, no significant correlation between miR-193a-3p and *RASSF1A* mRNA expression was found in ovarian or breast cancer (cohorts F and A). Moreover, any of the miRNAs that were selected for the analysis based on their negative correlation with *RASSF1A* mRNA expression in the MICMA breast cancer cohort were found not to directly regulate Rassf1a *in vitro* (III, Fig 1B and 2A). In conclusion, the degree of the target gene suppression *in vitro* does not seem to predict the interdependence *in vivo*. However, other gene regulation mechanisms may; for example *RASSF1A* is frequently silenced through hypermethylation early in tumorigenesis (Donninger *et al*., 2007; Hung *et al*., 1995; Klajic *et al*., 2013; Wistuba *et al*., 1997) and similar epigenetic regulation of miR-193a-3p has also been reported (Heller *et al*., 2012; Kozaki *et al*., 2008; Pronina *et al*., 2017; Seviour *et al*., 2016). Further, it is possible that in the tumors, miR-193a-3p targets one of the less redundant *RASSF1* isoforms, for example *RASSF1C*, which is not subjected to hypermethylation. Still, miR-193a-3p may be a biologically relevant regulator of Rassf1a expression *in vivo* in certain contexts. However, proving this notion correct would be challenging as it would require a specific cancer model in which *RASSF1* is not hypermethylated (Bräuer-Hartmann *et al*., 2015; Donninger *et al*., 2007) or alternatively, samples from very early stage tumors that are difficult to obtain.

6.2 Altered levels of mitosis-regulating miRNAs disturb mitotic timing (I-III)

Aurora B has a dual role in the SAC: the kinase is needed for resolving erroneous kt-mt attachments (Cheeseman *et al*., 2006; Hauf *et al*., 2003) but it also initiates MCC recruitment to unattached kinetochores (Ditchfield *et al*., 2003; Hauf *et al*., 2003). We observed an interesting transient mitotic arrest in a subset of non-drug treated cells overexpressing let-7b (II, Fig 1). The same was noted when cells were treated with low concentrations of the Aurora B inhibitor Barasertib. Let-7 also targets other cell cycle genes, Cdc25A and cyclin D1, however, suppression of

these genes should not cause M-phase arrest but rather a G2 and G1 arrest, respectively (Johnson *et al*., 2007; Schultz *et al*., 2008). Thus, the mitotic arrest in the let-7b overexpressing cells is most likely due to Aurora B suppression and the consequent presence of uncorrected, erroneous kt-mt attachments.

The effect of Mad2 depletion on mitotic timing is more straightforward: in unperturbed cells mitosis was accelerated after Mad2 reduction and prolonged in cells with excess Mad2, as was demonstrated by the manipulation of miR-493-3p levels (I, Fig 1F and 4D). The leaky SAC in Mad2 deficient cells is an obvious explanation for the accelerated mitosis. However, the kinetochore independent function of Mad2, as a mitotic timer, probably also plays a role (Meraldi *et al*., 2004; Rodriguez-Bravo *et al*., 2014). On the other hand, the mitotic delay in the Mad2 overexpressing cells (Hernando *et al*., 2004; Sotillo *et al*., 2007) may be induced by the presence of hyperstable kt-mt attachments (Kabeche and Compton, 2012).

A moderate prolongation of mitosis was also observed during miR-193a-3p overexpression (III, Fig 3), as has been reported before to occur in *RASSF1A* depleted cells (Guo *et al*., 2007; Tommasi *et al*., 2011). However, the mechanism behind this phenomenon remains unclear. We speculate that the observation relates to the centrosomal defects caused by reduced Rassf1a expression (III, Fig 5), possibly causing problems in the spindle assembly and chromosome alignment that delay mitotic progression (Gisselsson *et al*., 2008; Yang *et al*., 2008).

