# The role of B-Myb and its target genes in ES cell cycle progression

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Hohen Medizinischen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität
Bonn

Sarah Ann-Christin Bruweleit
aus Düren

2012

Angefertigt mit Genehmigung der
Medizinischen Fakultät der Universität Bonn

1. Gutachter: Prof. Dr. Georg Nickenig
2. Gutachter: Prof. Dr. rer. nat. Rainer Meyer

Tag der Mündlichen Prüfung: 30.10.2012

Medizinische Klinik und Poliklinik II – Innere Medizin des Universitätsklinikums Bonn
Direktor: Prof. Dr. Georg Nickenig
in Kooperation mit dem National Institute on Aging, Gerontology Research Center, Baltimore, Maryland, USA
Laboratory of Cardiovascular Science, Director: Prof. Ph.D. Kenneth Boheler

# Inhaltsverzeichnis

## Zusammenfassung

In der medizinischen Forschung wird große Hoffnung in embryonale Stammzellen und induzierte pluripotente Stammzellen gesetzt. Dies liegt vor allem an ihren besonderen Eigenschaften, wie die Fähigkeit sich zum Einen unbegrenzt selbsterneuern zu können sowie zum Anderen in Zellen aller drei Keimblätter differenzieren zu können. Daher stellen embryonale und induzierte pluripotente Stammzellen eine potentielle Grundlage in der Regenerativen Medizin in Form von Zellersatztherapien und Gewebezüchtung. Bevor diese möglichen Therapiestrategien allerdings Eingang in die klinische Versorgung von Patienten finden, ist zunächst von besonderer Bedeutung das komplexe regulatorische Netzwerk, das Pluripotenz und Selbsterneuerung in embryonalen Stammzellen ermöglicht, zu entschlüsseln und zu verstehen.

In diesem Zusammenhang erfährt der Transkriptionsfaktor B-Myb besondere Aufmerksamkeit, weil er in der Aufrechterhaltung von Pluripotenz, normaler Abfolge des Zellzyklus und chromosomaler Stabilität eine wichtige Rolle spielt. Da B-Myb die Expression von einigen Proteinen reguliert, die entweder mit dem Zellzyklus oder der Differenzierung von embryonalen Stammzellen in Verbindung gebracht werden, ergab sich die Fragestellung, ob einzelne dieser Zielgene eine Schlüsselfunktion ausüben oder ob B-Myb durch die Interaktion von mehreren Proteinen seine Funktion erfüllt. Aufgrund von vorangehenden Versuchen in somatischen Zellen, wurden die beiden Zielgene Aurora Kinase A und Cyclin B1 ausgewählt, um herauszufinden, ob sie in besonderer Weise zu der Funktion von B-Myb beitragen.

Im Rahmen meiner Doktorarbeit wurden Aurora Kinase A und Cyclin B1 mithilfe von microRNAs in embryonalen Stammzellen herunterreguliert und der Zellzyklus sowie der Spindelapparat während der Mitose untersucht. Besonderes Augenmerk lag darauf, ob der Phänotyp, der in früheren Experimenten durch Ausschaltung von B-Myb beobachtet wurde, durch Herunterregulierung eines der beiden Zeilgene nachgeahmt werden konnte. Im Gegensatz zu Ergebnissen aus somatischen Zellen führte ein partielles Ausschalten von Aurora Kinase A und Cyclin B1 in embryonalen Stammzellen allerdings nicht zu einem abnormen Zellzyklus oder Veränderungen während der Mitose. Der Phänotyp, der durch Abschalten von B-Myb erzielt wurde, konnte durch ein teilweises Herunterregulieren von Aurora Kinase A oder Cyclin B1 nicht reproduziert werden.

Demnach sind entweder mehrere Proteine für die Funktion des Transkriptionsfaktor B-Myb verantwortlich oder ein spezielles Zielgen, das bisher noch nicht untersucht wurde. Weitere Zielgene können in analoger Weise wie Aurora Kinase A und Cyclin B1 oder mithilfe der Ainv15 Zelllinie untersucht werden.

## List of Abbreviations

| | | |
|---|---|---|
| Aurka | - | Aurora Kinase A |
| bp | - | base pair(s) |
| Ccnb1 | - | Cyclin B1 |
| cdk | - | cyclin dependent kinase |
| ChIP | - | chromatin immunoprecipitation |
| CMV | - | Cytomegalovirus |
| Dapi | - | 4′,6-Diamidin-2-phenylindole |
| dsRNA | - | double-stranded RNA |
| DTT | - | Dithiothreitol |
| EDTA | - | Ethylenediaminetetraacetic acid |
| (Em)GFP | - | (Emerald) Green Fluorescent Protein |
| ES cell | - | embryonic stem cell |
| G1/G2-phase | - | gap1/gap2 phase |
| GAPDH | - | Glyceraldehyde-3-phosphate dehydrogenase |
| GTP | - | Guanosine-5'-triphosphate |
| h | - | hour |
| hES cell | - | human embryonic stem cell |
| HMG | - | high mobility group |
| Hprt | - | hypoxanthine-guanine phosphoribosyl-transferase |
| HRP | - | horse radish peroxidase |
| ICM | - | inner cell mass |
| IgG | - | Immunglobulin G |
| iPS cell | - | induced pluripotent stem cell |
| Kb | - | kilo base-pairs |
| LB medium | - | Lysogeny broth medium |
| LIF | - | Leukemia inhibitory factor |
| LINC | - | linker of nucleoskeleton and cytoskeleton |
| M-phase | - | mitosis phase |
| mES cell | - | mouse embryonic stem cell |
| miR, miRNA | - | microRNA |

| | | |
|---|---|---|
| PBS | - | Phosphate Buffered Saline |
| (p)RB | - | (phosphorylated) Retinoblastoma protein |
| Pre-miRNA | - | precursor-microRNA |
| Pri-miRNA | - | primary-microRNA |
| PVDF | - | Polyvinylidene Fluoride |
| R point | - | restriction point |
| Ripa buffer | - | Radioimmunoprecipitation assay buffer |
| RISC | - | RNA-induced silencing complex |
| RNAi | - | RNA interference |
| rtTA | - | reverse tetracycline transactivator |
| S-phase | - | synthesis phase |
| SDS | - | sodium dodecyl sulfate |
| shRNA | - | short hairpin RNA |
| TBE | - | Tris-Borate EDTA |
| TE | - | Tris-EDTA |
| TRE | - | tetracycline responsive element |

# 1. Introduction

## 1.1 Embryonic Stem Cells

### 1.1.1 Brief overview

Embryonic stem cells were first derived from mouse embryos in 1981 by two independent research groups (Evans & Kaufman, University of Cambridge, and Martin, University of California). These pluripotent cells are derived from the inner cell mass (ICM) of preimplantation embryos and give rise to all cell types evolved from the three primary germ layers, when differentiated in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Seventeen years after these initial discoveries, Thomson et al. (University of Wisconsin-Madison) established a breakthrough in human biomedical research by isolating embryonic stem cells from human blastocysts. Since this milestone, embryonic stem cells, with their unlimited self-renewal capacity and the ability to differentiate into all types of cells, have been hailed as a boon for regenerative medicine. This is because ES cells represent an experimental model system for the study of early mammalian and human development (Martin, 1981), and can function as a resource for in vitro screenings of pharmaceutical products and for toxicology studies. Most importantly, embryonic stem cells and their derivatives may serve as a possible source for cell replacement therapies that have the capacity to treat injuries and degenerative diseases such as heart failure, parkinsonism, diabetes or amyotrophic lateral sclerosis (Pera and Trounson, 2004; Stojkovic et al., 2004; Thomson et al., 1998).

Several problems must however be overcome before the goal of clinical applications can be realized. These include an understanding of how to efficiently direct and control differentiation into specific cell lineages in vitro. Moreover, problems of tumor formation or immunological rejection must also be overcome (Boheler, 2009). For this, ES cells may need to be fully differentiated into the desired cell type and residual pluripotent undifferentiated cells may need to be removed completely. Immunological rejection may also be obviated through the use of induced pluripotent stem (iPS) cells, which are largely indistinguishable from blastocyst-derived ES cells (Boheler, 2009). IPS cells can be prepared in vitro by transcription factor (mouse: Oct4/ Sox2/ c-Myc/ Klf4; human: Oct4/ Sox2/ Nanog/ LIN28) reprogramming of adult somatic cells isolated directly from the patient

(Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Since these cells are isolated from the patient, iPS cell derivatives should be immunologically compatible with the host. Finally and before achieving the goal of therapeutics, a more thorough understanding of the molecular mechanisms that form the basis of the pluripotent state of embryonic stem and induced pluripotent stem cells is required. In this regard it is important to unravel the interaction and regulatory network of pluripotency factors, transcription factors, cell cycle regulation and their target genes in ES cells. Towards this goal, my thesis deals with the role of B-Myb, an important transcription factor in embryonic stem cells, and its target genes Aurora Kinase A and Cyclin B1 in ES cells.

### 1.1.2 Pluripotency factors

At least three transcription factors, OCT4, SOX2 and NANOG, play critical roles in early mammalian development and are required for the maintenance of self-renewal and pluripotency (Boyer et al., 2005; Loh et al., 2006).

OCT 4, encoded by the gene *pou5f1*, belongs to a POU transcription factor family and is expressed in the inner cell mass (ICM) as well as in the epiblast (Chen et al., 2008). Appropriate levels of OCT4 are critical to maintain pluripotency in ES cells, because a greater than twofold increase causes differentiation into primitive endoderm and mesoderm, while a significant reduction leads to differentiation into trophoectoderm (Niwa et al., 1998). The transcription factor SOX2 contains a single HMG (high mobility group) DNA binding domain and is expressed in ES cells in a similar manner as OCT4 (Chen et al., 2008). SOX2 also heterodimerizes with OCT4 to regulate gene expression (Botquin et al., 1998). Last but not least the core regulator NANOG, a homeodomain containing protein, is expressed in the ICM and the epiblast. Additionally NANOG overexpression enables mouse ES (mES) cells to grow in culture without the Leukemia Inhibitor Factor (LIF), which is normally required to maintain self-renewal. Moreover, as OCT4 inhibits the formation of trophoectoderm it appears that NANOG inhibits differentiation into primitive endoderm (Boheler, 2009).

Besides OCT4, SOX2 and NANOG, which are all highly expressed in the inner cell mass of developing embryos, several other transcription factors are associated with ES cell

pluripotency, including but not limited to, c-Myc (Cartwright et al., 2005), Sall4 (Zhang et al.,2006), Tbx3 (Ogawa et al., 2007) and Zic3 (Lim et al., 2007). Like OCT4, SOX2 and NANOG, these are expressed in the ICM of blastocysts and often show decreased expression upon differentiation.

Moreover, the three principal pluripotency regulators co-occupy the promotors of at least 353 genes in hES cells (Boyer et al., 2005). Since OCT4, NANOG and SOX2 bind to DNA sequences, which are at times located within 25 bp of one another, the data suggest that they form tightly associated complexes (Marson et al., 2008). Importantly, they not only occupy target genes, these factors actually regulate them, which results in either gene activation or repression. Among the actively transcribed target genes are several transcription factors, known pluripotency regulators (Boheler, 2009), as well as components of signaling pathways that are involved in pluripotency and self-renewal. Repressed genes that bind these pluripotency regulators mostly encode transcription factors implicated in developmental processes (Boyer et al., 2005). OCT4, NANOG and SOX2 thus provide a regulatory network that consists of autoregulatory and feedforward loops (Boyer et al., 2005; Loh et al., 2006). Binding to the promotors of their own genes results in interconnected autoregulatory loops that confer several advantages. On the one hand gene expression becomes more stable, while on the other hand the regulatory network is capable of responding more rapidly to external signals.

The feedforward mechanism is based on a regulator that controls a second regulator, and both share a set of common target genes. In this case OCT4, SOX2 and NANOG act as those key regulators, which co-occupy and control a large set of genes encoding many other transcriptional regulators expressed in ES cells. The control of these secondary regulators leads to a hierarchical regulatory network structure that allows a rapid change in gene expression due to the fact that only three key regulators need to be targeted (Boyer et al., 2005; Cole and Young, 2008).

In conclusion this regulatory network enables ES cells to robustly maintain pluripotency and self-renewal, while being competent to respond appropriately to differentiation signals.

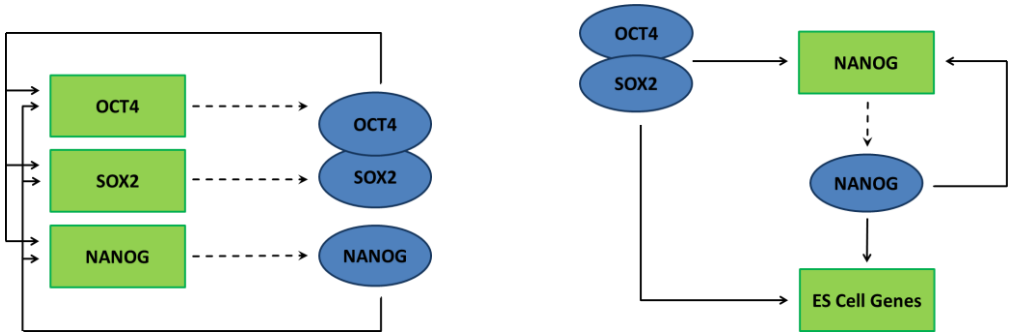



*Figure 1: Transcriptional Regulatory Motifs in Embryonic Stem Cells*
*(A) Interconnected autoregulatory loop provided by OCT4, SOX2 and NANOG in ES cells. The green rectangles represent promoters and the blue circles represent regulators. Solid arrows indicate binding of a regulator to a promoter and dashed arrows connect genes encoding regulators and their respective regulators.*
*(B) Feedforward loop formed by OCT4, SOX2 and NANOG.*

### 1.1.3 ES cell cycle

ES cells have a unique cell cycle that differs in total length, duration of the individual phases and cell cycle regulation when compared to differentiated somatic cells. The unusual rapid rate of cell division takes approximately 8-10 h in mES and 15-16 h in hES cells (vs. ~24-32 h in most actively proliferating somatic cells) (Becker et al., 2006; Stead et al., 2002). This extremely short generation time is achieved for the most part by shortening of the G1 phase, so that the cell cycle is composed mainly of DNA replication (S phase) and chromosome segregation (M phase) (Becker et al., 2006; Stead et al., 2002; White and Dalton, 2005). On the contrary, most somatic cell types stay typically in the main in G1, and need to be activated to pass the restriction (R) point in order to undergo a new round of cell division. This R checkpoint is also known as the 'point of no return', because once a cell has passed this, it will run through the cell cycle growth factor independently.

The molecular machinery required for cell cycle progression consists of a catalytic subunit, the cyclin-dependent protein kinases (Cdks), and a regulatory element, the cyclins (White and Dalton, 2005). These cyclins are unstable proteins, which are synthesized and degraded at different stages throughout the cell cycle. Since forming a complex with the correspondent Cdk leads to activation, the cell cycle contains periodic changes of phase-specific catalytic functions of Cdks. Normally Cdk4/6-cyclin D and Cdk2-cyclin E are activated in somatic cells to provide the transition from G1 to S phase (Stead et al.,

2002). In this connection the pRB-E2F pathway plays another decisive role due to the fact that phosphorylation of pRB by Cdk activity leads to derepression of E2F target genes in G1, which are required for DNA replication in S phase (White and Dalton, 2005).

By contrast, embryonic stem cells are characterized by a shortened G1 phase that lacks the normal restriction point (Stead et al., 2002). Furthermore they have a precocious activity of Cdks without the typical cell cycle dependent periodicity (Faast et al., 2004; Stead et al., 2002). Namely, except for the Cdk1-cyclin B complex, all cyclins (A, D, E) expressed in ES cells show comparable amounts throughout the whole cell cycle (Becker et al., 2006; Faast et al., 2004; Stead et al., 2002). The fact that cyclins have significantly elevated levels during the entire ES cell cycle compared to somatic cells as well as the constant activity of Cdks is responsible for their rapid rate of cell division (Becker et al., 2006; Stead et al., 2002; White and Dalton, 2005).

Additionally, the constitutive activation of these kinases holds pRB and p107 in a hyperphosphorylated state, which inactivates the pRB-E2F pathway (Savatier et al., 1994), so that their target genes, such as the important transcription factor B-Myb, are transcribed cell cycle independently (Stead et al., 2002). Since ES cells do not have the pRB/E2F switch that normally takes place at the R point and supports commitment to S phase entry, their cell cycle progression is independent of external factors e.g. exogenous growth factors or contact inhibition (Becker et al., 2010; White and Dalton, 2005). Recent studies have pointed out that, due to the lack of an E2F/pRB switch, hES cells are already determined in mitosis to run through the next S phase without the requirement of external regulatory signals (Becker et al., 2010). Furthermore, hES cells create a special medium by secreting autocrine factors in order to maintain their abbreviated G1 phase (Becker et al., 2010).

Differentiation of ES cells leads to an essential change in cell cycle regulation and structure, so that they show the characteristics normally seen in other mammalian cells.

At some point Cdk activity changes and becomes highly regulated as well as cell cycle dependent afterwards. In addition, the pRb/E2F pathway becomes activated, and therefore the restriction point is established. These changes yield to fully developed G1 and G2 phases, which are typically for differentiated somatic cells (White and Dalton, 2005).

Since the change in cell cycle regulation upon differentiation happens prior to the decrease in OCT4, NANOG and SOX2 protein abundance, provides support for the fact that the lack of cell cycle checkpoints is linked to the ability of maintaining pluripotency (Boheler et al., 2009).
In conclusion, these alterations connected to differentiation demonstrate that loss of G1 functions as well as precocious cell cycle regulatory mechanism are tightly linked to pluripotency and self-renewal (Becker et al., 2010; White and Dalton, 2005).

