Modulating the Activity of the c-Myc Oncoprotein

Implications for Therapeutic Treatment

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Abstract

The Myc oncoprotein regulates numerous cellular processes and is frequently deregulated in cancer due to genetic lesions. However, in addition to its tumor promoting activity, Myc and other oncoproteins induce intrinsic safe-guard mechanisms against tumorigenesis like apoptosis and cellular senescence, which have to be overcome by additional genetic lesions for cellular transformation.

In this work, we identify ways of reactivating these anti-tumorigenic pathways in cells with deregulated Myc. First, we uncover an unexpected capacity of transforming growth factor-β (TGF-β) to force hematopoietic cells with deregulated Myc into cellular senescence despite continuous Myc expression. This involved upregulation of the Myc antagonist Mad1, leading to repressed transcription of Myc target genes.

We further reveal a novel role of Myc in Myc/Ras dependent transformation. While Ras induced cellular senescence and suppressed Myc-activated apoptosis, we found that Myc repressed Ras-induced senescence. This required phosphorylation of Myc at Ser-62 by cyclin dependent kinase 2 (Cdk2). Further, pharmacological inhibitors of Cdk2 forced Myc+Ras expressing cells into senescence.

In addition, although redundant for cell cycle progression, Cdk2 was shown to have a unique role in suppressing Myc-induced senescence, and depletion of Cdk2 in a mouse Eμ-*myc* lymphoma model led to regression of tumor development. Taken together, this highlights Cdk2-targeting in Myc and Ras-driven tumors.

Finally, we uncover a novel interplay between Myc and the protein deacetylase SIRT1. While Myc induced SIRT1 expression and activity, SIRT1 fed back to Myc by stabilizing the Myc protein. Further, SIRT1 repressed Myc-induced apoptosis and senescence, pointing out SIRT1 as a promising target in neoplasia driven by Myc.

In summary, this thesis demonstrates potential new strategies for therapeutic intervention of tumors with deregulated Myc by targeting its essential cofactors and collaboration partners.

Keywords: Myc, cyclin E/Cdk2, $p27^{Kip1}$, SIRT1, transforming growth factor-β, interferon-γ, cell cycle, cellular senescence, phosphorylation, SA-β-Gal

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wu, S., Hultquist, A., **Hydbring, P**., Cetinkaya, C., Öberg, F., and Larsson, L.G. TGF-β induces senescence in Myc-transformed monocytic cells correlating with induction of Mad1 and repression of Myc-driven transcription (manuscript).

II **Hydbring, P**., Bahram, F., Su, Yingtao., Tronnersjö, S., Högstrand, K., von der Lehr, N., Lilischkis, R., Hein, N., Wu, S., Vervoorts, J., Henriksson, M., Grandien, A., Lüscher, B., and Larsson, L.G. Myc transforms by repressing Ras-induced senescence through Cdk2-mediated Ser-62 phosphorylation (manuscript).

III Campaner, S., Doni, M., **Hydbring, P**., Verrecchia, A., Bianchi, L., Sardella, D., Schleker, T., Perna, D., Tronnersjö, S., Barbacid, M., Larsson, L.G., and Amati, B. Cdk2 suppresses cellular senescence induced by the myc oncogene and oxidative stress (manuscript).

IV Menssen, A., **Hydbring, P**., Kapelle, K., Diebold, J., Bornkamm, G.W., Larsson, L.G., Lüscher, B., and Hermeking, H. Mutual regulation of c-MYC and SIRT1 constitutes a positive feed-back loop promoting cancer cell expansion (manuscript).