6.3 Cytokinesis perturbing miRNAs cause polyploidization and spindle abnormalities (II, III)

The first reports describing the role of the tumor suppressor Rassf1a in mitosis suggested that the protein functions as an early prometaphase timer, which binds to Cdc20, inhibits APC/C activity and controls the early phases of mitosis (Song *et al*., 2004, 2009b). However, later studies have questioned the Cdc20-binding capacity of Rassf1a and demonstrated that *RASSF1A*-depleted cells exhibit a mitotic delay along with the failure of cytokinesis (Guo *et al*., 2007; Liu *et al*., 2007; Song *et al*., 2009a; Tommasi *et al*., 2011). Our observations from cells that overexpress a *RASSF1A*-targeting miRNA (miR-193a-3p) are comparable to the latter phenotype (III, Fig 3). The severity of the miR-193a-3p-induced cytokinesis failure is also in line with the relatively low penetrance (c. 35%) of the defect even upon complete loss of *RASSF1A* (Guo *et al*., 2007; Tommasi *et al*., 2011). Deficiency of the downstream Rassf1a effector, STX16 (Song *et al*., 2009a), at the spindle midzone in miR-193a-3p overexpressing telophase cells reinforces the notion that the defect is linked with Rassf1a inhibition (III, Fig 4). Interestingly, *STX16* 3'UTR also harbors a predicted binding site for miR-193a-3p. Since no

decrease of *STX16* mRNA expression or overall signal intensity in immunofluorescence experiments was observed in miR-193a-3p overexpressing cells, it seems reasonable to speculate that the STX16 deregulation is a consequence of the Rassf1a suppression. Moreover, the observed decrease in STX16 protein levels in immunoblotting after miR-193a-3p overexpression may be due to the protein's destabilization after its delocalization from the Golgi/endosomal compartment in the interphase cells with reduced Rassf1a levels. However, the possibility of direct translational STX16 suppression by miR-193a-3p cannot be fully excluded since we did not test the miRNA binding to *STX16*. (III, Fig 4) Direct suppression of two cytokinesis regulators from the same signaling pathway (*RASSF1A*, *STX16*) might explain how the moderate suppression of Rassf1a expression by miR-193a-3p in HeLa cells results in a cytokinesis defect frequency that is nearly comparable to the *RASSF1A* knockout cells.

In addition, the let-7b target gene Aurora B localizes to the spindle midzone in telophase and, as a master regulator of cytokinesis, is involved in the contractile ring assembly (Basant *et al*., 2015), midzone microtubule stabilization (Landino and Ohi, 2016), and abscission (Steigemann *et al*., 2009). Phosphorylation by Aurora B also governs Rassf1a localization to the midzone (Song *et al*., 2009a). A very small proportion (5 %) of the unperturbed HeLa cells overexpressing let-7b failed in cytokinesis (II, Fig 2B). However, the observed slight increase of polyploidization by let-7b on its own, and more so in a combination with a chemical Aurora B inhibitor (II, Fig 2D and 6), is likely a consequence of an aberrant exit from mitosis without a proper execution of cytokinesis (Ditchfield *et al*., 2003; Kallio *et al*., 2002; Marxer *et al*., 2014).

Cytokinesis failure typically results in the presence of two centrosomes in the progeny cell. After their duplication in S-phase, the extra centrosomes can cause defects in the spindle assembly and chromosome segregation at the following M-phase (Gisselsson *et al*., 2008; Kwon *et al*., 2008; Yang *et al*., 2008). Accordingly, a significant increase of multipolar mitotic spindles was observed after let-7b (II, Fig 5) and miR-193a-3p transfection (III, Fig 5), as has been also reported upon Aurora B inhibition (Long *et al*., 2008) and *RASSF1A* depletion (Song *et al*., 2004). In the case of miR-193a-3p, it should be noted that probably *de novo* centrosome defects also contribute to the observed multipolarity, since half of the multipolar cells exhibited acentriolar pole(s). The centrosome fragmentation in miR-193a-3p overexpressing cells may be explained by the lack of Rassf1a at the centrosomes, as the phenotype is similar to the defect described upon the loss of the centrosomal Rassf1 binding partner, microtubule associated protein 1S (Dallol *et al*., 2007; Song *et al*., 2005). Additionally, prometaphase delay can induce precocious centriole disengagement and PCM fragmentation (Karki *et al*., 2017). This mechanism may contribute to the let-7b-induced multipolarity as a subset of the let-7b

overexpressing cells transiently arrest in mitosis, however, the moderate mitotic delay (<1h) in miR-193a-3p overexpressing cells is unlikely to affect centrosome integrity.