### 1.1.4 Epigenetic state

Multiple regulatory mechanisms are involved in the maintenance of the undifferentiated state of ES cells. On the one hand, binding of transcription factors plays an important role as previously mentioned. On the other hand, epigenetic factors such as DNA methylation or chromatin structure are connected with pluripotency and the initiation of differentiation (Bibikova et al., 2008).
The three different epigenetic states are euchromatin, constitutive heterochromatin and facultative heterochromatin.
Euchromatin contains the actively transcribed genes, whereas facultative heterochromatin is the result of gene silencing, but can again become transcriptionally active under specific circumstances. By contrast, constitutive heterochromatin is usually repetitive DNA that is highly condensed and supposed to be gene poor. In consequence of their different transcriptional activity, all three states vary in levels of DNA methylation, histone modification and timing of replication in S phase (Arney and Fisher, 2004).
Since embryonic stem cells differentiate without detectable changes in the DNA sequence, epigenetic factors are implicated in activation and inactivation of particular sets of genes. Cell differentiation is associated with methylation and demethylation of genomic DNA in order to establish a required formation of tissue-specific patterns of gene expression (Li, 2002; Shiota et al., 2002). Accordingly, the genes of the pluripotency factors OCT4 and NANOG are regulated by epigenetic mechanisms, and their silencing by methylation may play an important role in differentiation of embryonic stem cells (Hattori et al., 2007; Hattori et al., 2004).

### 1.1.5 B-Myb

Recently, the transcription factor B-Myb has been associated with maintaining pluripotency, normal cell cycle progression and chromosomal stability of embryonic stem cells (Tarasov et al., 2008). Furthermore it plays an important role in somatic cell differentiation, cell cycle progression and apoptosis (Joaquin and Watson, 2003; Oh and Reddy, 1999; Sala, 2005).

B-Myb, also known as avian myeloblastosis viral oncogene homolog-2 (mybl2), belongs to the myb gene family that includes A-Myb and c-myb. All three members encode nuclear proteins, which act as transcriptional transactivators in different tissues. C-myb is critical for hematopoiesis, and shows therefore high expression levels in immature hematopoietic cells, whereas A-Myb is important for spermatogenesis as well as mammary gland proliferation, and thus highly expressed in male germ cells and female breast ductal epithelium. In contrast to this tissue-specific expression, B-Myb is present in all proliferating cells. Furthermore, it is essential for formation and continued growth of the inner cell mass and, importantly, B-Myb is the only member of its family expressed in embryonic stem cells (Oh and Reddy, 1999; Tanaka et al., 2008).

As per description, B-Myb is directly regulated by E2F transcription factors, and therefore transcribed constitutively in embryonic stem cells due to the inactivation of the pRB-E2F pathway. By contrast, the expression of B-Myb in somatic cells is dynamically regulated in connection with the cell cycle, and is maximally induced at the G1/S boundary (Joaquin and Watson, 2003; Sala, 2005). For this reason, its functions were initially thought to be restricted to the progression from G1 to S phase, but in the meantime several studies have shown the important role for the G2/M transit.

In Zebrafish embryos, a loss-of-function mutation in B-Myb (crash&burn mutants) causes defects in mitotic progression, spindle formation and genomic instability (Shepard et al., 2005). Studies in Drosophila have shown that dMyb (homologue to B-Myb) mutants generate spindle defects and ploidy abnormalities (Manak et al., 2002). Furthermore mouse embryos lacking B-Myb die shortly after implantation because of inner cell mass defects (Tanaka et al., 1999).

Since B-Myb is highly expressed in ES cells, recent studies have focused on its function in those cells. However, previous publications conclude partially different results following B-Myb ablation.

Tarasov et al. state that knockdown of B-Myb in mES cells by short hairpin RNA leads to a transient but significant decreased transcription of SOX2 and OCT4, and subsequently to an upregulation in gene markers of differentiation. In contrast, Lorvellec et al. generated genetically modified embryonic stem cells lacking any expression of B-Myb. In these cells, they could not demonstrate any effect of B-Myb on the expression of pluripotency factors such as OCT4.

Apart from that, both studies show the influence of B-Myb in cell cycle progression. Ablation of B-Myb in mES cells results in abnormalities of S phase such as stalling of replication forks and superactivation of replication factories that causes disorganization of the replication program and an increase in double-strand breaks. These findings are partly the consequence of abnormal transcriptional regulation of proliferation factors like c-Myc and FoxM1, which are normally responsible for S phase progression (Lorvellec et al., 2010). B-Myb knockdown by shRNA leads to major cell cycle defects like a reduction of cells in G1 and S phase, and a delayed transit through G2/M with an accumulation of cells. These mitotic cells show centrosome and spindle defects, which causes an increase in polyploidy. This chromosomal constitution containing multiples of the normal number of chromosomes results either in programmed cell death or sustained aneuploidy (Tarasov et al., 2008). Apoptosis is only activated upon differentiation, and thus related to the establishment of robust cell cycle checkpoints in somatic cells (Mantel et al., 2007).

Since Cyclin B1 is normally activated by B-Myb in late S phase, the absence of this transcription factor causes decreased Cyclin B1 transcripts, and the observed mitotic defects in ES cell lacking B-Myb are at least partially due to this protein (Boheler, 2009; Tarasov et al., 2008).

In summary, the transcription factor B-Myb is required for maintenance of pluripotency, chromosomal integrity and normal cell cycle progression. Moreover, downregulation of B-Myb appears to be one of the earliest markers of pluripotent cell differentiation.

Recent studies in somatic cells have shown that B-Myb promotes its function as a part of the LINC complex. This dynamic DNA binding complex comprises a five protein core module (Lin-9, Lin-37, Lin-54, Lin-52 and RbAp48) that associates with p130, E2F4 and B-Myb in a cell cycle dependent manner in order to regulate gene expression (Litovchick et al., 2007; Schmit et al., 2007).

In G1 phase this multiprotein complex binds with p130 and E2F4 resulting in the repression of E2F target genes, which regulate the progression from G1 to S phase (Litovchick et al., Pilkinton et al., 2007).
Owing to the binding of B-Myb, the LINC complex is no longer transcriptionally repressive, but switches to a transcriptionally active complex in S-G2 phase (Knight et al., 2009). Beyond that, B-Myb recruits a Lin-9 complex to activate transcription of G2/M genes in undifferentiated embryonal carcinoma cells. Therefore both B-Myb and Lin-9 are required for transition through mitosis in pluripotent cells without a robust G1/S checkpoint (Knight et al., 2009).

The important role of the transcription factor B-Myb in regulating gene expression has been shown in preliminary ChIP on chip data (a genomescale location analysis by chromatin immunoprecipitation coupled with DNA microarrays). There it was described that B-Myb binds to over 240 promoter regions in mouse embryonic stem cells with a high degree of confidence. In the absence of B-Myb approx. 95 % of these genes possess reduced binding. Moreover a significant part of those proteins is either associated to the cell cycle or implicated in differentiation (64 out of 240). Target genes associated with the cell cycle include e.g. the members of the chromosomal passenger complex (Aurora Kinase B, INCENP, Borealin), centrosome associated kinases like Aurora Kinase A and signaling molecules such as Cyclin B1.
But it is still unclear whether the chromosomal aneuploidy that accompanies B-Myb deficiency is due to a generalized reduction in proteins required for G2/M progression or whether the dysregulation of individual factors like Cyclin B1 or Aurora Kinase A is responsible for the aneuploidy.
Therefore the ChIP-on-chip analyses have led to the hypothesis that the transcription factor B-Myb directly regulates gene expression of these proteins, and that their dysregulation leads directly to chromosomal instability, which can result in the generation of defective stem and somatic cell populations that have the potential to be pro-oncogenic
Based on depressed protein and RNA expression in ES cells lacking B-Myb, the attention is therefore focused on Aurora Kinase A and Cyclin B1.

### 1.1.6 Aurora Kinase A

The Aurora family of serine-threonine kinases consists of three members: Aurora Kinase A, B and C. They all share similar structures – highly conserved catalytic domains flanked by very short C-terminal tails and N-terminal domains of variable length. Besides, all three members vary in their expression patterns, subcellular localization and time of activation (Marumoto et al., 2005).

Aurora Kinase A plays a significant role in mitotic regulation and carcinogenesis. Its various functions include centrosome maturation and segregation, bipolar spindle assembly, chromosome alignment, the transition from prophase to metaphase and cytokinesis (Berdnik and Knoblich, 2002; Giet et al., 2002). In order to fulfill its task Aurora-A localizes to centrosomes during interphase of the cell cycle, and connected with the onset of mitosis Aurora-A is found at both spindle poles as well as spindle microtubules (Gopalan et al., 1997; Kimura et al., 1997; Zhou et al., 1998).

Due to its role in mitotic regulation the transcription of Aurora-A is cell-cycle dependent with a low expression in G1/S phase, maximum at G2/M phase and a rapidly decrease toward the end of mitosis (Bischoff et al., 1998; Kimura et al., 1997). Since the E2F family of transcription factors regulates the expression of genes that are required for DNA replication and mitosis, Aurora Kinase A is also one of their target genes. E2F3 modulates Aurora-A expression by upregulating its transcription during G2 and the beginning of mitosis (He et al., 2007). Moreover, the activity of Aurora Kinase is regulated by phosphorylation, and after mitosis is finished there are two ways of degradation: both the ubiquitin-mediated proteolysis and an ubiquitin-independent mechanism.

Furthermore previous studies have shown the importance of the accurate timing as well as the definite level of Aurora Kinase A expression to maintain a regular progression through mitosis. Aurora-A deletion in mouse embryonic fibroblast causes delayed mitotic entry, mitotic arrest with accumulation of cells in early prophase as well as defects in bipolar spindle formation. The fact that Aurora-A deficient cells were not observed in any phases of the cell cycle later than prometaphase indicates that those cells exit mitosis without cell division. Additionally, Aurora-A deletion in midgestation embryos results in an increased number of mitotic and apoptotic cells (Cowley et al., 2009). Germ line Aurora Kinase A deficiency leads to embryonic death at blastocyst stage due to mitotic

arrest and monopolar spindle formation (Cowley et al., 2009). This enzyme is thus essential for embryonic development.

On the contrary, overexpression of Aurora Kinase A leads to centrosome amplification and causes aneuploidy, which results in either growth arrest, cell death or survival through malignant transformation (Mao et al., 2007; Meraldi et al., 2004). Ectopic expression of Aurora-A causes increased centrosomes with associated loss or gain of chromosomes that finally leads to oncogenic transformation as well. In fact, this enzyme has been found frequently amplified and overexpressed in many human cancers, for instance breast, colorectal, pancreatic and bladder cancer, that correlates with clinically aggressive disease and poor prognosis (Katayama et al., 2003). Since aberrant Aurora-A expression is associated with tumorigenesis, Aurora Kinase Inhibitors have been developed in order to stop cancer cell growth, and have recently entered clinical trials (Boss et al., 2009).
A thorough understanding of the relevant molecular pathways involving Aurora Kinase A is therefore very important to understand its entire function in mitotic regulation, and the Aurora-A induced tumorigenic transformation process.

### 1.1.7 Cyclin B1

As mentioned previously, cell cycle transition is regulated by the periodic activation and inactivation of Cyclin/Cdk (Cyclin-dependent kinase) complexes at defined phases of the cell cycle.
In contrast to somatic cells, all Cyclin/Cdk-complexes in embryonic stem cells show constant activity throughout the cell cycle except for the Cyclin B1/Cdk1 complex. This complex regulates mitotic entry as well as progression through mitosis (Nurse, 1990).
Levels of Cyclin B1 increase during G2 phase resulting in an accumulation of Cyclin B1-Cdk1 complexes that are still inactivated (Pines and Hunter, 1989). Subsequently, dephosphorylation by Cdc25 phosphatases leads to gradually increased activity and, after a certain threshold activity is reached, further activation of Cyclin B1/Cdk1-complex is catalyzed by a positive-feedback loop (Boutros et al., 2006; Solomon et al., 1990). At first, this highly activated complex localizes to the centrosomes and triggers their sepa-

ration in late G2 or prophase (Blangy et al., 1997; Crasta et al., 2006; Jackman et al., 2003). Accordingly, owing to phosphorylation the Cyclin B1/Cdk1-complex is translocated into the nucleus, where it leads to enhanced chromosome condensation and nuclear envelope breakdown (Hagting et al., 1999; Li et al., 1997). Afterwards the two sister chromatids of each chromosome need to be correctly attached to opposite spindle poles (Pines, 2006; Taylor et al., 2004). This process is controlled by the mitotic spindle checkpoint, which inhibits Cyclin B1 destruction and Cdk1 inactivation and stops cell cycle progression as long as all chromosomes are connected to the correct mitotic spindles. If all requirements for the checkpoint are complied with, decreased Cyclin B1/Cdk1 activity is essential for chromosome segregation and completion of cell division (Pines, 2006).

Importantly, Cyclin B1 is a known target for the transcription factor B-Myb, it shows high expression levels in ES cells and is downregulated upon differentiation just like B-Myb. Additionally the observed mitotic defects in G2/M phase caused by B-Myb knockdown were in part ascribed to Cyclin B1.

## 1.2 Inducible Expression System

To accurately analyze the function of specific genes in mammalian cell culture systems, it is critical to tightly regulate their expression. Therefore the use of an inducible expression system employing regulatory elements of the tetracycline resistance operon is very helpful.
In this study the cell line Ainv15 was planned to generate for a stronger knockdown of B-Myb and its target genes (see results): however, the data generated with this system occurred after the allotted time for completion of my studies.

## 1.3 MicroRNA

MicroRNAs (miRNA) are a group of short ~22 nucleotide RNA molecules encoded in the genome of animals and plants. These nonprotein-coding RNAs regulate gene expres-

sion by binding to complementary sequences in the 3'-untranslated region (3' UTR) of specific mRNAs, resulting in their being silenced.

MicroRNAs were first discovered in 1993, and since then more than 700 miRNAs have already been identified in humans and several hundreds more are predicted to exist. They have a wide range of functions, and act as key regulators for instance in cell proliferation and cell death, early development, cell differentiation or brain development. Furthermore, miRNA dysregulation is also associated with different kinds of diseases such as viral infection, chronic lymphocytic leukemia or colonic adenocarcinoma.

MiRNAs have to undergo a multistep posttranscriptional modification before they can function as active single-stranded miRNAs. They are initially expressed as primary transcripts (pri-miRNA) containing 5'caps and 3'poly-A-tails by RNA-Polymerase II (Cai et al., 2004; Lee et al., 2002; Smalheiser, 2003). The miRNA part of the pri-miRNA transcript forms a hairpin loop structure, which is recognized by the dsRNA-specific ribonuclease Drosha. This enzyme digests the pri-miRNA in the nucleus and releases the hairpin, precursor miRNA (pre-miRNA) (Lee et al., 2003). Pre-miRNAs consist of approximately 70 nucleotides (nt) with 2 nt 3'overhangs and a stem loop structure. In the next step the pre-miRNA is transported to the cytoplasm by Exportin-5/Ran-GTP (Yi et al., 2003). Afterwards the endonuclease Dicer cleaves the pre-miRNA stem loop, so that two complementary short RNA molecules are formed (Bernstein et al., 2001; Hutvágner et al., 2001). Only one of the two strands associates with the RNA-induced silencing complex (RISC) (Hutvágner and Zamore, 2002). This mature mi-RNA guides the RISC to target genes in order to induce post-transcriptional gene silencing.

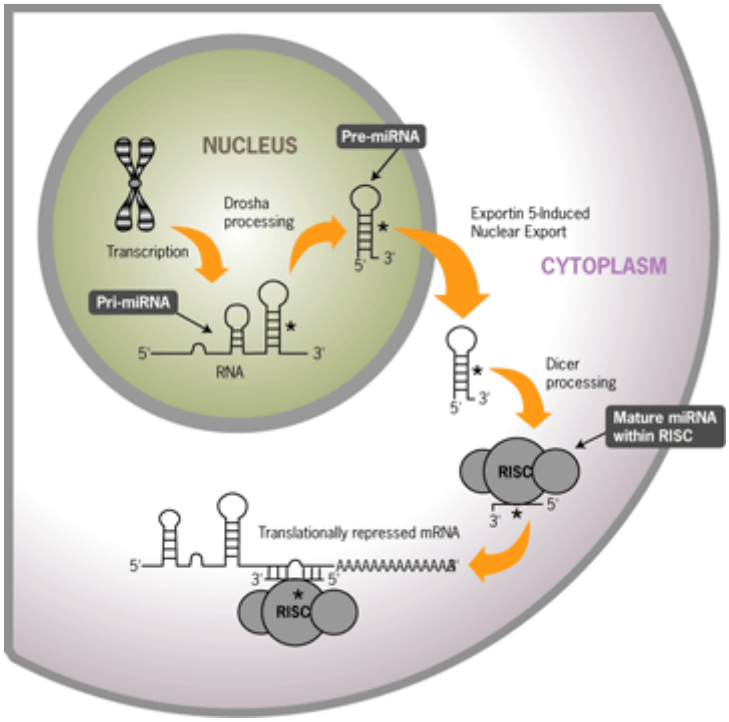



*Figure 2: MiRNA processing pathway (Applied Biosystems, 2010)*

To detect their targets, the 5' nucleotides 2-7 (known as 'seed region') of the miRNA have to be perfectly or near perfectly complementary to the messenger RNA.
The dimension of complementarity is implicated as an important factor in the mechanism of posttranscriptional repression by miRNAs. Perfect complementarity leads to mRNA cleavage, whereas near perfect binding excludes cleavage and causes inhibition of the mRNA translation (Carthew and Sontheimer, 2009).
Importantly, the miRNA pathway is a useful system to knockdown a gene of interest and study its function in cell culture.