Abbreviations

4-OHT	4-hydroxy-tamoxifen
ABC	ATP-binding cassette
AD	activation domain
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
Arf	alternative reading frame
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	atm related kinase
bHLHLZ	basic helix-loop-helix leucine zipper
Bim	Bcl-2 interacting mediator of cell death
Brd4	bromodomain protein 4
CIITA	class II transactivator
CAK	cyclin dependent activating kinase
CBP	CREB binding protein
Cdc	cell division cycle
Cdk	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
Chk	checkpoint transducer kinase
CKI	cyclin dependent kinase inhibitor
CML	chronic myeloid leukemia
CSC	cancer stem cell
CSL	CBF1/RBP-Jk, suppressor of hairless, LAG-1
CTCL	cutaneous T-cell lymphoma
CTD	carboxy terminal domain
DBC1	deleted in breast cancer 1
DBD	DNA binding domain
Disp	dispatched

DNMT3a	DNA methyl transferase 3a
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ER	estrogen receptor
ES	embryonic stem cell
FACS	fluorescence activated cell sorting
FGFR	fibroblast growth factor receptor
GAS	gamma activated sequence
GEF	guanidine nucleotide exchange factor
GNAT	general control non-depressable 5 related N-acetyl transferase
G-protein	guanidine nucleotide binding protein
GSK	glycogen synthase kinase
GTF	general transcription factor
HAT	histone acetyl transferase
HCE	human capping enzymes
HDAC	histone deacetylase
HDF	human diploid fibroblast
Hh	hedgehog
HMGA2	high-mobility group A2
HSC	hematopoietic stem cell
hPAF	human RNA polymerase II-associated factor
ICN	intracellular Notch
Ink4	inhibitors of Cdk4
INR	initiator
Jak	janus activating kinase
Kip	kinase inhibitory protein
LDH-A	lactate dehydrogenase-A
LEF	lymphoid enhancer-binding factor
LIF	leukemia inhibitory factor
LRP	low-density lipoprotein receptor-related protein
MAML	mastermind-like polypeptides
MAPK	mitogen activated protein kinase
Max	Myc associated protein X
MCM	mini chromosome maintenance
MEF	mouse embryo fibroblast
MPF	maturation promoting complex
Myc	myelocytomatosis
MycER	Myc-Estrogen receptor fusion
MYST	MOZ, Ybf2/Sas3, Sas2 and Tip60 family
NAD^+	nicotinamide adenine dinucleotide

NAM	nicotinamide
N-TEF	negative transcription elongation factors
PCAF	p300/CBP associated factor
PCP	planar cell polarity
PDGFR	platelet derived growth factor receptor
PI3-K	phosphatidylinositol-3 kinase
PIC	preinitiation complex
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
pRb	retinoblastoma protein
Ptc	patched
P-TEFb	positive transcription elongation factor b
RAR	retinoic acid receptor
REF	rat embryo fibroblast
RTK	receptor tyrosine kinase
SA-β-Gal	senescence associated β-galactosidase
SAGA	SPT/ADA/Gcn5/acetyltransferase
SCF	skp1-cullin-f-box protein
SIRT	silent information regulator
Ski	skinny hedgehog
Skp2	s-phase associated kinase associated protein 2
Smo	smoothened
STAT	signal transducer and activator of transcription
TAD	transcription activation domain
TBP	TATA-binding protein
TBP-1	thrombospondin-1
TCF	T-cell specific transcription factor
TERT	telomerase reverse transcriptase
TF	transcription factor
TGF-α	transforming growth factor α
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor-α
TNF-R	tumor necrosis factor receptor
TRRAP	transformation/transcription domain associated protein
TSA	trichostatin A
Ub	ubiquitin
VDR	vitamin D3 receptor
VEGFR	vascular endothelial growth factor receptor

1 Background

During the lifespan of a higher ordered multi-cellular organism such as humans, there is a tight control of the smallest living building blocks that compose it. These building blocks are referred to as cells. Humans consist of approximately 10^{14} cells that originate from the fertilized egg cell. To build up a complex organism depends on numerous biological pathways creating a huge number of different cell types during embryonic development. The extremely complex network build up by all these cells could be compared with a metropolis where a number of citizens have to cooperate to make the city function. To control the citizens, there will be laws for them to follow. However, apart from "good" citizens there will also be "bad" citizens corresponding to cells not following the set laws. A common violation is that these cells, due to genetic lesions, will not follow the set number of cell divisions and thereby making themselves immortal. This is one of the major reasons why cancer arises.