6.4 Anti-mitotic miRNAs' effect on genomic balance and tumorigenesis (I-III)

Both up- and downregulation of Mad2 expression have been shown to lead to genomic instability in various *in vitro* and mouse models (Hernando *et al*., 2004; Lentini *et al*., 2012; Michel *et al*., 2001; Rowald *et al*., 2016; Schvartzman *et al*., 2011; Sotillo *et al*., 2007, 2010). The Mad2 deficiency-induced aneuploidy results from chromosome segregation defects, which we also observed occurring in miR-493-3p overexpressing cells (I, Fig 3B). As expected, the frequency of aneuploidy was elevated in the cells with excess miR-493-3p (I, Fig 3C). Moreover, inhibition of miR-493-3p, which upregulated Mad2 levels, caused a significant increase in the number of lagging chromosomes and chromatin bridges (I, Fig 4E). We did not measure how the suppression of miR-493-3p and upregulation of Mad2 affects the genomic balance but, based on an earlier study (Kabeche and Compton, 2012), we speculate that the frequency of aneuploidy would be elevated in these cells. Aneuploidy induced by excess Mad2 is relevant for cancer biology because Mad2 overexpression is common in tumors, especially in the high-grade subtypes (Hernando *et al*., 2004; Li *et al*., 2003; Tanaka *et al*., 2001; Zhang *et al*., 2008b). Furthermore, our finding that the high-grade ovarian tumors exhibit elevated *MAD2* and decreased miR-493-3p compared to the clear cell tumors or normal epithelium (I, Fig 5), support the view that Mad2 overexpression is more relevant for *in vivo* tumorigenesis than Mad2 deficiency. The reason for this remains unclear, but it has been hypothesized that Mad2 deficiency *in vivo* induces severe genomic imbalance, intolerable for cells, resulting in suppression of tumorigenesis (Dobles *et al*., 2000; Michel *et al*., 2004). Although this is contradictory to the genomic imbalance observed in miR-493-3p overexpressing cultured cells, it should be noted that the severely aneuploid cells may have been eliminated from the cell population. This notion is supported by the fact that we detected very few cells with more than four or zero copies of a chromosome (I, Table S2). Instead, cell death and senescence were increased in cell populations with excess miR-493-3p (Fig 8; I, Fig 3D and S4). Moreover, it is possible that tumor cells *in vivo* are more prone to die in response to elevated aneuploidy than cultured human cancer cell lines.

Interestingly, fairly similar frequency of aneuploidy was also observed in HCT116 cell population with excess let-7b and diminished Aurora B expression (II, Fig 4C). Similar to Mad2 and certain other checkpoint proteins, up- and

downregulation of *Aurora B* induces CIN and promotes tumorigenesis in mouse models (Fernández-Miranda *et al*., 2011; González-Loyola *et al*., 2015). The potential mechanisms for Aurora B-mediated aneuploidy are diverse. In cells, reduced Aurora B activity causes problems in chromosome alignment and segregation due to improper correction of kt-mt attachment errors, as well as a cytokinesis defect leading to polyploidization and centrosome amplification (Ditchfield *et al*., 2003; Hauf *et al*., 2003; Kallio *et al*., 2002). On the other hand, Aurora B overexpression also destabilizes correct kt-mt attachments at least in yeast cells, and has been shown to impair cytokinesis and cause aneuploidy in cultured yeast and human cells (Munoz-Barrera and Monje-Casas, 2014; Tatsuka *et al*., 1998). In the studied breast cancer cohorts, let-7b expression was found to be low and *AURKB* expression high in the poorly differentiated (grade 2 and 3) and aggressive (basal-like, HER2 positive) tumors in comparison to the low-grade and luminal-type neoplasms (II, Fig 7 and S4). Moreover, low let-7b levels were associated with poorer survival of breast cancer patients. These findings are in accordance with the reported Aurora B overexpression in different types of tumors and the correlation of this feature with patients' poor prognosis (Chen *et al*., 2009; Pannone *et al*., 2011; Takeshita *et al*., 2013). The effect of Aurora B overexpression on genomic balance in cultured cell could not be directly assessed in this thesis work due to the high toxicity of the excess Aurora B in our experimental settings.