## 1.4 Aim of the study

The aim of the study was to determine how B-Myb regulates transit through the G2/M phase of ES cells to maintain euploidy. More specifically, previous data has shown that knockdown of the transcription factor B-Myb in ES cells causes major cell cycle defects and polyploidy accompanied by reduced levels of target genes such as Aurora Kinase A and Cyclin B1. Therefore the question has been raised whether those target genes contribute to the phenotype seen by B-Myb knockdown.

To clarify the roles of putative B-Myb target genes, miRNAs directed against Aurora Kinase A and Cyclin B1 have been designed and tested in transient transfections. Since miRNAs can affect other target genes, the ones with the best knockdown efficiency and the least promiscuity were chosen. In order to mimic the results seen with B-Myb knockdown, protein levels of Aurora Kinase A and Cyclin B1 were decreased by miRNA knockdown to levels comparable to those seen in B-Myb deficient cells. Subsequently the best miRNAs were nucleofected into mES cells to study their function by cell cycle and spindle assembly analysis. The main objective at this juncture was to explore whether knockdown of Aurora Kinase A and/or Cyclin B1 leads to similar effects compared to the B-Myb knockdown, such as aneuploidy, changes in cell cycle and centrosome/ spindle defects.

At the same time the B-Myb shRNA, which was already designed in previous experiments by others, was nucleofected in mES cells as well in order to compare cell cycle and spindle analysis.

Finally, it was planned to generate the constructs for the Ainv15 cells that are regulated by the reverse tetracycline transactivator to drive expression of transgenes that knockdown B-Myb or one of the target genes using miRNAs. Thus, a more complete knockdown of Aurora Kinase A and Cyclin B1 would be achieved, since shRNA and miRNA only partially decrease their targets. Secondly, different target genes of B-Myb detected with the help of microarray analysis could be tested similarly.

Since the time working on my project at the National Institute on Aging was limited to six months, it was not possible to complete the planned generation of the Ainv15 cells during my stay. Anyhow, I finished cell cycle and spindle assembly analysis using the designed miRNAs for Aurora Kinase A as well as Cyclin B1 and compared the results to B-Myb knockdown.

The experiments concerning Ainv15 cells have been completed by others since I left the laboratory.

# 2. Methods

## 2.1 DNA Plasmid Preparation

Plasmid DNA was cultured overnight at 37°C in LB media containing Spectinomycin. In order to isolate a high quantity of Plasmid DNA from maxi-cultures the GenElute HP Endotoxin-Free Plasmid Maxiprep Kit was used, and to extract Plasmid DNA from mini-cultures for analytical purposes the QuickGene Plasmid kit S II (Fujifilm) was employed.
The functional principle of both Kits is to lyse the cells under alkaline conditions and neutralize the solution in the next step to precipitate chromosomal DNA and proteins. In contrast Plasmid DNA has the ability to renature rapidly after denaturation and to stay in solution, so that it can be easily extracted from the bacterial chromosomal DNA. Therefore the Plasmid DNA was absorbed by a binding column and contaminants were eluted by two washing steps.
Finally, the isolated DNA was quantified with the help of a spectrophotometer e.g. Genequant pro, which measures the absorbance at 260 and 280 nm, to determine the concentration.

## 2.2 Gel electrophoresis

Agarose gel electrophoresis is a common technique to separate DNA fragments according to their sizes. Upon application of an electric current, the negatively charged DNA moves through the gel pores toward the positive pole (anode) of the electrophoresis chamber. Smaller molecules move more easily through the porous lattice than larger molecules. Therefore the fragments can be separated into distinct bands according to their molecular weight.
Basically, fragments of linear DNA migrate with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. In comparison, circular forms of DNA migrate much differently to linear DNA of the same mass. The migration of an uncut plasmid will appear more rapidly than the same vector when linearized by restriction enzymes. Most preparations of uncut plasmid contain at least two different forms of DNA: the supercoiled form and nicked circles. When these plasmids are cut and thus linearized they all become identical, and one then observes only one band on the gel.

In order to visualize the gel with UV light, the DNA has to be stained with a fluorescent dye. Ethidium bromide is commonly used for detecting DNA, because it intercalates between bases of nucleic acids and therefore strongly enhances the staining.
The DNA was separated by 0.5-2 % agarose gel electrophoresis (Ultra Pure Agarose by Invitrogen) and 0.2 µg/ml ethidium bromide in TBE Buffer (Tris-Borate EDTA). The gels were run at 80-120 V for 45-90 min.

## 2.3 Transfection

Transfection describes the process of bringing foreign DNA into cells. There are several different methods for introducing DNA into cells concerning e.g. the type of carrier, which is used. FuGENE 6 Transfection Reagent (Roche) consists of a blend of lipids that forms a complex with the Plasmid DNA. After fusing with the cell membrane the Plasmid DNA is released into the cell where it can be expressed with the help of the cell's transcription apparatus.
Protocol:
35mm culture dishes containing a monolayer of C2C12 cells, that is 50-80 % confluent were used for transfection. The Ratio of µl Transfection Reagent : µg DNA was chosen at 3:1. First of all, the Transfection Reagent was diluted with serum-free medium and subsequently the DNA was added. The complexes were allowed to form during an incubation period of 15 minutes at room temperature before being added to the cells.

## 2.4 Nucleofection

Nucleofection is a transfection method with which the substrate, such as Plasmid DNA, is transferred directly into the cell nucleus and the cytoplasm. Contrary, other transfection methods depend on cell division for the purpose of transferring DNA into the nucleus. Predominantly, nucleofection is considered for cell lines that are very difficult to transfect, for instance stem cells.
The Amaxa Nucleofector technology is based on electroporation, and contains various profiles as well as several nucleofector solutions, which are defined for specific cell types.

Protocol:

Cells were harvested by trypsinization, and then pelleted via centrifugation. In order to resuspend cells the appropriate nucleofection solution was chosen, and afterwards the Plasmid DNA was added. Subsequently, the electroporation was carried out either by using the Nucleofector or the 96 well shuttle system depending on the number of samples. Cells were transferred into culture dishes, incubated at 37°C and selection reagents were added 24h after nucleofection. Puromycin was used for cells nucleofected with B-Myb shRNA and pSuper, and Blasticidin was taken as selection reagent for cells nucleofected with miR neg, Aurka and Ccnb1.

## 2.5 Western Blot

As an analytical technique the Western immunoblotting is used to detect a specific protein in a given sample. Furthermore the molecular weight of a protein can be determined and relative amounts of the protein can be compared in different samples.

The isolated proteins are separated using SDS-polyacrylamide gel electrophoresis, which primarily denatures all proteins to their primary structure. For the purpose of making the proteins accessible to antibody detection, they are then transferred onto a PVDF membrane. An electric field is applied to the gel, and the separated proteins move to the positive charged membrane, retaining the same pattern of separation as they had on the gel. Afterwards the membrane is placed in a blocking solution to prevent non-specific binding between the membrane and the antibody. In order to detect the desired antigen, the primary antibody is added and forms a complex with the antigen. Subsequently the membrane is exposed to the secondary antibody to detect the bonded primary antibody. The secondary antibody is a species-specific anti-immunoglobulin that contains the reporter enzyme, such as horseradish peroxidase. Finally, the substrate for the reporter enzyme is added; luminescence is produced wherever there is an antigen-antibodies-complex. By exposing the membrane to photographic film one can visualize the bands.

Protocol:

The Cells were trypsonized, washed with PBS (pH 7.4) and harvested. According to the size of the pellet 50-500 µl Cell ‘RIPA’ buffer was added to resuspend the cells. After

incubation on ice with occasional vortexing for 30 min, the samples were centrifuged at 4°C and 10,000 g for 10 min and the supernatant including the protein lysate was transferred to a new tube. In order to determine the protein concentration Bio-Rad DC Protein Assay was used.

Subsequently the samples were diluted to a total protein amount of 25-50 µg, solubilized by heating for 5 min at 95°C and loaded along with the molecular weight marker onto a precast Criterion 18 well 7.5 %/4 % Tris-HCl SDS-Page mini-gel (Bio-Rad). To separate proteins the gel was run at constant voltage, initially at 60 V for 30 min and then at 120 V for 1.5 hours. Afterwards the protein bands were blotted onto a PVDF membrane presoaked in 100 % methanol using Bio-Rad Criterion Mini Trans-Blot Electrophoretic Transfer Cell Apparatus and Accessories. To transfer the proteins to the membrane, the gel was run at a constant current of 250 mA for 2.5 hours.

Since both antibodies are proteins as well, and the membrane serves the purpose of binding proteins, all sorts of other interactions must be prevented. Therefore the membrane was placed for 1 hour into a blocking solution containing 5 % dried milk, so that the protein of this solution can bind to the membrane and cover all the free places to which the target proteins have not attached themselves.

Next, the membrane was incubated overnight at 4°C rocking in primary antibody solution. After washing three times for 10 minutes in PBS/T, it was incubated for 1 hour at room temperature in horse radish peroxidase (HRP) conjugated secondary antibody solution.

Subsequently, the membrane was washed again three times in PBS/T, and then incubated for 30 seconds in Pierce Supersignal ECL Horse Radish Peroxidase substrate solution. This substrate is oxidized by HRP, producing luminescence in proportion to the amount of protein present, which can be detected on a photographic film.

## 2.6 Cell Cycle Analysis

Cell Cycle Analysis is a quick method to quantify the relative nuclear DNA content and ploidy level of a cell population by flow cytometry.

First of all, single cells are stained with a fluorescent dye e.g. Propidium Iodide, which binds stoichiometrically to the DNA. Afterwards the stained material is measured by flow cytometry, and the replication state of the cells can be analyzed.

Protocol:

Cells were trypsonized, washed with PBS (pH 7.4) and harvested. Subsequently the cells were fixed in methanol and stored at -20°C. After this, the cells were washed with three times with PBS, one million of the cells were stained with Propidium Iodide, and measured by flow cytometry using Becton Dickinson FACs CANTO II.

# 3. Materials

## 3.1 Laboratory apparatus

Daigger Vortex Genie 2 (A. Daigger & Co, Inc)
Centrifuge 5415C (Eppendorf)
Sorvall RC 5C Plus (Heraeus & Sorvall)
GPKR Centrifuge (Beckman)
IEC Clinical Centrifuge (International Equipment Company)
Type 16500 Dri-Bath (Thermolyne)
All Stainless Steel Water Bath Model 181 (Precision)
Gel Pump GP 110 (Savant)
Gene Quant pro (Pharmacia Biotech)
Spectrophotometer DU-640 (Beckman)
Gel Doc 1000 (Bio Rad)
Power Pac 3000 (Bio Rad)
Model 200/2.0 Power Supply (Bio Rad)
AE 240 Analytical Balance (Mettler)
BP 210D (Sartorius)
Hot Shaker (Bellco Glass, Inc)
Mini Trans-Blot Electrophoretic Transfer Cell Apparatus and Accessories (Bio Rad)
Axio Observer A1 (Zeiss)
Axiovert 35 (Zeiss)
Mini Medicla Series (AFP Imaging)
Fixer ChemBlend (H.R. Simon and Company Inc.)
$CB^2$ Developer (H.R. Simon and Company Inc.)
Becton Dickinson FACs CANTO II (BD Biosciences)

## 3.2 Kits

BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen)
QuickGene Plasmid Kit S II (Fujifilm)
Gen Elute HP Endotoxin-free Plasmid Maxiprep Kit (Sigma-Aldrich)

FuGENE 6 Transfection Reagent (Roche)
Mouse ES Cell Nucleofector Kit (Amaxa)
Mouse ES Cell 96-well Nucleofector Kit (Amaxa)
DC Protein Assay Kit (Bio Rad)

## 3.3 Enzymes

Xho I (Promega)
Sal I (Promega)

## 3.4 Antibodies

Mouse monoclonal to Aurora A (Abcam)
Rabbit polyclonal to Cyclin B1 (Santa Cruz Biotechnology)
Rabbit polyclonal to B Myb (Abcam)
Rabbit polyclonal to Oct4 (Abcam)
Mouse to GAPDH (Sigma)
Goat anti-mouse IgG HRP Conjugate (Invitrogen)
Goat anti rabbit IgG HRP Conjugate (Invitrogen)
α-Tubulin (Sigma)
γ-Tubulin (Sigma)

## 3.5 Reagents

TE pH 7.4 (Quality Biological Inc)
Tris-Borate EDTA (TBE) 10X Solution (Fisher Scientific)
Ethyl Alcohol 200 Proof (The Warner-Graham Company)
Isopropanol (J.T. Baker)
Methanol (Fisher Scientific)
Acetone (Sigma)
Sodium Acetate pH 5.2 (Quality Biological Inc)

Glycerol (Life Technologies)
PBS pH 7.4 (GIBCO)
0.25 % Trypsin-EDTA (GIBCO)
Buffer D 10X (Promega)
Ultra Pure Agarose (Invitrogen)
Ethidium Bromide Solution 10mg/ml (Research Genetics)
10X Blue Juice Gel Loading Buffer (Invitrogen)
Sodium Chloride (Fisher Scientific)
Bacto Tryptone (BD)
Bacto Yeast Extract (BD)
Bacto Agar (BD)
1 kb DNA Ladder (Invitrogen)
50 bp DNA Ladder (Invitrogen)
25 bp DNA Step Ladder (Promega)
Precision Pure Protein Dual Color Standards #161-0374 (Bio Rad)
Blasticidin S HCl (GIBCO)
Spectinomycin (Invitrogen)
Puromycin (Invitrogen)
Ribonuclease A (Sigma)
Dapi (Sigma)
Vectashield mounting medium for fluorescence (VECTOR)
Propidium iodide (Sigma)
Paraformaldehyde (Fisher Scientific)
Glycine (Sigma)
1 % Triton X100 (Sigma)
Cell RIPA Buffer (Sigma)
Nupage LDS Sample Buffer 4x (Invitrogen)
DTT (Sigma)
Precision Plus Protein All Blue Standards (Bio-Rad)
10x Tris/ Glycine/ SDS Buffer (Bio-Rad)
Blotting Grade Blocker non-fat dry milk (Bio-Rad)
Tween 20 (Sigma-Aldrich)
SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific)

Restore Western Blot Stripping Buffer (Thermo Scientific)

## 3.6 Cell lines

C2C12

R1

## 3.7 Cell culture media

2X LIF media (Sigma)

# 4. Results

## 4.1 Nucleofections

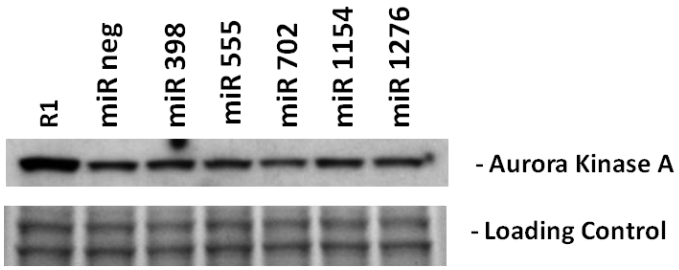
### 4.1.1 Testing miRNAs for Aurora Kinase A knockdown

The purpose of my study was to mimic the results observed by B-Myb knockdown in mES cells. Since protein levels of specific target genes such as Aurora Kinase A or Cyclin B1 were reduced in B-Myb deficient cells, miRNAs were designed and used initially to knockdown those target genes to similar levels in order to detect whether an identical phenotype could be reproduced.

Therefore miRNA vectors directed against Aurora Kinase A and Cyclin B1 were generated; these are described in detail in a following chapter of the result section.

In order to identify whether the designed miRNA constructs achieve a successful knockdown of the target gene Aurka, R1 cells were nucleofected with each of the five vectors (pc DNA 6.2-GW/EmGFP miR Aurka 398, 555, 702, 1154 and 1276). R1 cells without nucleofection served as a negative control as well as the pc DNA 6.2-GW/EmGFP miR negative plasmid, because its insert processed into mature miRNA does not target any known vertebrate gene.

The cells were harvested 24, 48 and 72h after selection and protein expression was detected by Western immunoblotting.



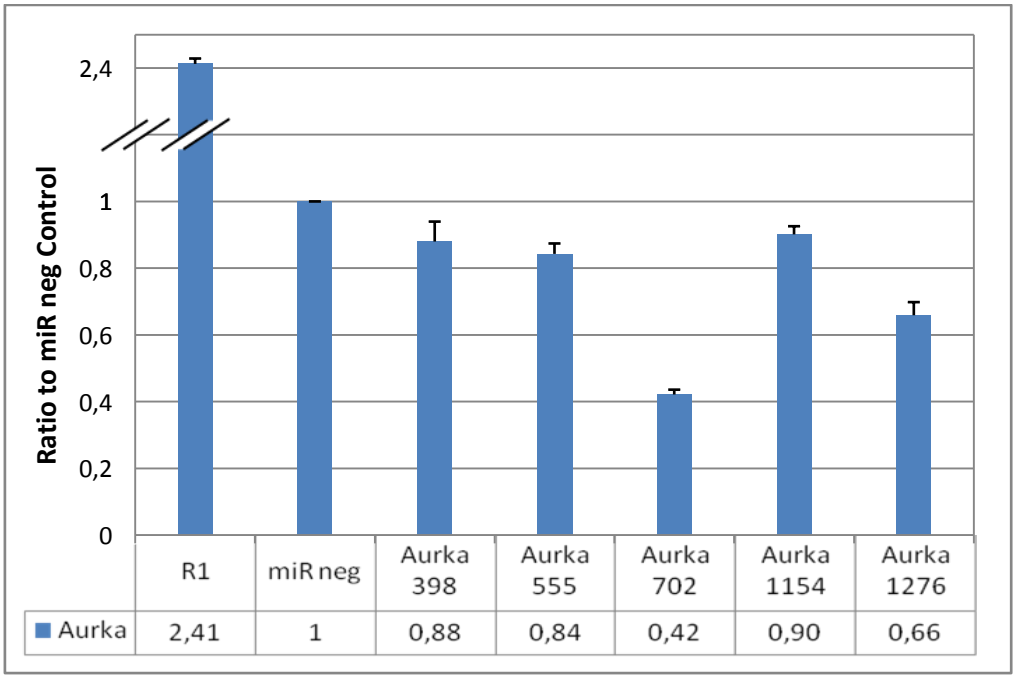



*Figure 3: Western Blot showing knockdown of Aurora Kinase A at 48h after selection.*

Western immunoblotting enables a comparison of efficiency of Aurora Kinase A knockdown achieved by the five different miRNAs.