The mechanisms controlling the life-cycles and interactions of normal cells involve a complex machinery starting at the level of gene transcription. The genome of living cells consists of coding genes which are transcribed into RNA which in turn serves as template for protein translation. The mature proteins coming out of these processes are involved in fundamental cellular processes as growth, proliferation and cell death. The growth promoting proteins are encoded by genes often referred to as "proto-oncogenes", since genetic lesions affecting these genes give rise to proteins that eventually give rise to cancer cells. These proteins are counteracted by growth restraining proteins encoded by "tumor suppressor genes". The Myc oncoprotein studied in this thesis is one of the major proteins giving rise to cancer when deregulated. Myc serves as an essential transcription factor controlling the expression of a huge number of genes in the mammalian

genome and Myc depletion is lethal for embryonic development. The fact that many oncoproteins are essential for development and cell division in general has been considered a problem in previous and existing cancer therapy. How do you target such oncogenes, without hitting the healthy cells as well?

A way of achieving this is to utilise the fact that mammalian cells have evolved both intrinsic and extrinsic safe-guard mechanisms making the non-behaving cells either commit suicide or entering a permanent growth arrest. These mechanisms are active in living multi-cellular organisms but are overcome by genetic lesions targeting multiple oncogenes or tumor suppressor genes, and subsequently lead to aggressively dividing cancer cells that eventually form tumors. However, recently it has been discovered that cancer cells may still harbor intrinsic safe-guard pathways that can be reactivated. Reactivation of these pathways may lead to tumor regression with minimal damage to the healthy cells.

This thesis is based on studies describing certain modulations of the Myc oncoprotein. After the introductory part, describing basic processes such as cell division and transcription, my thesis work is outlined in three parts based on four papers. The first part deals with the interplay between Myc and a signaling tumor suppressive pathway initiated by transforming growth factor β (TGF-β). The second part uncovers how Myc cooperates with another oncoprotein named Ras in tumorigenesis and also identifies an important mediator in this cooperation. The last part describes a novel interplay between Myc and a protein deacetylase, SIRT1, also found to be important for Myc's tumor-promoting activity in metabolism.

1.1 The mammalian cell cycle

Cell division was established as early as mid-nineteenth century. Today the mammalian cell cycle is generally defined as a sequence of changes starting when a new cell is produced by cell division, continuing with the duplication of its DNA and ending with a new cell division creating the daughter cells. These events can be illustrated by a circle divided into four phases where the main phases, the synthetic- (S) and mitotic- (M) phase, are separated by two gap phases (G1 and G2).

The first phase, G1, ranges from the prior cell division until initiation of DNA-synthesis. In this phase, critical decisions are being made whether the

cell should continue to cycle or enter growth arrest. During the following S-phase, all DNA is replicated. The S-phase is followed by the next gap phase (G2) where the cell prepares itself for the subsequent entry of mitosis and cell division that will terminate one round of the cell cycle. The last phase, M, consists of four sub phases; prophase, metaphase, anaphase and telophase preceding cytokinesis where the cytoplasm is divided and one mother cell results in two daughter cells. The different sub phases in M represent stages in condensation, alignment, separation and decondensation of the DNA-content, respectively.

This model is based on extensive work performed during the last half century with major breakthrough discoveries in the 1950s and 1960s focusing on DNA-replication and mitosis-progression (for review, see(Nurse, 2000)).