In contrast to the SAC abrogating miRNAs, no gross structural or numerical aneuploidy was detected in cells with excess of the *RASSF1A* targeting miR-193a-3p. This is a fairly unexpected finding considering the high frequency of multinuclear interphase cells and multipolar mitotic cells in miR-193a-3p overexpressing population (III, Fig 3C and 5). The answer may rely in the mitosis-independent apoptosis-promoting function of miR-193a-3p: miR-193a-3p increased cell death also in this study (III, Fig 5D). We speculate that this could be caused by suppression of the anti-apoptotic Mcl-1 protein that is a direct target of the miRNA (Kwon *et al*., 2013). Thus, the potentially aneuploid cells may be eliminated from the cell population through cell death induction. In this context, it is important to note that even *Rassf1a* null mice do not exhibit any gross genomic aberrations (van der Weyden *et al*., 2005). However, simultaneous loss of another tumor suppressor protein may be enough to increase aneuploidy, as has been demonstrated with *Rassf1a/p53* knock-out mouse models (Tommasi *et al*., 2011).

6.5 Mitosis-regulating miRNAs and the response of tumor cells to MTA treatment (I, II, IV)

In cultured human cancer cells, excess of miR-493-3p or let-7b impairs the SAC and promotes slippage from MTA treatment induced mitotic block (I, Fig 1; II, Fig 1). The same has been previously reported to occur upon reduction of Mad2 levels (Li and Benezra, 1996; Michel *et al*., 2004, 2001) or Aurora B activity (Ditchfield *et al*., 2003; Hauf *et al*., 2003; Kallio *et al*., 2002; Marxer *et al*., 2014). Divergent endogenous miR-493-3p expression also correlates with the intrinsic sensitivity to paclitaxel in ovarian carcinoma cells (I, Fig 6 and 7). In addition to the SAC override, miR-493-3p promotes senescence via suppressing Mad2, which may help the cancer cells to escape the drug-induced cell death (Prencipe *et al*., 2009; Weiner-Gorzel *et al*., 2015). In ovarian and breast carcinoma cohorts, high miR-493-3p significantly correlated with poor disease-specific survival of patients with high and grade III tumors, respectively (I, Fig 7). Together with the results from cell-based studies, this finding can be interpreted as an indication of miR-493-3p induced therapy resistance against paclitaxel, which is routinely used as a part of adjuvant chemotherapy for advanced breast and ovarian carcinomas (Frasci *et al*., 2009; Henderson *et al*., 2003; McGuire *et al*., 1996; Vasey *et al*., 2004). Furthermore, in the breast cancer cohort the miR-493-3p association to survival was seen only in the paclitaxel treated patients and not in the epirubicin therapy group (I, Fig 7), reinforcing the hypothesis of miRNA-mediated MTA resistance in these patients. Previous studies regarding the correlation of Mad2 expression with cancer patient survival have yielded variable results (Furlong *et al*., 2012; Hernando *et al*., 2004; Zhang *et al*., 2008b). The reason for high Mad2 in some studies and low Mad2 levels in others being associated with a poor prognosis remains elusive. A possible explanation may lie in the treatment of the patients (MTA or non-MTA) or other clinicopathological features of the tumors; for example, in ovarian carcinoma, the correlation of low Mad2 expression with reduced survival has been established in several independent studies (Furlong *et al*., 2012; Park *et al*., 2013; Sumi *et al*., 2012). Cancer patient survival is also greatly affected by tumor metastasis. However, although miR-493-3p has been previously reported to suppress the invasive capacity of cancer cells through different target genes (Gu *et al*., 2014; Okamoto *et al*., 2012; Sakai *et al*., 2014; Ueno *et al*., 2012), the possible increase in the invasion potency does not appear related to the high miR-493-3p correlation with poor survival.