R1 cells without nucleofection and R1 cells nuclefected with the empty vector control clearly displayed regular expression of Aurora Kinase A. In contrast, all five miRNAs decreased the quantity of Aurora Kinase A compared to control cells, but knockdown with miRNA 702 caused lowest protein levels with a significant reduction of Aurora Kinase A transcripts by 60 % relative to miR neg. This miRNA proved most effective for knockdown of Aurora Kinase A, and was used for further analysis.

### 4.1.2 Testing miRNAs for Cyclin B1 knockdown

In an analogous way, it was checked whether the designed miRNA constructs achieve a successful knockdown of the target gene Ccnb1. R1 cells were nucleofected with each of the three vectors (pc DNA 6.2-GW/EmGFP miR Ccnb1 682, 744, 826). Again, R1 cells without nucleofection served as a negative control as well as the pc DNA 6.2-GW/EmGFP miR negative plasmid. The cells were harvested 24, 48 and 72 h after selection and protein expression was detected by Western immunoblotting.

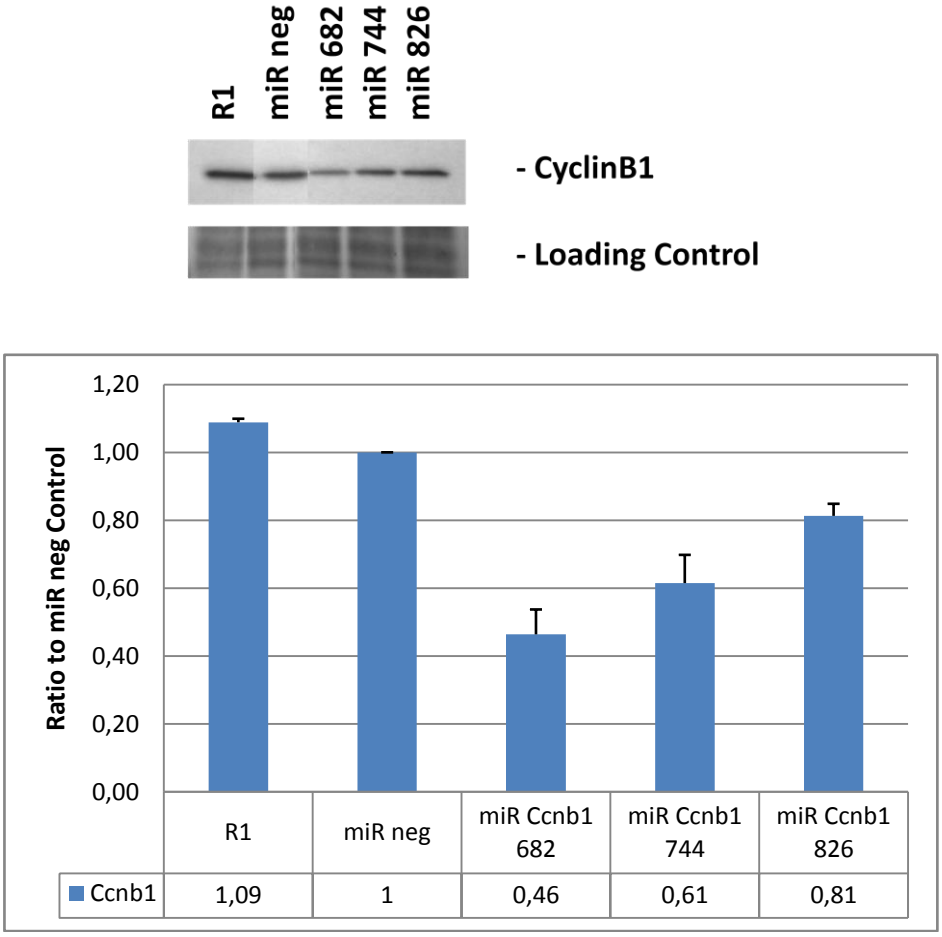



*Figure 4: Western Blot showing knockdown of Cyclin B1 at 48h after selection.*

R1 cells without nucleofection as well as R1 cells nucleofected with the empty vector control showed regular expression of Cyclin B1. By comparison, all R1 cells nucleofected with the three pcDNA 6.2-GW/EmGFP miR Ccnb1 vectors displayed lower levels of the target gene, but knockdown by miRNA 682 reduced Cyclin B1 expression most efficiently, leading to a significant reduction of Cyclin B1 transcripts by 55 % relative to miR neg. Therefore this miRNA was used for further analysis.

## 4.2 Comparing levels of B-Myb, Aurora Kinase A and Cyclin B1 by knockdown

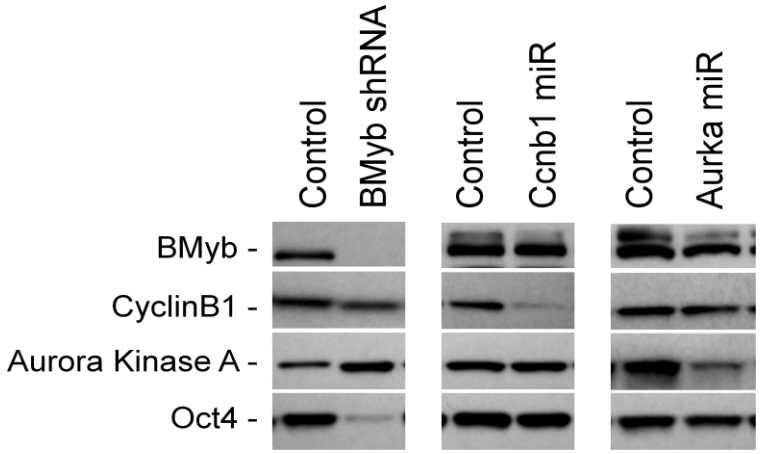
Silencing gene expression of a certain target gene via RNA interference does not only lead to a reduced level of that specific protein, but can also cause decreased expression of other genes. For instance, previous experiments have shown that knockdown of B-

Myb by shRNA results in decreased levels of target genes such as Cyclin B1, due to the fact that B-Myb regulates gene expression of those target genes.

Thus it was important to check whether knockdown of Aurora Kinase A or Cyclin B1 by the designed miRNAs leads to equal protein levels compared to those seen in B-Myb deficient cells.

In order to compare knockdown efficiency, R1 cells were nucleofected with vectors containing miR Aurka 702, miR Ccnb1 682 as well as a vector containing shRNA for B-Myb knockdown. (*This shRNA was already employed by Tarasov et al. in previous experiments to knockdown B-Myb.)* R1 cells nucleofected with the miR neg vector served as a negative control for Aurka and Ccnb1. The empty vector, pSuper, was nucleofected into R1 cells as well and served as a negative control for B-Myb shRNA.

Cells were harvested 48 h after selection and protein expression was detected by Western immunoblotting.



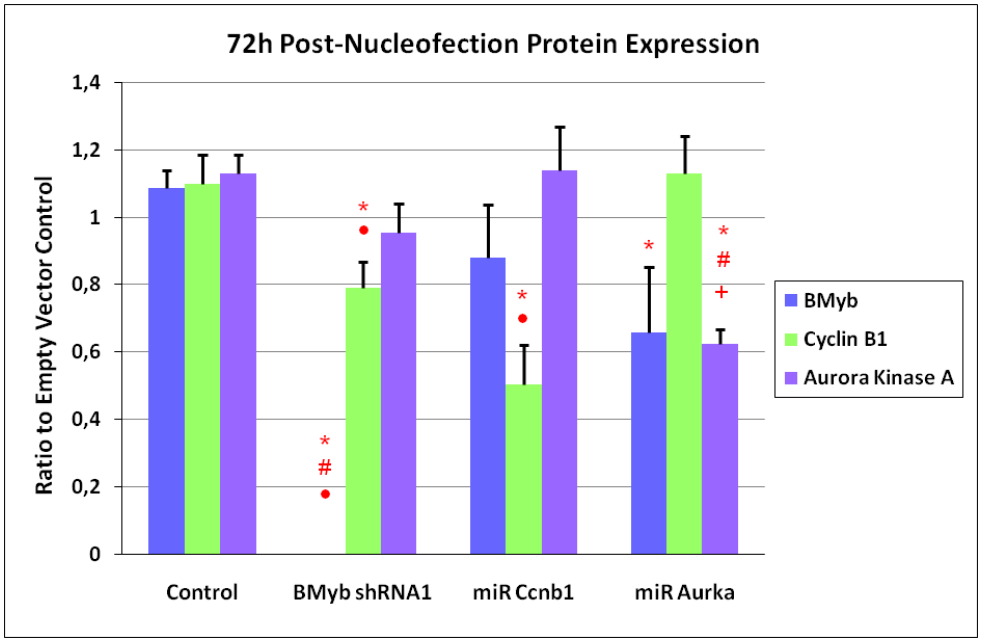



*Figure 5: Protein Expression 72 hours after nucleofection. Significance levels p<0,05:* * *= compared to control,* + *= compared to B-Myb shRNA,* # *= compared to miR Ccnb1,* • *= compared to miR Aurka.*

In R1 cells nucleofected with B-Myb shRNA, B-Myb expression was completely eliminated. Additionally, Cyclin B1 levels were also decreased to 80 % whereas Aurora Kinase A protein expression was not significantly reduced.

By comparison, nucleofection with miR Ccnb1 led to Cyclin B1 reduction of 50 %. B-Myb and Aurora Kinase A levels were not significantly changed. R1 cells nucleofected with miR Aurka showed significantly decreased levels of Aurora Kinase A to 60 %. Loss of Aurora Kinase A also led to significantly reduced levels of B-Myb. Expression of Cyclin B1 was not affected in this regard.

In summary, knockdown with the help of miRNA caused lower levels of Cyclin B1 and Aurora Kinase A compared to levels seen with B-Myb knockdown.

## 4.3 Cell Cycle Analysis

Cell cycle analysis is a useful technique to quantify the relative nuclear DNA content of a cell population and consequently distinguish cells in different phases of the cell cycle.

After staining the cells with Propidium Iodide, which binds stoichiometrically to the DNA, the stained material is measured by flow cytometry, and the replication state of the cell population can be analyzed.

A regular cell cycle analysis displays three populations of cells representing G1-, S- and G2/M phase. G1 cells have one copy of DNA (2N) and thus show 1X fluorescence intensity. Whereas cells in G2/M phase are ready to go into mitosis and contain two copies of DNA (4N) resulting in a 2X fluorescence intensity. Since cells in S phase are replicating the DNA, they have fluorescence values somewhere between 1X and 2X populations. The resulting histogram shows those three populations, in which the G1 and G2/M cells are represented by Gaussian curves (see Figure 6).

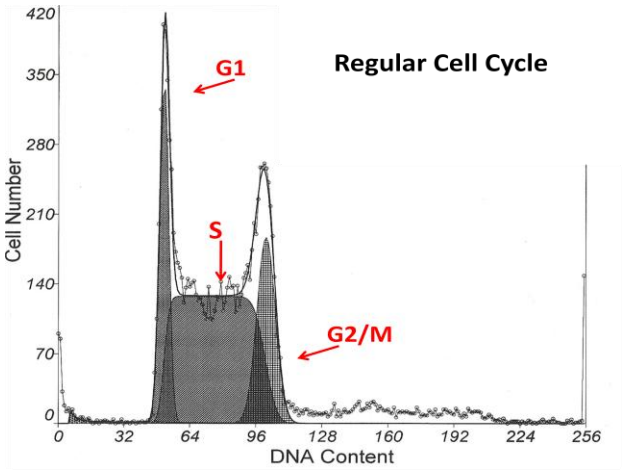



*Figure 6: Cell Cycle Analysis.*

Furthermore flow cytometry can also detect changes in the number of chromosomes in a population of cells and is therefore competent to reveal aneuploidy, which is characterized by an abnormal number of chromosomes, or polyploidy, which describes cells with more than two homologous sets of chromosomes.

Since polyploid cells have at least one full extra complement of chromosomes, one can observe an additional population in the histogram with a fluorescence intensity that is some multiple of 1X. In the case of polyploidy, there is also a population of cells representing a second S phase, when DNA is replicated from 4N to 8N (Figure 7).

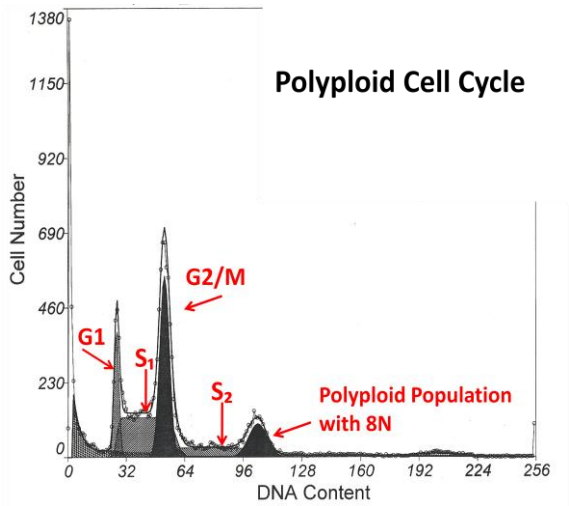



*Figure 7: Cell Cycle Analysis.*

Moreover, in all cell cycle analyses there is usually a population of cells to be seen in the S2 region. These cells have a PI staining higher than 4N, which can occur when doublets (two cells stuck together) are read as a single cell by flow cytometer. The number of readings in this region is usually insignificantly small and thus left out in analysis, but since polyploidy was expected in this study the S2 phase is included.

Furthermore it is possible to detect aneuploid cell populations by flow cytometry. Due to the fact that aneuploid cells have an abnormal number of chromosomes, the G1 or G2/M curve can either show two peaks or a broader side that is described as a shoulder.

With the help of special software the percentages of cells occupying the different phases of the cell cycle can be calculated and thus analyzed.

In this study, cell cycle analysis was used to test whether knockdown of Aurora Kinase A or Cyclin B1 leads to major cell cycle defects or polyploidy.

R1 cells were nucleofected with both vectors (pcDNA 6.2-GW/EmGFP miR Aurka 702 and Ccnb1 682). As a negative control for Aurora Kinase A and Cyclin B1, R1 cells were nucleofected with the miR neg vector.

In order to compare resulting data to the phenotype seen by B-Myb knockdown, R1 cells were nucleofected with a B-Myb shRNA vector as a positive control. The empty vector, pSuper, was nucleofected into R1 cells as well and served as a negative control for B-Myb shRNA.

Cells were harvested 24, 48 and 72 h after selection, stained with Propidium Iodide and cell cycle analysis was run afterwards.

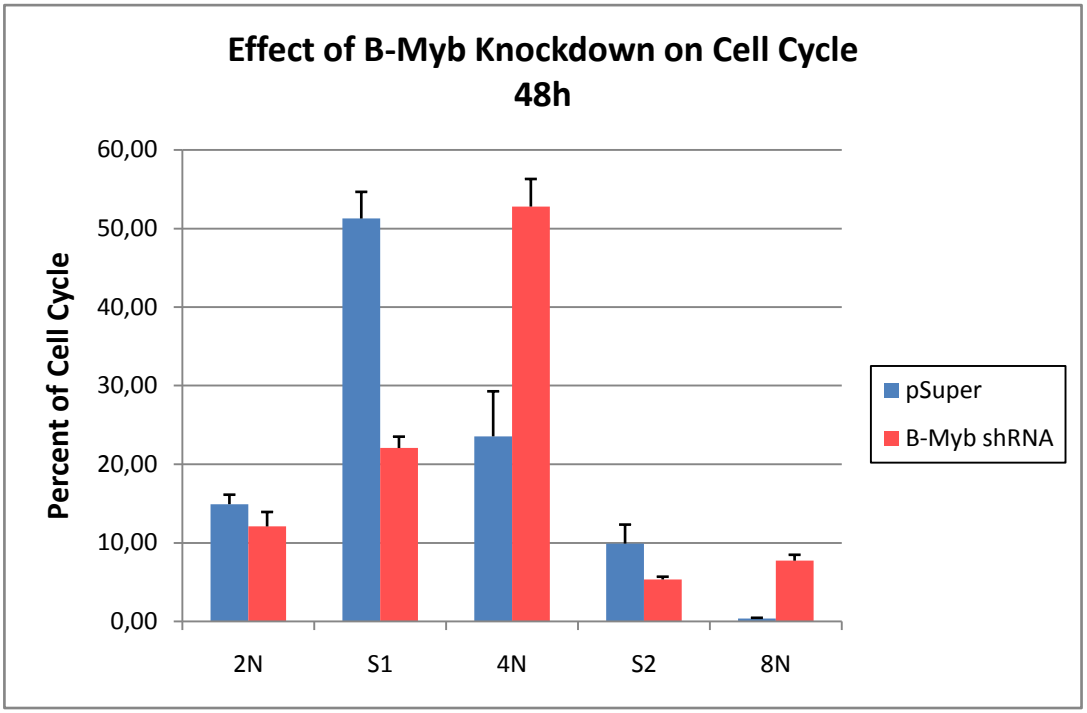



*Figure 8: Cell cycle analysis: Percentages of cells occupying the different phases of the cell cycle. 2N = normal set of chromosomes in non-dividing cells, representing G1 phase. S1 = S phase, DNA replication. 4N = duplicated set of chromosomes, representing G2/M phase. S2 = cells having fluorescence values higher than 4N. 8N = 4 sets of chromosomes, polyploid cells.*

R1 cells nucleofected with the pSuper control showed a regular cell cycle for embryonic stem cells. The rapidly dividing mES cells have short cell cycle duration (~8-10hrs) and tend to have relatively short G1 and G2/M durations with the majority of cells in S phase. These characteristics are shown for pSuper (Figure 8): more than 50 % of the cells are in S phase and the amount of cells in G1- and G2/M phase is clearly lower. No abnormal polyploidy cell population can be observed for pSuper.