1.1.1 Cyclins and Cyclin Dependent Kinases

25 years ago, Tim Hunt and colleagues discovered the first cyclins in sea urchins, which were named cyclin A and B (Murray, 2004; Evans *et al.*, 1983). The name "cyclin" came from the observed oscillating levels of these proteins. Due to this, they hypothesized that the purpose of synthesizing these novel proteins was to force cells into mitosis while their degradation allowed cells to complete one round of the cell cycle before entering the next (Murray, 2004). Cyclin was several years later found to associate with a kinase partner, cell division cycle 2 (Cdc2), after the characterization of the two components of maturation-promoting factor (MPF) (Gautier *et al.*, 1990; Draetta *et al.*, 1989; Gautier *et al.*, 1988). The MPF-complex was found by Masui and Smith in 1971 to induce meiotic maturation of frog oocytes (Murray, 2004) while Cdc2 was originally discovered in fission yeast by Paul Nurse and colleagues (Nurse *et al.*, 1976). However, the term "Cell Division Cycle" had its origin from earlier work by Leland Hartwell in budding yeast where cell division cycle mutants were isolated and characterized with their respective effect on the cell cycle. This work of Hartwell and colleagues also identified the *cdc28* gene as a necessary gene for initiation of DNA synthesis in budding yeast and Cdc28 was later found to be the homolog of fission yeast Cdc2 (Murray, 2004; Nurse, 2000; Hartwell, 1974). The human homolog of Cdc2 was identified in 1987 (Lee & Nurse, 1987) and renamed to cyclin dependent kinase 1 (Cdk1) a few years later. This was preceded by the discovery that mammalian Cdc2 homologs also bound cyclins (Doree & Hunt, 2002; Pines & Hunter, 1990) and the discovery of a second mammalian kinase, which also associated with

these proteins (Elledge & Spottswood, 1991; Tsai *et al.*, 1991). Since then, a number of additional cyclins and Cdks have been discovered in mammals of which the most important ones for cell cycle progression are depicted in Figure 1 and described later. It should be noted that yeast, unlike higher eukaryotes, only possess one Cdk (Cdc2 or Cdc28).

The binding of cyclin to Cdk leads to a conformational change that will make the catalytic site of the Cdk accessible for ATP-binding. This is followed by phosphorylation of a Threonine-residue in the so called activation segment by Cyclin-Dependent Activating Kinase (CAK) which in turn will promote binding of a substrate and subsequent phosphorylation of the substrate. (Lolli & Johnson, 2005). Cyclin/Cdks phosphorylate numerous substrates to ensure continuous cell cycle progression by promoting the G1-S transition and G2-M transition of the cell cycle. Apart from a role in direct activation of the Cdk, cyclins also play roles in directing the substrate specificity of Cdks. This is performed either through interactions between the cyclin and target proteins or cyclin-dependent subcellular localization (Blain, 2008; Miller & Cross, 2001). In addition to this, cyclins also control Cdk activity by their own degradation. Proteolysis of cyclins via the ubiquitin proteasome system leads to a drop in Cdk activity and is involved in the control of phase-transitions of the cell cycle. The process involving ubiquitin (Ub) molecules attached to lysine residues requires a three-step process consisting of an Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzyme. Ub contains seven lysine residues where lysine 48 (K48)-linked chains leads to proteasomal degradation of its targets (Haglund & Dikic, 2005; Nurse, 2000).

Shortly after the findings that cyclin and mammalian Cdc2-homologs cooperate in the same complex, three additional cyclins (cyclin C, D and E) were discovered (Murray, 2004; Koff *et al.*, 1991; Lew *et al.*, 1991). Cyclin E interacted with another Cdc2 family kinase named Cdk2 which was identified around the same time (Elledge & Spottswood, 1991; Tsai *et al.*, 1991), while the G1-specific cyclins D1-D3 were shown to interact with the kinases Cdk4 and 6. These complexes were further shown to bind the retinoblastoma (pRb) tumor suppressor with subsequent phosphorylation of pRb and G1 progression, due to release of pRb from E2F, see chapter 1.1.2 and 1.1.3 (Bates *et al.*, 1994; Sherr, 1993; Matsushime *et al.*, 1992). Later, another isoform of cyclin E, cyclin E2, was discovered (Lauper *et al.*, 1998; Zariwala *et al.*, 1998). A simplified view of the mammalian cell cycle

containing these players is visualized below (Figure 1). Additional phase-specific players depicted in this figure will be described below.