miR-433 has also been validated as targeting *MAD2* and high expression of the miRNA, as well as low expression of Mad2, induce paclitaxel resistance in ovarian cancer cells and correlate with poor survival in high-grade ovarian tumors (Furlong *et al*., 2012; Weiner-Gorzel *et al*., 2015). Interestingly, *MIR433* together with *MIR493*, is located in a big miRNA cluster that is often deregulated in cancer

(Manodoro *et al*., 2014; Zehavi *et al*., 2012; Zhang *et al*., 2008a). Potential simultaneous genomic upregulation of both *MAD2* targeting miRNAs may yield an immense effect on drug response in, for example, ovarian tumors. The possible role of the third validated *MAD2* targeting miRNA, miR-28-5p (Hell *et al*., 2014; Schneider *et al*., 2014), in drug sensitivity remains to be studied.

Compared to the miR-493-3p, the connection of let-7b with breast cancer patient survival is not as straightforward. In an analysis of the MICMA breast cancer cohort data, low let-7b levels significantly correlated with poor patient prognosis (II, Fig 7). This finding is not in concordance with the MTA-resistance observed in let-7b overexpressing cultured human cancer cells. On the other hand, it correlates with previous reports indicating high Aurora B expression as a predictor of a poor prognosis (Chen *et al*., 2009; Pannone *et al*., 2011; Takeshita *et al*., 2013) as well as with our findings from two breast cancer cohorts showing decreased let-7b levels aggressive tumors (II, Fig 7 and S4). No detailed information about the treatment of the patients in these cohorts was available for the analyses (Naume *et al*., 2001) but it is likely that many of them received targeted therapeutics (Herceptin, tamoxifen) in combination with other chemotherapeutics such as taxanes and DNA damaging drugs. Thus, the let-7b correlation to patient survival in this context may portray more the malignancy of the tumors, which may depend on Aurora B, as well as other known let-7b target oncogenes (Johnson *et al*., 2007, 2005). Interestingly, let-7b may actually improve MTA response and patient prognosis through an SAC-independent mechanism. The let-7 family has been reported to suppress the anti-apoptosis protein Bcl-xL (Shimizu *et al*., 2010), suppression or inhibition of which promotes death in MTA-treated cells (Bennett *et al*., 2016; Topham *et al*., 2015; Wong *et al*., 2012). On the other hand, let-7 also suppresses c-Myc (Sampson *et al*., 2007), which has an opposite effect on cell fate upon MTA treatment (Topham *et al*., 2015). In contrast to causing resistance to MTAs through SAC override *in vitro*, let-7b had a synergistic effect on polyploidy induction with another anti-mitotic compound, the Aurora B inhibitor Barasertib. The likely explanation for this controversy is the different mechanism of action of the drugs: Barasertib drives the cells through an aberrant mitosis rather than causing, as MTAs do, a SAC-dependent mitotic arrest (Yang *et al*., 2007). Interestingly, let-7b did not enhance cell death upon Barasertib treatment (II, Fig 6). Likewise, the chemical inhibition of the let-7 target Bcl-xL does not sensitize cells to drugs that induce a forced exit from abnormal mitosis, such as Aurora kinase inhibitors, but only to drugs that induce a mitotic arrest (Bennett *et al*., 2016).

The anti-apoptosis protein Bcl-xL has been reported to be a critical mediator of c-Myc induced cell fate choice after anti-mitotic drug treatment (Topham *et al*., 2015). Accordingly, we observed the *BCL2L1* targeting miRNA, miR-203b-3p, to increase cell death frequency in cultured human cancer cells after treatment with

low dose paclitaxel (IV, Fig 1 and 2). Despite the consistent *in vitro* result from two breast and two ovarian carcinoma cell lines, only a modest trend toward high miR-203b-3p expression in patient groups with better survival or good response to paclitaxel therapy was found in the two analyzed breast cancer cohorts (IV, Fig 2C-E). Concurrently, high *MYC* expression was significantly associated with better survival of taxane-treated breast cancer patients (IV, Fig 4E-F).