By contrast, R1 cells nucleofected with B-Myb shRNA showed major cell cycle defects such as a decrease of cells in G1 and S, while having a significant increase of cells in G2/M with more than 50 % of cells in this phase of the cell cycle. The observed cell cycle characteristics could reveal a delayed transit through G2/M with an accumulation of cells as this was seen by Tarasov et al. 2008 as well.

Since there was also a polyploid cell population (8N), in addition to a delay, the higher amount of cells containing a duplicated set of chromosomes (4N) also indicated an increase in cells undergoing abnormal cell division.

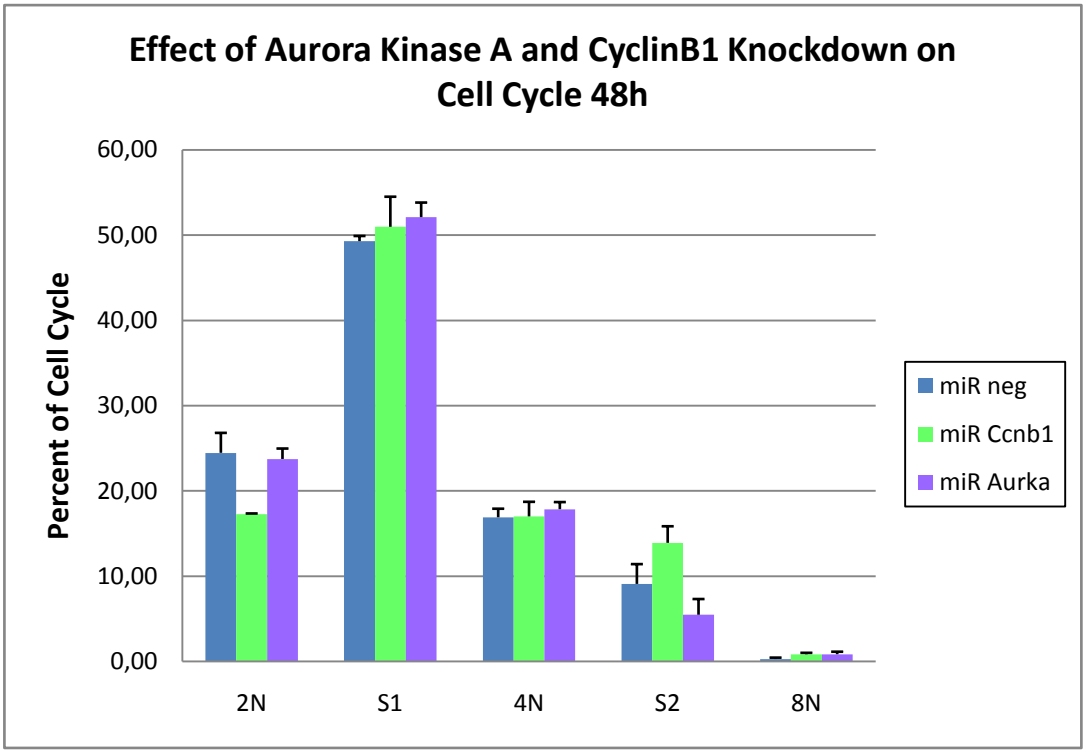



*Figure 9: Cell cycle analysis: Percentages of cells occupying the different phases of the cell cycle.*

R1 cells nucleofected with the miR neg as a negative control displayed a regular cell cycle for mouse embryonic stem cells as seen for pSuper as well. Most of the cells were in S phase and due to short generation times with almost eliminated gap phases the quantity of cells in G1 and G2/M is smaller.

The embryonic stem cells nucleofected with miR Aurka 702 and miR Ccnb1 682 respectively both showed a regular cell cycle that was comparable to the negative control. Major cell cycle defects such as a delay in a specific phase or a significant polyploid population were not observed.

## 4.4 Spindle-Assembly Analysis

Spindle-assembly analysis is a method to visualize by immunofluorescence microscopy whether the spindle apparatus and chromosomes are arranged properly during mitosis. Moreover, the relative quantity of cells undergoing the different stages of mitosis can be counted and analyzed by this technique.

The process of mitosis can be divided into four principal stages and each stage can be identified with the help of defined characteristics such as centrosome and spindle formation or chromosome alignment:

In prophase chromatin condenses into chromosomes, the two centrosomes begin moving to opposite sides of the cell and the nucleolus disappears. In prometaphase, which is sometimes considered part of prophase, the nuclear envelope breaks down and microtubules emanating from the centrosomes attach to the kinetochores of the chromosomes. Subsequently, in metaphase, centrosomes start pulling the chromosomes toward the two ends of the cell. As a result, all chromosomes become aligned in the metaphase plate at the cell´s equator. Afterwards the cell proceeds to anaphase where paired chromosomes separate at the kinetochores and the single chromatids thus can now be considered individual chromosomes, and are pulled to opposite ends of the cell. When the separating chromosomes reach the poles in telophase, new nuclear envelopes form around each set. Chromosomes and spindle fibers disperse and the division of cytoplasm, cytokinesis, starts during this phase.

For this purpose, R1 cells were nucleofected with both vectors (pcDNA 6.2-GW/EmGFP miR Aurka 702 and Ccnb1 682). In addition, R1 cells nucleofected with the miR neg vector served as a negative control.

To compare resulting data to the phenotype seen by B-Myb knockdown in previous experiments, R1 cells nucleofected with B-Myb shRNA vector were used as a positive control. The empty vector, pSuper, was nucleofected into R1 cells as well and served as a negative control for B-Myb shRNA.

Cells were fixed with 4 % paraformaldehyde at 48 h after selection, and via α-tubulin, γ-tubulin and DNA staining, the state of mitosis was analyzed by immunofluorescence microscopy.

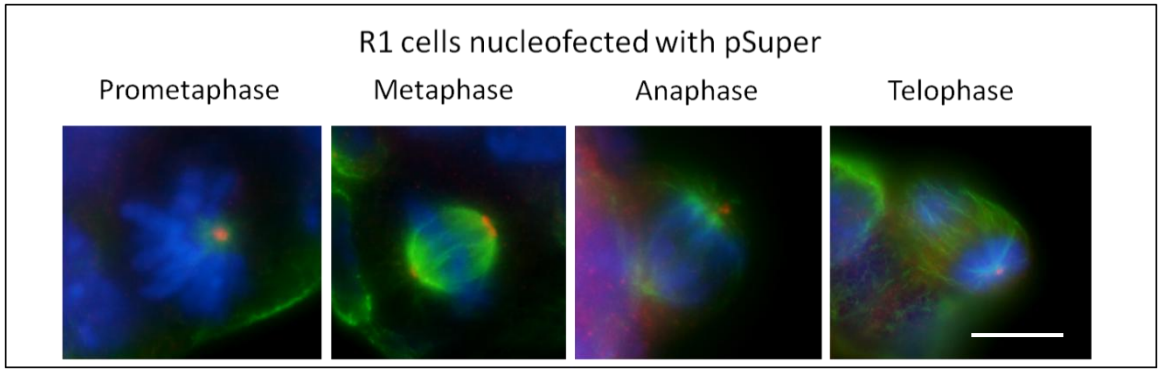



*Figure 10: Mitotic cells 72h after nucleofection. Cells are stained with DAPI (blue), gamma tubulin (red) and alpha tubulin (green). Bar = 20 µm.*

R1 cells nucleofected with miR neg and pSuper, as a negative control, showed regular characteristics in all stages of mitosis: normal bipolar centrosomes in prophase, regular alignment of chromosomes in the metaphase plate, bipolar mitotic spindle formation in anaphase and separating chromosomes reaching opposite poles in telophase (see Figure 10).

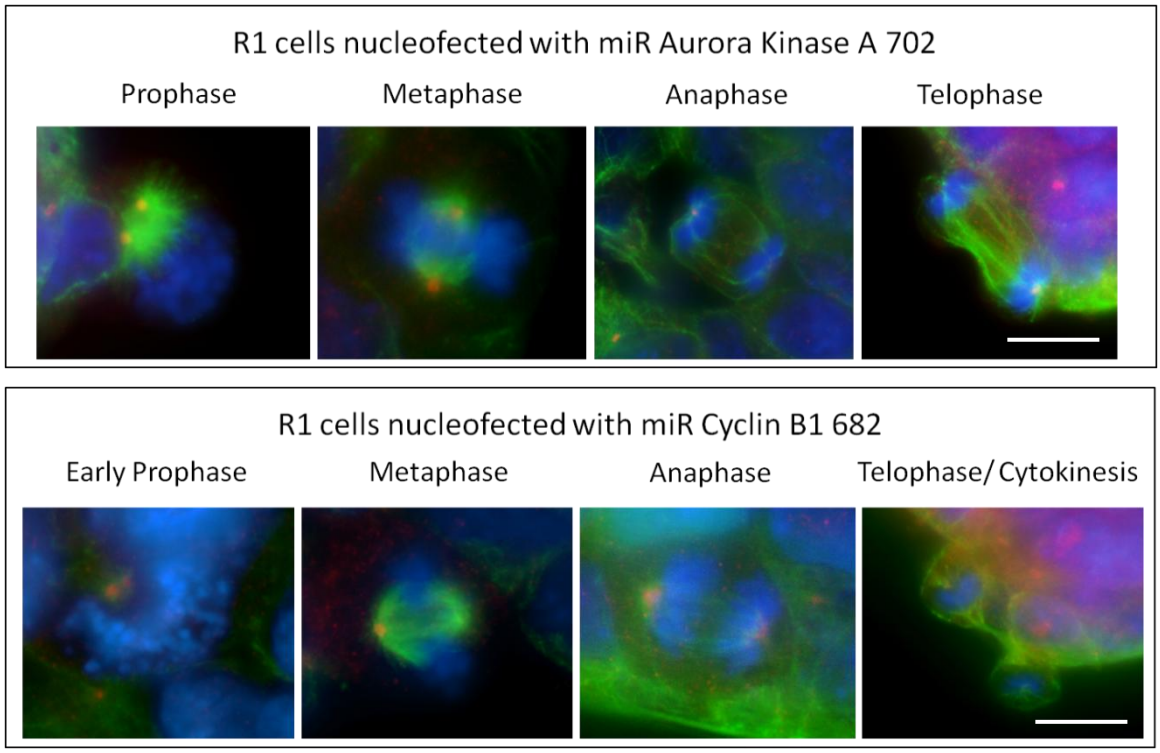



*Figure 11: Mitotic cells 72h after nucleofection. Cells are stained with DAPI (blue), gamma tubulin (red) and alpha tubulin (green). Bar = 20 µm.*

Similarly, R1 cells nucleofected with miR Aurora Kinase A and Cyclin B1 vectors displayed normal centrosome-/ spindle formation and chromosome alignment with the

regular characteristics as explained for miR neg and pSuper (see Figure 11). No mitotic spindle or centrosome defects could be observed.

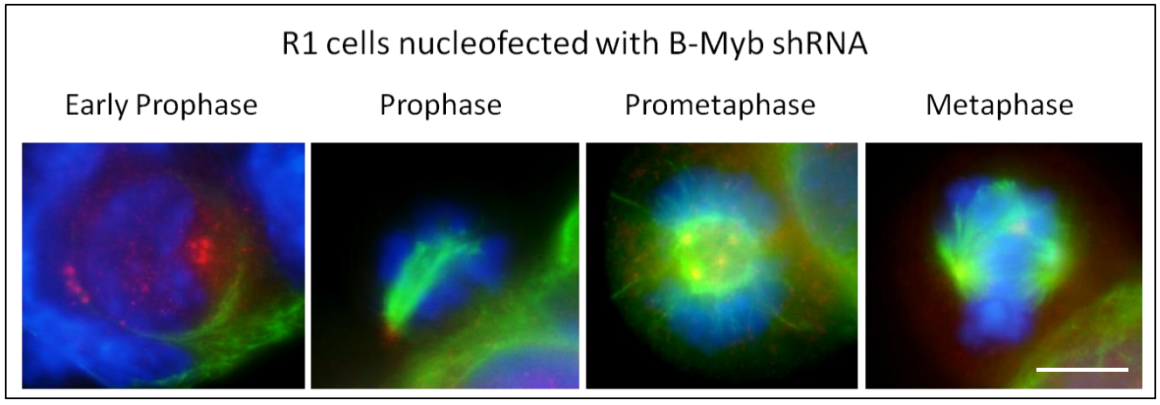



*Figure 12: Mitotic cells 72h after nucleofection. Cells are stained with DAPI (blue), gamma tubulin (red) and alpha tubulin (green). Abnormalities in spindle assembly analysis are apparent e.g. multiple, irregular centrosomes (red) in early prophase; abnormal monopolar spindle formation (green) in prophase; abnormal number of centrosomes (red) and abnormal spindle formations (green) in prometaphase; multiple centrosomes (red) and mulitpolar spindle formation (green) in metaphase. Bar = 20 µm.*

On the contrary, in mitotic cells lacking B-Myb, several abnormalities were detected, such as multiple centrosomes (red) and abnormal monopolar or multipolar spindle formations (green). There were only very few cells seen in anaphase or telophase.

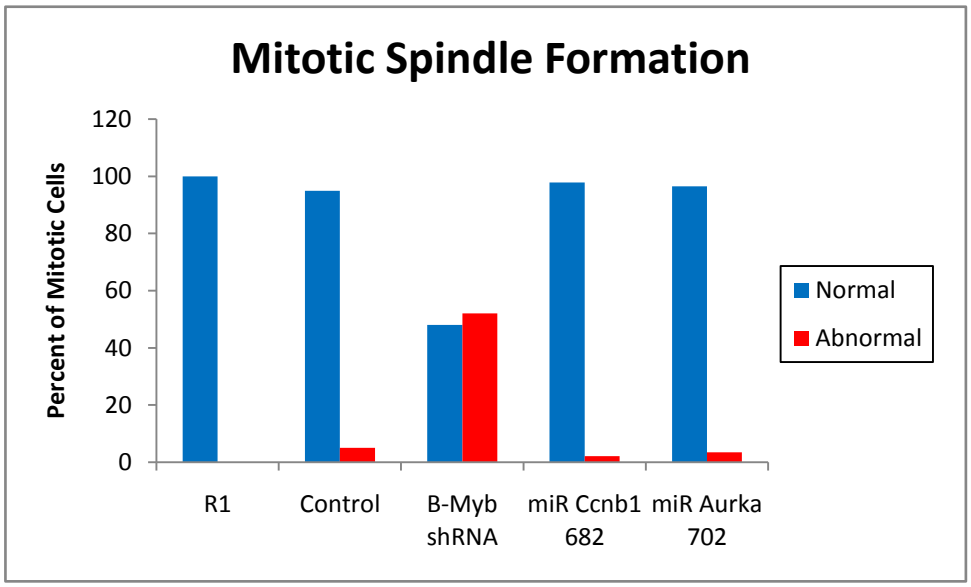



*Figure 13: Mitotic spindle formation analysis as percent of mitotic cells. Cells with regular mitotic spindle apparatus (blue). Amount of cells with mono- or multipolar spindle formation or an irregular configuration of mitotic spindles is displayed as abnormal cells (red). Cells nucleofected with pSuper served as negative control. No error bars are given due to aggregate analysis.*

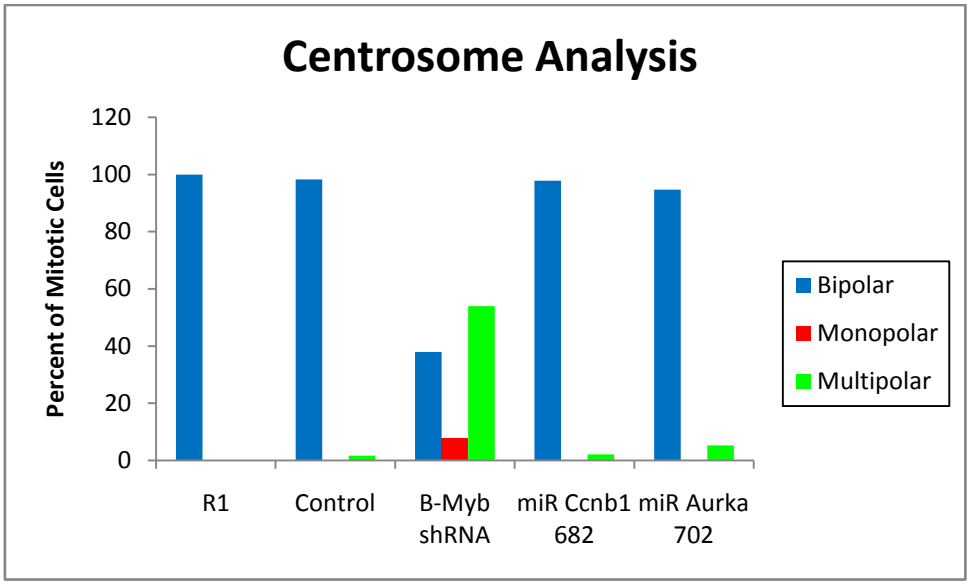



*Figure 14: Centrosome analysis as percent of mitotic cells. Amount of cells containing regular bipolar centrosomes (blue), irregular monopolar (red) or mulitpolar centrosomes (green) are shown. Cells nucleofected with pSuper served as a negative control. No error bars are given due to aggregate analysis.*

In summary, a total of 312 cells were analyzed with regard to spindle and centrosome formation (see Figure 13, 14) observing that abnormal spindle or centrosome formations could only be detected in a small amount of R1 cells lacking Aurora Kinase A or Cyclin B1, which was comparable to negative control cells. In contrast, loss of B-Myb led to irregular spindle formations, such as mono- or multipolar spindle apparatus and an irregular amount of centrosomes in a significant part of R1 cells.