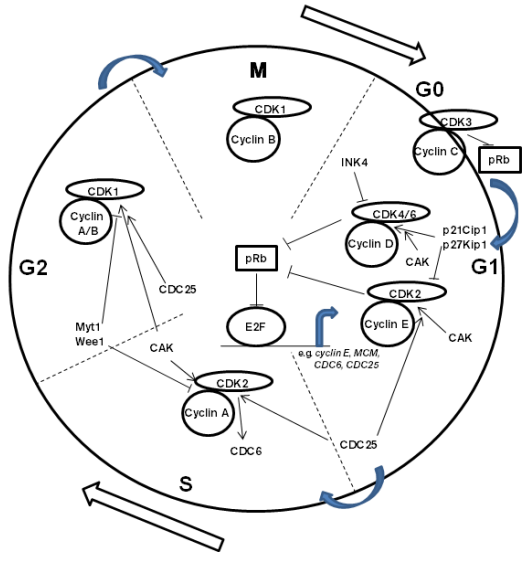

Figure 1. The classic model of cyclins and Cdks with their major targets and regulators in the mammalian cell cycle

There seemed to be some functional differences in cellular functions between the different cyclins. While cyclin B was shown to strictly support entry of mitosis and cyclin E to support duplication of centrosomes and DNA during interphase, cyclin A was able to promote both these processes (Strausfeld *et al.*, 1996). In contrast to the other cyclins, cyclin C in complex with Cdk8 showed to have cell cycle progression independent roles and instead function in phosphorylation of the Carboxy Terminal Domain (CTD) of RNA polymerase II, thereby regulating transcription (Rickert *et al.*, 1996). More recently a role for cyclin C in complex with Cdk3 has been suggested for pRb phosphorylation relevant for G0-G1 transition (Ren & Rollins, 2004)(Figure 1). A role in transcription was also shown for the later discovered cyclin H and T which complex with Cdk7 and 9, respectively (Napolitano *et al.*, 2002; Shuttleworth, 1995). Importantly, cyclin H/Cdk7 also constitutes the Cdk-activating kinase (CAK) which performs the

activating phosphorylations of cyclin/Cdk complexes (Kaldis, 1999), see chapter "1.2.5 CAK and Cdc25". The role of the G1-specific D-type cyclins will be described in detail below (Sherr, 1995).

1.1.2 G1-S transition and Cyclin Dependent Kinase Inhibitors (CKIs)

The model of how cells progress through the G1 phase of the cell cycle and into S-phase has been revised during the recent years. The old model presented mammalian cells to be dependent on D- and E-type cyclins, which regulate Cdk4/6 and Cdk2 activity, respectively. Here, mitogenic signaling, like Ras-signaling (see chapter 1.4.1), upregulates D-type cyclins. Synthesized cyclin D will then assemble with Cdk4/6, thereby contributing to G1 phase progression and G1-S transition through phosphorylation of the pRb protein (Peeper *et al.*, 1997; Lavoie *et al.*, 1996; Winston *et al.*, 1996; Albanese *et al.*, 1995). Cyclin D/Cdk4/6 will phosphorylate pRb in the mid-G1 and the process will be completed by activated cyclin E/Cdk2 phosphorylating pRb on additional sites (Sherr & Roberts, 1999; Lundberg & Weinberg, 1998). This will in turn trigger the release of the transcription factor E2F from pRb. The E2F-family of transcription factors promotes S-phase entry by facilitating transcription of E2F-responsive genes of importance for G1- to S-phase transition and DNA-synthesis. Such genes include *cyclin E*, *cyclin A*, *Cdk2*, *Cdk1*, *orc1*, *cdc6* and multiple *MCM* genes (Dyson, 1998). The synthesis of cyclin E has previously been shown to contribute to the DNA prereplication complex (Coverley *et al.*, 2002) and it was reported that quiescent cyclin E-null cells were unable to re-enter the cell cycle and complete G0-S transition due to their defect in MCM replicative helicase loading (Geng *et al.*, 2003). More recently, it was shown that this action of cyclin E is kinase independent. Cyclin E was shown to be loaded onto chromatin in a kinase independent fashion during progression from G0-S phase where it formed a bridge between Cdt1 and MCM loading. This was however only seen in quiescent cells and not in cycling cells (Geng *et al.*, 2007).