A previous study showed that repression of one pro-apoptotic c-Myc target gene is not enough to fully mimic the effect of *MYC* silencing on MTA-response (Topham *et al*., 2015). Thus, the modest Bcl-xL suppression by an miRNA would probably require a concomitant depletion of another anti-apoptosis protein or up-regulation of a pro-apoptosis factor to display a strong association with MTA sensitivity *in vivo*, as in the case of c-Myc. Accordingly, only the broader Bcl-2 family inhibitors, ABT-263 and ABT-737, and not the more Bcl-2 specific inhibitor, ABT-199, have shown promising results in combination with taxane treatment in pre-clinical cancer models (Oakes *et al*., 2012; Wali *et al*., 2017; Wang *et al*., 2015). Since according to the target gene prediction softwares, miR-203b-3p may also suppress the levels of several pro-apoptosis proteins (*e.g.* Noxa and Bid), it is probably the balance of the miRNA-regulated anti- and pro-apoptosis proteins that determines the impact of miR-203b-3p on MTA-sensitivity in different contexts. Still, if the levels of redundant anti-apoptosis proteins, such as Mcl-1, are low and Bcl-xL expression is relatively high in a tumor, Bcl-xL inhibition alone may sensitize the tumor cells to anti-mitotic drugs (Bennett *et al*., 2016; Wong *et al*., 2012). Altogether, the results of this thesis effort suggest that c-Myc may be superior to Bcl-xL and miR-203a/b in predicting the MTA-response *in vivo*, yet inhibition or suppression of Bcl-xL can increase the cancer cells' sensitivity to taxanes.

An earlier study has shown that miR-203a-3p controls Bcl-xL expression and sensitizes colon cancer cells to paclitaxel (Li *et al*., 2011). These findings were elaborated by our observations that identified miR-203b-3p as a novel regulator of Bcl-xL expression and taxane sensitivity in breast and ovarian cancer cells (IV, Fig 1-2). Importantly, we identified a new potential component of this regulatory axis; we found that c-Myc may boost miR-203b-3p and miR-203a-3p expression to suppress Bcl-xL expression and enhance taxane efficacy (IV, Fig 3-4). Interestingly, c-Myc can also induce let-7 expression, which in turn represses Bcl-xL (Adams *et al*., 2016). Together these findings raise the possibility of the existence of a c-Myc mastered miRNA-signaling route that controls the fate of taxane-treated cells via Bcl-xL. This could be part of the unidentified mechanism of c-Myc induced Bcl-xL downregulation (Eischen *et al*., 2001) that is important for the c-Myc dependent coordination of taxane sensitivity (Topham *et al*., 2015). Still, more direct evidence on the c-Myc mediated transcriptional control of *MIR203A/B* is needed to fully prove the function of this regulatory axis.

6.6 The net-effect of mitosis- and taxane sensitivity regulating miRNAs in cancer (I-IV)

According to the data presented here, the effect of SAC protein targeting miRNAs seems to be tumor suppressive while overexpression of SAC proteins is associated with malignant behavior *in vivo* (I, II). Information about the status and degree of CIN in tumors might help to distinguish the underlying mechanism(s) of tumorigenesis. The effect of SAC targeting miRNAs on genomic balance *in vivo* is interesting also from the therapy angle since many anti-mitotic drugs under development aim for induction of high frequency aneuploidy (Hoar *et al*., 2007; Tardif *et al*., 2011).