Furthermore spindle assembly analysis enables the relative amount of cells in the four different phases of mitosis to be compared.

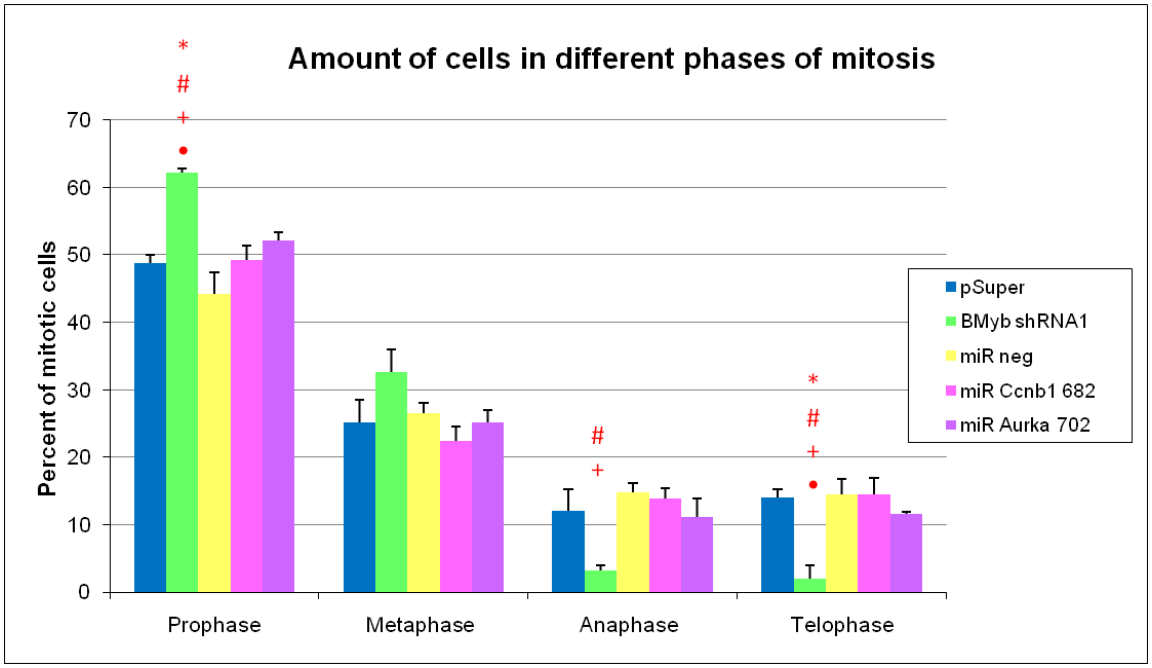



*Figure 15: Phases of mitosis characterized by alpha tubulin and DNA IF staining. Prophase includes both prophase and prometaphase. pSuper and B-Myb shRNA n=2 counts, miR neg, Ccnb1 682 and Aurka 702 n=3 counts. * = p value compared to pSuper, # = p value compared to miR neg, + = p value compared to Ccnb1 miR, • = p value compared to Aurka. Significance is given for within phase only. (i.e. prophase to prophase, not prophase to telophase)*

When comparing negative control cells to R1 cells lacking Aurora Kinase A or Cyclin B1, there were similar numbers of cells in all stages of mitosis. Whereas B-Myb knockdown cells were mostly in prophase and metaphase with only few cells in anaphase and telophase (see Figure 15), which can be due to the observed mitotic spindle and centrosome defects and a resulting delay in mitosis.

In conclusion, spindle assembly analyses have shown that neither Aurora Kinase A nor Cyclin B1 have a significant effect on mitotic progression in embryonic stem cells. The phenotype seen by B-Myb knockdown could not be reproduced by knockdown of a single target gene.

## 4.5 Generation of the inducible expression system Line Ainv15

The genetically modified embryonic stem cells Line Ainv15 contain a reverse tetracycline transactivator (rtTA) under control of a CMV (Cytomegalovirus) promoter. This rtTA is inserted at the Rosa26 locus of chromosome 6 to allow a stable and efficient expression. The transcribed tetracycline-controlled transactivation proteins bind to the tetracycline

responsive element (TRE) at the hprt (hypoxanthine-guanine phosphoribosyltransferase) locus of the X-chromosome only in the presence of tetracycline or derivatives such as doxycycline. Since binding to the TRE activates transcription of the gene of interest, the activation of transgenes can thus be regulated in vivo in a dose-dependent manner.

Furthermore the ES cells employed comprise the EmGFP (Emerald Green Fluorescent Protein) that has been engineered to allow insertion of a miRNA in its 3'UTR. The EmGFP sequence contains flanking and loop sequences that facilitate miRNA excision and function. An miRNA can thus be expressed co-cistronically with EmGFP, which essentially permits 100 % correlation of EmGFP expression with the knockdown activity of miRNA.

ES cell lines are being generated that can inducibly activate the expression of EmGFP-containing miRNA to downregulate the specific target.

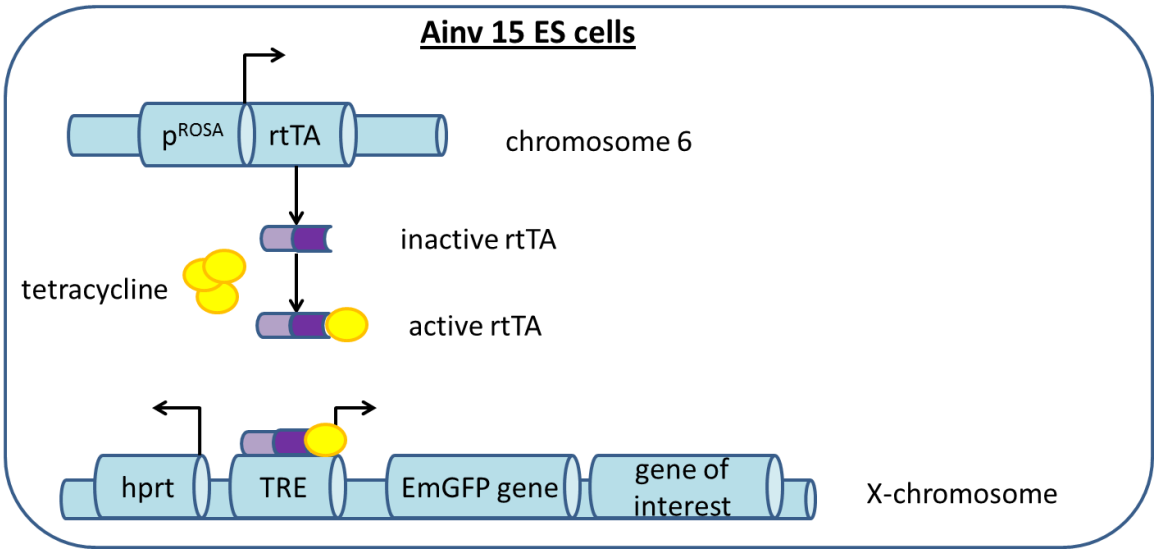



*Figure 16: Inducible RNAi in Ainv 15 embryonic stem cells. Schematic illustration of genetically modified Ainv 15 cells.*

As explained in the aim of this study, it was initially planned to use the inducible expression system Ainv15 as the last step to get a stronger knockdown and therefore a more reliable phenotype compared to knockdown by miRNA. Since my research project was limited to six months, I only contributed to the design and preparation of this cell line, but was unable to run a detailed analysis in the Ainv15 cells during my stay. This work was completed by others after I left the NIA, and the results are not included here at the request of my supervisor, Prof. Kenneth Boheler.

## 4.6 Design of a miRNA vector directed against Aurora Kinase A

To complete the results section, the design of the miRNA vectors directed against Aurora Kinase A and Cyclin B1, which provide the basis for my study, is described in detail.

The BLOCK-iT Pol II miR RNAi Expression Vector Kit was chosen for vector-based expression of miRNA to achieve stable suppression of Aurora Kinase A and Cyclin B1.

This system was used to clone ds oligos encoding a specific miRNA target sequence into a vector containing the human cytomegalovirus (CMV) promoter for use in RNAi analysis. The engineered miRNAs produced by this Kit fully complement their target site, and thus lead to cleavage of the desired messenger RNA (mRNA).

The CMV promoter is recognized by RNA Polymerase II and allows a high-level constitutive miRNA expression in mammalian cells. Since the vector includes the EmGFP gene, one can visually detect the cells in which knockdown is occurring. The Green Fluorescent Protein is a bioluminescent protein derived from the Jellyfish Aequorea Victoria and emits fluorescence upon excitation, which can easily be measured by fluorescence microscopy. The Emerald Green Fluorescent Protein is a variant of enhanced GFP that increases the intensity of the fluorescence signal.

By placing the EmGFP gene downstream of the CMV promoter and upstream of the pre-miR, it can be used as a marker of gene expression, because one can track those cells in which the EmGFP protein and miRNA are expressed simultaneously.

In order to select positive transformants, the vector contains a Spectinomycin and Blasticidin resistance gene, which both allow selection in E.coli. Moreover, the Blasticidin resistance gene is suitable for selection in mammalian cells to generate stable cell lines.

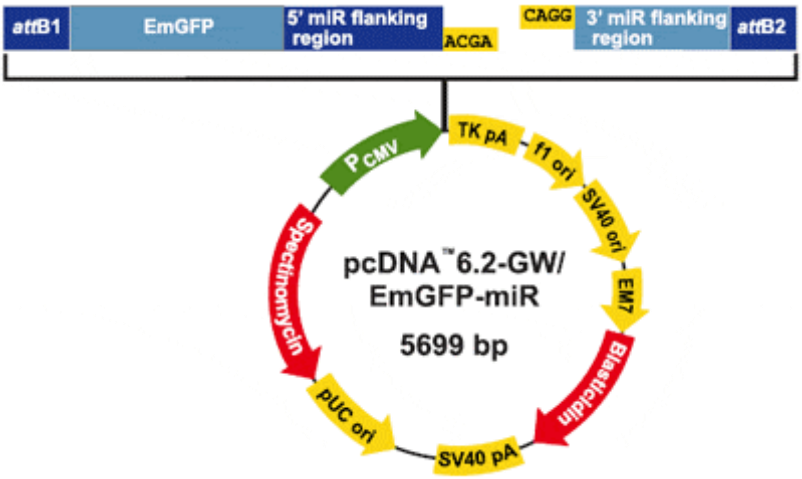



*Figure 17: Map and Features of the pcDNA 6.2-GW/EmGFP-miR (Invitrogen).*

In the first step of the pc DNA 6.2-GW/EmGFP vector construction, the pairs of complementary DNA oligonucleotides were designed with the help of the BLOCK-iT™ RNAi Designer (Invitrogen).

The top-strand oligo encodes the target pre-miRNA and has the following structural features:

- A 5' overhang (TGCT), which is complementary to the vector and required for directional cloning (Linker)
- A 5' G + a short 21 nucleotide antisense sequence (mature miRNA) derived from the target gene
- A short spacer of 19 nucleotides to form a terminal loop
- A short sense target sequence with 2 nucleotides removed (Δ2) to create an internal loop
- A 4 nucleotide, 5’ overhang (CAGG) complementary to the vector and required for directional cloning (Linker)


Five pairs of pre-miRNAs were generated for knockdown of Aurora Kinase A:

### miRNA Aurka 398

| | |
|---|---|
| top strand | 5'-TGCTGAATCATTTCCGGAGGCTGCAGGTTTTGGCCACTGACTGACCTGCAGCCCGGAAATGATTCAGG-3' |
| bottom strand | 5'-CCTGAATCATTTCCGGGCTGCAGGTCAGTCAGTGGCCAAAACCTGCAGCCTCCGGAAATGATTC-3' |
| ds oligo | 5'-TGCTGAATCATTTCCGGAGGCTGCAGGTTTTGGCCACTGACTGACCTGCAGCCCGGAAATGATTCAGG-3'<br>3'-CTTAGTAAAGGCCTCCGACGTCCAAAACCGGTGACTGACTGGACGTCGGGCCTTTACTAAGTCC-5' |

### miRNA Aurka 555

| | |
|---|---|
| top strand | 5'-TGCTGTTCAGAGCCAGGATGAACTTGGTTTTGGCCACTGACTGACCAAGTTCACTGGCTCTGAACAGG-3' |
| bottom strand | 5'-CCTGTTCAGAGCCAGTGAACTTGGTCAGTCAGTGGCCAAAACCAAGTTCATCCTGGCTCTGAAC-3' |
| ds oligo | 5'-TGCTGTTCAGAGCCAGGATGAACTTGGTTTTGGCCACTGACTGACCAAGTTCACTGGCTCTGAACAGG-3'<br>3'-CAAGTCTCGGTCCTACTTGAACCAAAACCGGTGACTGACTGGTTCAAGTGACCGAGACTTGTCC-5' |

### miRNA Aurka 702

| | |
|---|---|
| top strand | 5'-TGCTGTCTAGAATCAGATAAACTCGGGTTTTGGCCACTGACTGACCCGAGTTTCTGATTCTAGACAGG-3' |
| bottom strand | 5'-CCTGTCTAGAATCAGAAACTCGGGTCAGTCAGTGGCCAAAACCCGAGTTTATCTGATTCTAGAC-3' |
| ds oligo | 5'-TGCTGTCTAGAATCAGATAAACTCGGGTTTTGGCCACTGACTGACCCGAGTTTCTGATTCTAGACAGG-3'<br>3'-CAGATCTTAGTCTATTTGAGCCCAAAACCGGTGACTGACTGGGCTCAAAGACTAAGATCTGTCC-5' |

### miRNA Aurka 1154

| | |
|---|---|
| top strand | 5'-TGCTGTTGAAATGAGGTCCCTGGCTCGTTTTGGCCACTGACTGACGAGCCAGGCCTCATTTCAACAGG-3' |
| bottom strand | 5'-CCTGTTGAAATGAGGCCTGGCTCGTCAGTCAGTGGCCAAAACGAGCCAGGGACCTCATTTCAAC-3' |
| ds oligo | 5'-TGCTGTTGAAATGAGGTCCCTGGCTCGTTTTGGCCACTGACTGACGAGCCAGGCCTCATTTCAACAGG-3'<br>3'-CAACTTTACTCCAGGGACCGAGCAAAACCGGTGACTGACTGCTCGGTCCGGAGTAAAGTTGTCC-5' |

### miRNA Aurka 1276

| | |
|---|---|
| top strand | 5'-TGCTGTTTGCTGGTTGGCTCTTTGCTGTTTTGGCCACTGACTGACAGCAAAGACAACCAGCAAACAGG-3' |
| bottom strand | 5'-CCTGTTTGCTGGTTGTCTTTGCTGTCAGTCAGTGGCCAAAACAGCAAAGAGCCAACCAGCAAAC-3' |
| ds oligo | 5'-TGCTGTTTGCTGGTTGGCTCTTTGCTGTTTTGGCCACTGACTGACAGCAAAGACAACCAGCAAACAGG-3'<br>3'-CAAACGACCAACCGAGAAACGACAAAACCGGTGACTGACTGTCGTTTCTGTTGGTCGTTTGTCC-5' |

The top- and bottom-strand oligonucleotides were annealed, cloned into the linearized pc DNA 6.2-GW/EmGFP-miR vector using T4 DNA Ligase and afterwards transformed into competent E.coli One Shot TOP10. 50 and 100 µl from each transformation were spread on pre-warmed LB agar plates containing 50 µg/ml spectinomycin and incubated overnight at 37°C. 10 spectinomycin-resistant colonies were chosen and cultured overnight in LB medium containing 50 µg/ml spectinomycin. After isolation of the plasmid DNA by QuickGene Plasmid kit S II, the correct insertion of the pre-miRNA was confirmed by digestion reaction using the restriction enzymes XhoI and SalI (data not shown).

In order to check the expression of the miRNA, positive transformants were transfected into C2C12-cells and fluorescence of the EmGFP protein was detected 24 h later.

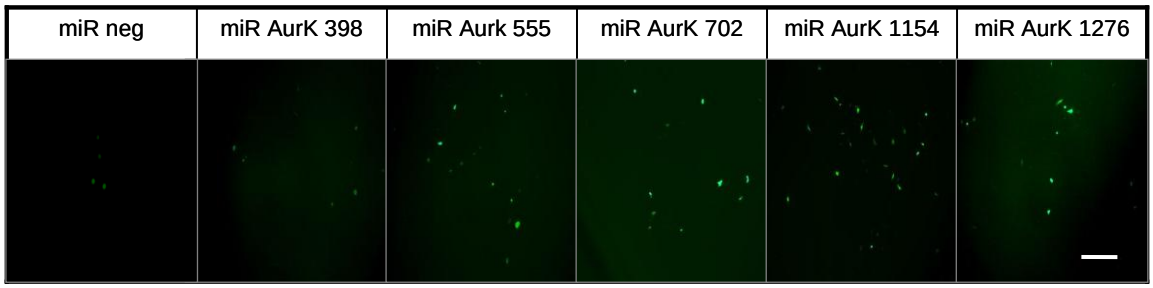



*Figure 18: C2C12 cells transfected with positive transformants. Green fluorescent cells show EmGFP expression. Bar = 400 µm.*

## 4.7 Design of a miRNA vector directed against Cyclin B1

Three pairs of pre-miRNAs were designed with the help of the BLOCK-iT™ RNAi Designer (Invitrogen) for knockdown of Cyclin B1.


### miRNA Ccnb1 682

| | |
|---|---|
| top strand | 5'-TGCTGTAGCCAGTCAATGAGGATAGCGTTTTGGCCACTGACTGACGCTATCCTTTGACTGGCTACAGG-3' |
| bottom strand | 5'-CCTGTAGCCAGTCAAAGGATAGCGTCAGTCAGTGGCCAAAACGCTATCCTCATTGACTGGCTAC-3' |
| ds oligo | 5'-TGCTGTAGCCAGTCAATGAGGATAGCGTTTTGGCCACTGACTGACGCTATCCTTTGACTGGCTACAGG-3' |
| | 3'-CATCGGTCAGTTACTCCTATCGCAAAACCGGTGACTGACTGCGATAGGAAACTGACCGATGTCC-5' |

### miRNA Ccnb1 744

| | |
|---|---|
| top strand | 5'-TGCTGATAATGGACACAGTCATGTACGTTTTGGCCACTGACTGACGTACATGAGTGTCCATTATCAGG-3' |
| bottom strand | 5'-CCTGATAATGGACACTCATGTACGTCAGTCAGTGGCCAAAACGTACATGACTGTGTCCATTATC-3' |
| ds oligo | 5'-TGCTGATAATGGACACAGTCATGTACGTTTTGGCCACTGACTGACGTACATGAGTGTCCATTATCAGG-3' |
| | 3'-CTATTACCTGTGTCAGTACATGCAAAACCGGTGACTGACTGCATGTACTCACAGGTAATAGTCC-5' |

### miRNA Ccnb1 826

| | |
|---|---|
| top strand | 5'-TGCTGTTTGCTTGCAATAAACATGGCGTTTTGGCCACTGACTGACGCCATGTTTTGCAAGCAAACAGG-3' |
| bottom strand | 5'-CCTGTTTGCTTGCAAAACATGGCGTCAGTCAGTGGCCAAAACGCCATGTTTATTGCAAGCAAAC-3' |
| ds oligo | 5'-TGCTGTTTGCTTGCAATAAACATGGCGTTTTGGCCACTGACTGACGCCATGTTTTGCAAGCAAACAGG-3' |
| | 3'-CAAACGAACGTTATTTGTACCGCAAAACCGGTGACTGACTGCGGTACAAAACGTTCGTTTGTCC-5' |

As mentioned above, the BLOCK-iT Pol II miR RNAi Expression Vector Kit was used for vector construction as well. The procedure is identical to that described for the miRNA vector directed against Aurora Kinase A (see 4.6).

In order to check the expression of the miRNA, positive transformants were transfected into C2C12-cells and fluorescence of the EmGFP protein was detected 24 h later.

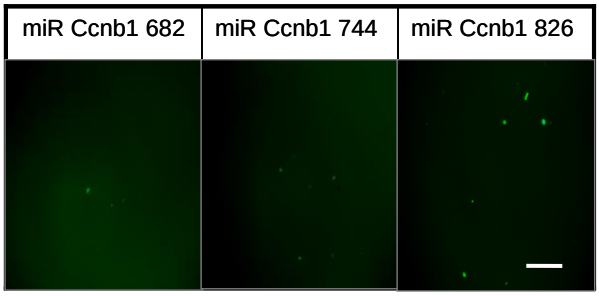



*Figure 19: C2C12 cells transfected with positive transformants. Green fluorescent cells show EmGFP expression. Bar = 400 µm.*

## 5. Discussion

Embryonic stem cells and induced pluripotent stem cells have the capacity for unlimited self-renewal and the ability to differentiate into all cell types of the three embryonic germ layers. Owing to those unique characteristics, ES and iPS cells are considered to serve as a possible source for regenerative medicine, including tissue engineering and cell replacement therapy.

The field of regenerative medicine holds great potential to treat injuries and degenerative diseases such as heart failure (Cao et al., 2008), diabetes (D'Amour et al., 2006) or Parkinson´s disease (Yang et al., 2008). However, before the translation of stem cell applications to the clinic can be realized, several problems like e.g. tumor formation must be overcome, and a more thorough understanding of the molecular mechanisms that form the basis of the pluripotent state of ES and iPS cells is required. In order to understand how pluripotency is established and maintained, it is important to determine the role of specific transcription factors operating in this regulatory network.

In this regard, the transcription factor B-Myb was recently spotlighted, because it plays an important role in maintaining pluripotency, normal cell cycle progression and chromosomal stability of embryonic stem cells. Previous experiments have demonstrated that B-Myb is required for proper S phase progression, as B-Myb ablation leads to a significant slowdown of the proliferation rate, stalling of replication forks and a superactivation of active replication foci causing disorganization of the replication program and an increase in double-strand breaks. These defects seen with loss of B-Myb are partly due to c-Myc and FOXM1 regulatory mechanisms (Lorvellec et al. 2010). Furthermore Tarasov et al. have shown the role of B-Myb in regular progression through G2/M phase, since knockdown of B-Myb by shRNA leads to major cell cycle defects and ES cell aneuploidy.

As preliminary ChIP on chip data has indicated, the transcription factor B-Myb regulates gene expression of several proteins, which are either cell cycle associated or implicated in differentiation. To understand its precise role in cell cycle regulation, it is very important to establish whether a generalized reduction of its target genes or dysregulation of individual factors causes the phenotype seen by B-Myb knockdown. Therefore the function of specific target genes needs to be determined in order to detect major or minor contributors of B-Myb.

Due to the fact that B-Myb regulates gene expression of Aurora Kinase A and Cyclin B1, this project was focused on those target genes to find out if they serve as major or minor contributors of B-Myb.

Aurora Kinase A was chosen because previous experiments in mouse embryonic fibroblasts have shown a delayed mitotic entry, defects in spindle formation as well as polyploidy relating to Aurora Kinase A ablation (Cowley et al., 2009). That phenotype resulting from loss of Aurora Kinase A function in somatic cells is similar to the phenotype caused by B-Myb knockdown in embryonic stem cells.

Since B-Myb regulates gene expression of Aurora Kinase A, this protein could be responsible for the observed B-Myb knockdown phenotype. If this hypothesis could be proved, the target gene Aurora Kinase A would be a major contributor of B-Myb.

In order to study Aurora Kinase A function in embryonic stem cells, five miRNAs for its knockdown were established and tested. With the help of Western Immunoblotting miRNA Aurka 702 was chosen, since it achieved lowest protein levels of Aurora Kinase A. By contrast, knockdown of B-Myb caused only an insignificant reduction of Aurora Kinase A levels, whereas knockdown of Aurora Kinase A by miRNA 702 resulted in a relevant decrease in expression at the 72 hours timepoint.

MiRNA 702 was thus used for cell cycle- and spindle assembly analysis in order to investigate on the one hand whether the phenotype resulting from Aurora Kinase A ablation in somatic cells could be reproduced in ES cells, and on the other hand whether reduced expression of Aurora Kinase A is responsible for the phenotype seen by B-Myb knockdown and accordingly serves as a major contributor for B-Myb.

However, cell cycle analysis of R1 cells nucleofected with miRNA Aurka 702 showed a regular cell cycle for mES cells containing G1-, S- and G2/M populations with most of the cells in S-phase. Neither major cell cycle defects nor polyploid or aneuploid cell populations were observed.

In contrast, cell cycle analysis of B-Myb deficient cells displayed a shift of cells in cell cycle with a reduction of cells in G1- and S phases and a significant increase of cells in G2/M. That change indicates an increase in cells undergoing abnormal cell division, which is confirmed by the observed polyploid population.

Furthermore, spindle assembly analysis of R1 cells nucleofected with miR Aurka 702 showed comparable numbers of cells in each stage of mitosis (prophase, metaphase,

anaphase, telophase), and no significant amount of abnormally formed spindles when compared to miR neg controls. In contrast, Cowley et al. have shown in previous data that loss of Aurora Kinase A in mouse embryonic fibroblasts leads to a delayed mitotic entry with an accumulation of cells in early prophase as well as defects in bipolar spindle formation. This data reveals that the phenotype resulting from Aurora Kinase A ablation in somatic cells could not be reproduced in embryonic stem cells with the partial knockdown in this study. A complete knockdown or knockout of Aurora Kinase A would be expected to lead to such a phenotype, unless other members of the Aurora Kinase family are able to compensate its function.

The positive control cells (nucleofected with B-Myb shRNA) had very few cells in post-metaphase phases of the cell cycle and featured several cells with abnormally formed spindles such as multiple centrosomes and a spindle apparatus with several central points.

In summary, the data from cell cycle- and spindle assembly analysis clarified that incomplete knockdown of Aurora Kinase A using miRNA to levels lower than that seen with B-Myb knockdown failed to produce the same phenotype.

The target gene Cyclin B1 was chosen for testing because it plays a decisive role in promoting several events in early phases of mitosis and due to its decreased expression in embryonic stem cells following B-Myb knockdown, the observed mitotic defects have been ascribed in part to Cyclin B1 (Boheler, 2009; Tarasov et al., 2008). This assumption is in accordance with preliminary data from megakaryocytes, where transient loss of Cyclin B1 causes polyploidy (Zhang et al., 1996).

To study Cyclin B1 function in mES cells, three miRNAs were established and tested for knockdown. Western immunoblotting showed that miRNA 682 was most effective for Cyclin B1 knockdown, and accordingly used for further analysis. Levels of Cyclin B1 are also decreased both by B-shRNA and by miR Ccnb1 682, but knockdown by miRNA leads to lower levels.

Thus, miRNA 682 was tested in cell cycle and spindle assembly analysis in order to detect whether the phenotype seen by B-Myb knockdown could be reproduced with loss of Cyclin B1. If so, the target gene Cyclin B1 would be a major contributor of B-Myb.

However, cell cycle analysis of R1 cells nucleofected with miRNA Ccnb1 682 showed a regular cell cycle for mouse embryonic stem cells. The amount of cells occupying the

different phases of cell cycle is in accordance with the typical characteristics of mES cells. Contrary to loss of B-Myb, major cell cycle defects such as a delay in a specific phase or an additional polyploid or aneuploid cell population were not observed with Cyclin B1 knockdown.

Furthermore, R1 cells nucleofected with miRNA Ccnb1 682 show regular centrosome and spindle formation in spindle assembly analysis. In contrast to B-Myb knockdown, there was no significant amount of cells containing monopolar/multipolar centrosomes or spindle formation defects. In addition, the distribution of cells by phases of mitosis equates the negative control.

Thus, cell cycle and spindle assembly analysis have shown that partial knockdown of Cyclin B1 by miRNA in mouse embryonic stem cells does not lead to the same phenotype seen with B-Myb knockdown.

In conclusion, the characteristic aneuploid phenotype observed by loss of B-Myb could not be reproduced by a partial knockdown of either Aurora Kinase A or Cyclin B1. Thus, the incomplete knockdown of these target genes cannot account for the cell cycle, spindle or centrosome defects observed in B-Myb deficient cells. Aurora Kinase A and Cyclin B1 may be in fact major contributors of B-Myb, but only in association with other essential regulatory factors, such as Plk1, Borealin, INCENP, c-Myc or FOXM1.

There are several different approaches to explain this data.

1. In the described experiments, miRNAs and shRNAs were used to knockdown Aurora Kinase A and Cyclin B1 to levels seen in B-Myb deficient cells in order to test whether the observed phenotype following loss of B-Myb can be mimicked.

   Therefore, five miRNAs were designed and tested for Aurora Kinase A knockdown and three miRNAs for Cyclin B1. The miRNAs Aurka 702 and Ccnb1 682, which were used for further analysis, achieved protein levels lower than those seen with B-Myb knockdown.

   But nevertheless, knockdown by miRNAs caused decreased protein levels of approx. 50 % of Aurora Kinase A and Cyclin B1 compared to negative control, whereas use of B-Myb shRNA led to totally reduced protein levels of B-Myb. It could be reasonable that lower or totally eliminated protein levels of Aurora Kinase A and Cyclin B1 would cause different effects on cell cycle and spindle assembly.

It is possible to design and test several other miRNAs, and maybe one would be found that leads to a more efficient knockdown of Aurora Kinase A or Cyclin B1 compared to those used in this work. Furthermore, combinations of two or more miRNAs could possibly improve the knockdown efficiency as well.

Another way of achieving a stronger knockdown is through the use of an inducible expression system such as the Ainv15 cells, which would permit a more titratable and complete response.

2. Since the transcription factor B-Myb regulates gene expression of many different target genes, Aurora Kinase A and Cyclin B1 were picked because of promising data from preliminary experiments in differentiated cells, namely mouse embryonic fibroblasts (Aurora Kinase A) and megakaryocytes (Cyclin B1).

   By contrast, in embryonic stem cells incomplete knockdown of both B-Myb target genes failed to reproduce the phenotype seen in previous experiments in somatic cells, i.e. it did not cause aneuploidy or mitotic spindle defects. This contrary data could be related to the huge differences between undifferentiated embryonic stem cells and somatic cells: ES cells possess three unique molecular properties – a unique transcriptional hierarchy, a poised epigenetic state, and a short cell cycle transit time (Boheler, 2009). Therefore it is possible that molecular characteristics and pathways may vary between those two different types of cells, and specific proteins such as the B-Myb target genes Aurora Kinase A or Cyclin B1 function in a different way in ES cells than in somatic cells. In this study, it is has been shown that partial knockdown of Aurora Kinase A and Cyclin B1, which are greatly involved in mitotic regulation in differentiated cells, did not lead to obvious cell cycle defects in embryonic stem cells.

3. Previous experiments in ES cells have shown that knockdown of the transcription factor B-Myb by shRNA causes a characteristic phenotype including a delayed transit through G2/M, severe mitotic spindle and centrosome defects, and polyploidy (Tarasov et al., 2008). Additionally, knockout of B-Myb results in a disturbance of S phase progression with stalling of replication forks and superactivation of replication factories (Lorvellec et al.). Since B-Myb regulates gene expression of several genes implicated in cell cycle, it is of great interest to detect whether dysregulation of a specific target gene leads to the observed phenotype. Although loss of B-Myb results in decreased protein levels of Aurora

Kinase A and Cyclin B1, this study has shown that incomplete knockdown of both to levels lower than that seen with B-Myb did not cause the specific phenotype.

In order to find out if dysregulation of another critical B-Myb target gene leads to severe cell cycle defects and aneuploidy, other individual factors like Plk1, INCENP or Borealin could be tested in an analogous manner. Apart from that, it is also possible that the observed B-Myb knockdown phenotype is due to a generalized reduction in proteins required for cell cycle progression in embryonic stem cells.

In conclusion, the effects of B-Myb loss on cell cycle progression in ES cells occur either through deficiency of a critical B-Myb target gene that does not depend exclusively on Cyclin B1 or Aurora Kinase A or through a multitude of events whose combined dysfunction lead to the cell cycle abnormalities observed following genetic loss or knockdown of B-Myb.

## 6. Summary

Embryonic stem cells and induced pluripotent stem cells represent a unique cell type that features the capacity of unlimited self-renewal and the ability to differentiate into all derivatives of the three primary germ layers. ES and iPS cells are therefore a promising source for regenerative therapies such as tissue engineering or cell replacement therapy. Nevertheless, before widespread stem cell applications in regenerative medicine can be realized, a detailed understanding of the regulatory pathways of pluripotency and self-renewal is necessary.

In this connection, the transcription factor B-Myb plays a decisive role in the regulatory network of ES cells and is involved in maintaining pluripotency, normal cell cycle progression and chromosomal integrity. Since B-Myb regulates gene expression of many proteins that are either associated with the cell cycle or implicated in differentiation, the question has been raised whether individual target genes act as major contributors of B-Myb or if the interaction of several proteins fulfills this task.

Taking previous data in somatic cells into account, the target genes Aurora Kinase A and Cyclin B1 were chosen to detect whether they serve as major contributors for B-Myb in embryonic stem cells.

In the present study, miRNAs for knockdown of Aurora Kinase A and Cyclin B1 were used for cell cycle and spindle assembly analysis in order to test whether the phenotype described for B-Myb knockdown could be reproduced by one of those target genes. Contrary to preliminary experiments in somatic cells, incomplete knockdown of neither Aurora Kinase A nor Cyclin B1 in ES stem cells led to abnormalities or defects in cell cycle or mitotic progression. Partial knockdown of both proteins did not reproduce the phenotype caused by B-Myb knockdown.

The effects of B-Myb in embryonic stem cells must involve multiple factors or another critical factor that has to be identified.

Other B-Myb target genes than Aurora Kinase A or Cyclin B1 can be tested in an analogous manner or by using the Ainv15 cells. Furthermore, dysregulation of several target genes could cause the aneuploidy observed with loss of B-Myb. Thus, it is still unclear, how B-Myb regulates cell cycle progression precisely in embryonic stem cells.