We found that *MAD2* levels are higher in the more aggressive ovarian tumors but controversially high expression of the *MAD2* targeting miR-493-3p was found to be a marker of poorer prognosis for ovarian and breast cancer patients (I, Fig 5 and 7C-F). This disagreement may relate to the fact that an individual miRNA may have opposing effects on tumor initiation and treatment sensitivity depending on its target gene(s) (Svoronos *et al*., 2016). For the same reason it is often difficult to determine whether an miRNA has tumor suppressor or oncogenic role in cancer. In addition, the *RASSF1A* targeting miRNA, miR-193a-3p, displays controversial functions: high miR-193a-3p may promote malignant cell growth via inducing Rassf1-dependent cell division errors (III, Fig 3), although it has been previously reported to suppress cancer spreading via several target genes (Pu *et al*., 2016; Seviour *et al*., 2016; Yu *et al*., 2015). The *in vivo* effect of Bcl-xL or other cell death signaling proteins regulating miRNAs may be more straightforward and only related to treatment response, as inhibition of *e.g.* Bcl-2 proteins does not alter cell survival in untreated cell populations but only upon drug treatment (Oakes *et al*., 2012; Shi *et al*., 2011; Wang *et al*., 2015). Nevertheless, more pre-clinical research is needed to elucidate the potential of mitosis/MTA sensitivity regulating miRNAs for clinical use as biomarkers.

7 SUMMARY

Novel mitotic functions were discovered for three human miRNAs in this thesis research effort. Altered levels of miR-493-3p abrogated mitotic timing and chromosome segregation, as the miRNA directly controlled the expression of the SAC component, Mad2. The let-7b miRNA was found to regulate another mitotic protein, Aurora B kinase. Excess of let-7b also disturbed SAC function and caused an increased frequency of multipolar mitotic spindles. The third anti-mitotic miRNA that was identified, miR-193a-3p, was observed to regulate directly the expression of the tumor suppressor protein, Rassf1a. Excess of miR-193a-3p perturbed the last phase of mitosis, cytokinesis, leading to polyploidization and spindle abnormalities in the following M-phase. Overexpression of the SAC targeting miR-493-3p and let-7b, but not the cytokinesis controlling miR-193a-3p, induced significant aneuploidy in the cultured human cancer cells. This finding underscores the importance of the SAC in the maintenance of genomic balance.

Human tumors are characterized by genomic imbalance, which is also one of the potential factors contributing to initial tumor formation. Accordingly, the expression of miR-493-3p and let-7b was found to be deregulated in breast and ovarian tumors. Moreover, high or low expression of miR-493-3p and let-7b, respectively, correlated with a poor survival of ovarian and breast cancer patients. The connection of low let-7b expression to poor patient survival may be due to the tumor suppressor role of the miRNA. However, high expression of miR-493-3p in those patients with poor prognosis is possibly linked to taxane resistance and impaired SAC function, as was demonstrated occurring in miRNA overexpressing cultured cancer cells.

In addition to the three mitosis-regulating miRNAs, a fourth miRNA was discovered to regulate taxane sensitivity via a mechanism that is independent of the pathways that normally control mitosis; miR-203b-3p directly suppressed the expression of the anti-apoptotic protein Bcl-xL and sensitized breast and ovarian cancer cells to clinically relevant doses of paclitaxel. High expression of the miRNA was also associated, to some degree, with better paclitaxel therapy response and patient survival in breast cancer *in vivo*. Importantly, the results of this work introduce a prospect that miR-203b-3p together with another Bcl-xL regulating miRNA, namely miR-203a-3p, may be mediators for the clinically relevant c-Myc-induced sensitization to taxane therapy.

Based on the results presented here, it can be concluded that specific miRNAs are relevant regulators of mitosis, genomic balance, and the taxane sensitivity of cancer cells. However, careful studies with cell and animal models, as well as retrospective analyses of cancer cohorts, are still needed to reveal the precise

mechanisms of actions of these miRNAs and their biological relevance to cancer. Only then can their diagnostic value in tumor subtyping and the prediction of drug responses be further assessed in clinical settings. In the future, if the therapeutic development of miRNAs succeeds in bringing miRNA mimics and inhibitors to clinical use, the therapeutics that target these mitosis-regulating miRNAs could be useful tools in the fight against cancer.

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