## 7. Literaturverzeichnis

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol. 2000; 227: 271-278

Andersson ER, Lendahl U. Regenerative medicine: a 2009 overview. J Intern Med. 2009; 266: 303-310

Applied Biosystems, 2010: Getting Started with MiRNA Research. http://www.ambion.com/techlib/tn/123/5.html (08.02.2010)

Arney KL, Fisher AG. Epigenetic aspects of differentiation. J Cell Sci. 2004; 117: 4355-4363

Becker KA, Ghule PN, Therrien JA, Lian JB, Stein JL, van Wijnen AJ, Stein GS. Self-renewal of human embryonic stem cells is supported by a shortened G1 cell cycle phase. J Cell Physiol. 2006; 209: 883-893

Becker KA, Stein JL, Lian JB, van Wijnen AJ, Stein GS. Establishment of histone gene regulation and cell cycle checkpoint control in human embryonic stem cells. J Cell Physiol. 2007; 210: 517-526

Becker KA, Stein JL, Lian JB, van Wijnen AJ, Stein GS. Human embryonic stem cells are pre-mitotically committed to self-renewal and acquire a lengthened G1 phase upon lineage pro-gramming. J Cell Physiol. 2010; 222: 103-110

Berdnik D, Knoblich JA. Drosophila Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. Curr Biol. 2002; 12: 640-647

Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001; 409: 363-366

Bibikova M, Laurent LC, Ren B, Loring JF, Fan JB. Unraveling epigenetic regulation in embryonic stem cells. Cell Stem Cell. 2008; 2: 123-134

Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B, Schryver B, Flanagan P, Clairvoyant F, Ginther C, Chan CS, Novotny M, Slamon DJ, Plowman GD. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J. 1998; 17: 3052-3065

Blangy A, Arnaud L, Nigg EA. Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150. J Biol Chem. 1997; 272: 19418-19424

Boheler KR. Stem cell pluripotency: a cellular trait that depends on transcription factors, chromatin state and a checkpoint deficient cell cycle. J Cell Physiol. 2009; 221: 10-17

Boss DS, Beijnen JH, Schellens JH. Clinical experience with aurora kinase inhibitors: a review. Onco-logist. 2009; 14: 780-793

Botquin V, Hess H, Fuhrmann G, Anastassiadis C, Gross MK, Vriend G, Schöler HR. New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. Genes Dev. 1998; 12: 2073-2090

Boutros R, Dozier C, Ducommun B. The when and wheres of CDC25 phosphatases. Curr Opin Cell Biol. 2006; 18: 185-191

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122: 947-956

Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA. 2004; 10: 1957-1966

Cao F, Wagner RA, Wilson KD, Xie X, Fu JD, Drukker M, Lee A, Li RA, Gambhir SS, Weissman IL, Robbins RC, Wu JC. Transcriptional and functional profiling of human embryonic stem cell-derived cardiomyocytes. PLoS One. 2008; 3: e3474

Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. Cell. 2009; 136: 642-655

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development. 2005; 132: 885-896

Chen X, Vega VB, Ng HH. Transcriptional regulatory networks in embryonic stem cells. Cold Spring Harb Symp Quant Biol. 2008; 73: 203-209

Cole MF, Young RA. Mapping key features of transcriptional regulatory circuitry in embryonic stem cells. Cold Spring Harb Symp Quant Biol. 2008; 73:183-193

Cowley DO, Rivera-Pérez JA, Schliekelman M, He YJ, Oliver TG, Lu L, O'Quinn R, Salmon ED, Magnuson T, Van Dyke T. Aurora-A kinase is essential for bipolar spindle formation and early development. Mol Cell Biol. 2009; 29: 1059-1071

Crasta K, Huang P, Morgan G, Winey M, Surana U. Cdk1 regulates centrosome separation by restraining proteolysis of microtubule-associated proteins. EMBO J. 2006; 25: 2551-2563

D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol. 2006; 24: 1392-1401

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292:154-156

Faast R, White J, Cartwright P, Crocker L, Sarcevic B, Dalton S. Cdk6-cyclin D3 activity in murine ES cells is resistant to inhibition by p16(INK4a). Oncogene. 2004; 23: 491-502

Giet R, McLean D, Descamps S, Lee MJ, Raff JW, Prigent C, Glover DM. Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. J Cell Biol. 2002; 156: 437-451

Gopalan G, Chan CS, Donovan PJ. A novel mammalian, mitotic spindle-associated kinase is related to yeast and fly chromosome segregation regulators. J Cell Biol. 1997; 138: 643-656

Hagting A, Jackman M, Simpson K, Pines J. Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr Biol. 1999 Jul 1; 9: 680-689

Hattori N, Imao Y, Nishino K, Hattori N, Ohgane J, Yagi S, Tanaka S, Shiota K. Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells. Genes Cells. 2007; 12: 387-396

Hattori N, Nishino K, Ko YG, Hattori N, Ohgane J, Tanaka S, Shiota K. Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. J Biol Chem. 2004; 279: 17063-17069

He L, Yang H, Ma Y, Pledger WJ, Cress WD, Cheng JQ. Identification of Aurora-A as a direct target of E2F3 during G2/M cell cycle progression. J Biol Chem. 2008; 283: 31012-31020

Hemmati-Brivanlou A, Spagnoli FM. Guiding embryonic stem cells towards differentiation: lessons from molecular embryology. Curr Opin Genet Dev. 2006; 16: 469-475

Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. Nat Biotechnol. 2005; 23: 699-708

Hohenstein KA, Pyle AD, Chern JY, Lock LF, Donovan PJ. Nucleofection mediates high-efficiency stable gene knockdown and transgene expression in human embryonic stem cells. Stem Cells. 2008; 26: 1436-1443

Hutvágner G, McLachlan J, Pasquinelli AE, Bálint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science. 2001; 293: 834-838

Hutvágner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science. 2002; 297: 2056-2060

Invitrogen Corporation, 2009: BLOCK-iT RNAi Designer. https://rnaidesigner.invitrogen.com/rnaiexpress/ (10.09.2009)

Jackman M, Lindon C, Nigg EA, Pines J. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat Cell Biol. 2003; 5: 143-148

Joaquin M, Watson RJ. Cell cycle regulation by the B-Myb transcription factor. Cell Mol Life Sci. 2003; 60: 2389-2401

Katayama H, Brinkley WR, Sen S. The Aurora kinases: role in cell transformation and tumorigenesis. Cancer Metastasis Rev. 2003; 22: 451-464

Kimura M, Kotani S, Hattori T, Sumi N, Yoshioka T, Todokoro K, Okano Y. Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. J Biol Chem. 1997; 272: 13766-13771

Knight AS, Notaridou M, Watson RJ. A Lin-9 complex is recruited by B-Myb to activate transcription of G2/M genes in undifferentiated embryonal carcinoma cells. Oncogene. 2009; 28: 1737-1747

Kooreman NG, Wu JC. Tumorigenicity of pluripotent stem cells: biological insights from molecular imaging. J R Soc Interface. 2010; 6: 753-763 kein Punkt am Ende

Lakshmipathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS, Verfaillie CM. Efficient transfection of embryonic and adult stem cells. Stem Cells. 2004; 22: 531-543

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates miRNA processing. Nature. 2003; 425: 415-419

Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 2002; 21: 4663-4670

Li CC, Chu HY, Yang CW, Chou CK, Tsai TF. Aurora-A overexpression in mouse liver causes p53-dependent premitotic arrest during liver regeneration. Mol Cancer Res. 2009; 7: 678-688

Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet. 2002; 3: 662-673

Li J, Meyer AN, Donoghue DJ. Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. Proc Natl Acad Sci U S A. 1997; 94: 502-507

Lim LS, Loh YH, Zhang W, Li Y, Chen X, Wang Y, Bakre M, Ng HH, Stanton LW. Zic3 is required for maintenance of pluripotency in embryonic stem cells. Mol Biol Cell. 2007; 18: 1348-1358

Litovchick L, Sadasivam S, Florens L, Zhu X, Swanson SK, Velmurugan S, Chen R, Washburn MP, Liu XS, DeCaprio JA. Evolutionarily conserved multisubunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence. Mol Cell. 2007; 26: 539-551

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet. 2006; 38: 431-440

Lorvellec M, Dumon S, Maya-Mendoza A, Jackson D, Frampton J, García P. B-Myb is critical for proper DNA duplication during an unperturbed S phase in mouse embryonic stem cells. Stem Cells. 2010; 28: 1751-1759

Lukasiewicz KB, Lingle WL. Aurora A, centrosome structure, and the centrosome cycle. Environ Mol Mutagen. 2009; 50: 602-619

Manak JR, Mitiku N, Lipsick JS. Mutation of the Drosophila homologue of the Myb protooncogene causes genomic instability. Proc Natl Acad Sci USA. 2002; 99: 7438-7443

Mannefeld M, Klassen E, Gaubatz S. B-MYB is required for recovery from the DNA damage-induced G2 checkpoint in p53 mutant cells. Cancer Res. 2009; 69: 4073-4080

Mantel C, Guo Y, Lee MR, Kim MK, Han MK, Shibayama H, Fukuda S, Yoder MC, Pelus LM, Kim KS, Broxmeyer HE. Checkpoint-apoptosis uncoupling in human and mouse embryonic stem cells: a source of karyotpic instability. Blood. 2007; 109: 4518-4527

Mao JH, Wu D, Perez-Losada J, Jiang T, Li Q, Neve RM, Gray JW, Cai WW, Balmain A. Crosstalk between Aurora-A and p53: frequent deletion or downregulation of Aurora-A in tumors from p53 null mice. Cancer Cell. 2007; 11: 161-173

Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hannett N, Sharp PA, Bartel DP, Jaenisch R, Young RA. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell. 2008; 134: 521-533

Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981; 78: 7634-7638

Marumoto T, Zhang D, Saya H. Aurora-A - a guardian of poles. Nat Rev Cancer. 2005 Jan; 5: 42-50

Meraldi P, Honda R, Nigg EA. Aurora kinases link chromosome segregation and cell division to cancer susceptibility. Curr Opin Genet Dev. 2004; 14: 29-36

Meraldi P, Honda R, Nigg EA. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. EMBO J. 2002; 21: 483-492

Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol. 2001; 2: 21-32

Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. 1998; 12: 2048-2060

Nurse P. Universal control mechanism regulating onset of M-phase. Nature. 1990 ; 344: 503-508

Ogawa K, Shimosato D, Takahashi K, Yagi R, Toyooka Y, Masui S, Matoba R, Ko MS. Forced expression of Tbx3 promotes LIF-independent self-renewal of mouse ES cells. Dev Biol. 2007; 306: 391–392

Oh IH, Reddy EP. The myb gene family in cell growth, differentiation and apoptosis. Oncogene. 1999; 18: 3017-3033

Ouchi M, Fujiuchi N, Sasai K, Katayama H, Minamishima YA, Ongusaha PP, Deng C, Sen S, Lee SW, Ouchi T. BRCA1 phosphorylation by Aurora-A in the regulation of G2 to M transition. J Biol Chem. 2004; 279: 19643-19648

Pera MF, Trounson AO. Human embryonic stem cells: prospects for development. Development. 2004;131: 5515-5525

Pilkinton M, Sandoval R, Colamonici OR. Mammalian Mip/LIN-9 interacts with either the p107, p130/E2F4 repressor complex or B-Myb in a cell cycle-phase-dependent context distinct from the Drosophila dREAM complex. Oncogene. 2007; 26: 7535-7543

Pines J, Hunter T. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell. 1989; 58: 833-846

Pines J. Mitosis: a matter of getting rid of the right protein at the right time. Trends Cell Biol. 2006; 16: 55-63

Ptashne M. On the use of the word 'epigenetic'. Curr Biol. 2007; 17: 233-236

Qin L, Tong T, Song Y, Xue L, Fan F, Zhan Q. Aurora-A interacts with Cyclin B1 and enhances its stability. Cancer Lett. 2009; 275: 77-85

Rajagopalan H, Lengauer C. Aneuploidy and cancer. Nature. 2004; 432: 338-341

Sala A. B-MYB, a transcription factor implicated in regulating cell cycle, apoptosis and cancer. Eur J Cancer. 2005; 41: 2479-2484

Sasai K, Parant JM, Brandt ME, Carter J, Adams HP, Stass SA, Killary AM, Katayama H, Sen S. Targeted disruption of Aurora A causes abnormal mitotic spindle assembly, chromosome misalignment and embryonic lethality. Oncogene. 2008; 27: 4122-4127

Savatier P, Huang S, Szekely L, Wiman KG, Samarut J. Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. Oncogene. 1994; 9: 809-818

Schmit F, Cremer S, Gaubatz S. LIN54 is an essential core subunit of the DREAM/LINC complex that binds to the cdc2 promoter in a sequence-specific manner. FEBS J. 2009; 276: 5703-5716

Schmit F, Korenjak M, Mannefeld M, Schmitt K, Franke C, von Eyss B, Gagrica S, Hänel F, Brehm A, Gaubatz S. LINC, a human complex that is related to pRB-containing complexes in invertebrates regulates the expression of G2/M genes. Cell Cycle. 2007; 6: 1903-1913

Shepard JL, Amatruda JF, Stern HM, Subramanian A, Finkelstein D, Ziai J, Finley KR, Pfaff KL, Hersey C, Zhou Y, Barut B, Freedman M, Lee C, Spitsbergen J, Neuberg D, Weber G, Golub TR, Glickman JN, Kutok JL, Aster JC, Zon LI. A zebrafish bmyb mutation causes genome instability and increased cancer susceptibility. Proc Natl Acad Sci USA. 2005; 102: 13194-13199

Shiota K, Kogo Y, Ohgane J, Imamura T, Urano A, Nishino K, Tanaka S, Hattori N. Epigenetic marks by DNA methylation specific to stem, germ and somatic cells in mice. Genes Cells. 2002; 7: 961-969

Smalheiser NR. EST analyses predict the existence of a population of chimeric microRNA precursor-mRNA transcripts expressed in normal human and mouse tissues. Genome Biol. 2003; 4: 403

Solomon MJ, Glotzer M, Lee TH, Philippe M, Kirschner MW. Cyclin activation of p34cdc2. Cell. 1990; 63: 1013-1024

Stead E, White J, Faast R, Conn S, Goldstone S, Rathjen J, Dhingra U, Rathjen P, Walker D, Dalton S. Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. Oncogene. 2002; 21: 8320-8333

Stojkovic M, Lako M, Strachan T, Murdoch A. Derivation, growth and applications of human embryonic stem cells. Reproduction. 2004; 128: 259-267

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131: 861-872

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126: 663-676

Tanaka M, Ueda A, Kanamori H, Ideguchi H, Yang J, Kitajima S, Ishigatsubo Y. Cell-cycle-dependent regulation of human aurora A transcription is mediated by periodic repression of E4TF1. J Biol Chem. 2002; 277: 10719-10726

Tanaka Y, Patestos NP, Maekawa T, Ishii S. B-myb is required for inner cell mass formation at an early stage of development. J Biol Chem. 1999; 274: 28067-28070

Tarasov KV, Tarasova YS, Tam WL, Riordon DR, Elliott ST, Kania G, Li J, Yamanaka S, Crider DG, Testa G, Li RA, Lim B, Stewart CL, Liu Y, Van Eyk JE, Wersto RP, Wobus AM, Boheler KR. B-MYB is essential for normal cell cycle progression and chromosomal stability of embryonic stem cells. PLoS One. 2008; 3: e2478

Taylor SS, Scott MI, Holland AJ. The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation. Chromosome Res. 2004;12(6):599-616.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282: 1145-1147

Wang XX, Liu R, Jin SQ, Fan FY, Zhan QM. Overexpression of Aurora-A kinase promotes tumor cell proliferation and inhibits apoptosis in esophageal squamous cell carcinoma cell line. Cell Res. 2006; 16: 356-366

White J, Dalton S. Cell cycle control of embryonic stem cells. Stem Cell Rev. 2005; 1: 131-138

Yang D, Zhang ZJ, Oldenburg M, Ayala M, Zhang SC. Human embryonic stem cell-derived dopaminergic neurons reverse functional deficit in parkinsonian rats. Stem Cells. 2008; 26: 55-63

Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003; 17: 3011-3016

Zhang D, Hirota T, Marumoto T, Shimizu M, Kunitoku N, Sasayama T, Arima Y, Feng L, Suzuki M, Takeya M, Saya H. Cre-loxP-controlled periodic Aurora-A overexpression induces mitotic abnormalities and hyperplasia in mammary glands of mouse models. Oncogene. 2004; 23: 8720-8730

Zhang J, Tam WL, Tong GQ, Wu Q, Chan HY, Soh BS, Lou Y, Yang J, Ma Y, Chai L, Ng HH, Lufkin T, Robson P, Lim B. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nat Cell Biol. 2006; 8: 1114-1123

Zhang Y, Wang Z, Ravid K. The cell cycle in polyploid megakaryocytes is associated with reduced activity of cyclin B1-dependent cdc2 kinase. J Biol Chem. 1996; 271: 4266-4272

Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR, Sen S. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet. 1998; 20: 189-193

## Danksagung

Ganz besonders bedanken möchte ich mich bei Herrn Prof. Ph.D. Kenneth Boheler, der mir den Forschungsaufenthalt am National Institute on Aging (NIA) im Rahmen meiner Doktorarbeit ermöglicht hat. Die Betreuung, fachliche Unterstützung und Einbindung ins Team war von Beginn an herausragend.

Mein besonderer Dank gilt Herrn Prof. Dr. Nickenig für die Übernahme des Erstgutachtens an der Uni Bonn.

Weiterhin bedanken möchte ich mich bei Boehringer Ingelheim Fonds, die meine Doktorarbeit und den Aufenthalt am NIA durch ein Stipendium unterstützt haben.

Nicht zuletzt geht mein herzlicher Dank an meine Familie, meinen Freund und meine engen Freunde für die Motivation und Unterstützung.