The absolute dependency of the players for cell cycle progression in the classic model (Figure 1) started to be questioned over 10 years ago in reports demonstrating that individual D-type cyclin knockout mice were viable (Sicinski *et al.*, 1996; Sicinski *et al.*, 1995), followed by a report that cyclin E could compensate the function of cyclin D1 (Geng *et al.*, 1999), indicating redundancy of these cyclins in many cell types. The challenge of the model was intensified by the reports that mice expressing a single D-type cyclin

developed until late gestation or were viable (Ciemerych *et al.*, 2002) followed by the striking report that mice lacking all D-type cyclins developed until mid/late gestation (Kozar *et al.*, 2004). Previous to the latter report, a double knockout of cyclin E1/E2 was also shown to be largely dispensable for mitotic division (Geng *et al.*, 2003; Parisi *et al.*, 2003) and phosphorylation of pRb and induction of E2F target genes were carried out normally in cyclin E-null cells indicating that this function can be facilitated by another cyclin, presumably cyclin A (Geng *et al.*, 2003).

Supporting the redundancy of cyclins in G1-S transition, individual Cdk2, 4 and 6 knockout mice were all viable (Malumbres *et al.*, 2004; Berthet *et al.*, 2003; Ortega *et al.*, 2003; Rane *et al.*, 1999). All these findings culminated with the recent report that mice lacking all interphase Cdks (Cdk2, 3, 4 and 6) develop until midgestation. In these mice, Cdk1 substitutes for all interphase Cdks and interacts with all interphase cyclins, indicating that Cdk1, like in yeast, is the only essential Cdk for mammalian cell proliferation. This was further strengthened by the fact that Cdk1 ablation was embryonic lethal at an early stage (Santamaria *et al.*, 2007). A picture of the mammalian cell cycle summarizing these reports is illustrated in Figure 2.

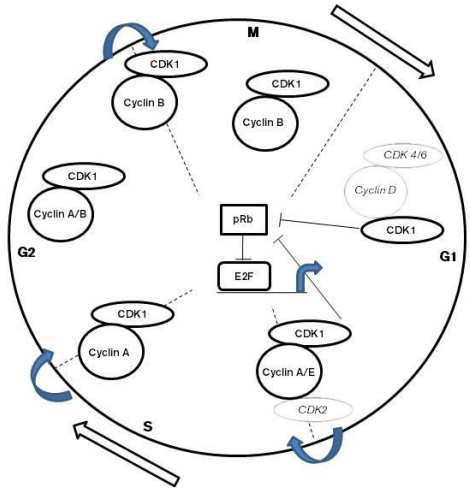

Figure 2. A compensatory model of the mammalian cell cycle based on mice knock-out experiments showing the essential cyclins and Cdk1 in bold

These reports do however not say that the previous cell cycle model is incorrect but rather that cyclins and Cdks play different roles in specific tissues and compensate each others functions if necessary. It should also be emphasized that mice expressing only Cdk1 or deficient for all D-cyclins fail to complete their embryo development (Santamaria *et al.*, 2007; Kozar *et al.*, 2004) arguing that the classic model, which is shown in Figure 1, likely reflects what is going on in most cells but with space for compensatory mechanisms, if necessary (Figure 2).

The classic model of G1 progression also involves cyclin dependent kinase inhibitors, CKIs. These inhibitors are separated into two families, the Ink4- and Cip/Kip-families, targeting cyclin D-, or E/A-dependent kinases, respectively. The Ink4-family consists of p15Ink4b, p16Ink4a, p18Ink4c and p19Ink4d while the Cip/Kip-family contains the p21Cip1, p27Kip1 and p57Kip2 proteins. These CKIs function by inhibiting the ATP-binding site of their bound Cdk, thereby inactivating the cyclin/Cdk complex. Whereas Ink4-proteins bind strictly to Cdk4 and 6, Cip/Kip proteins can bind to both cyclin and Cdk subunits. In cycling cells, Cip/Kip proteins have been shown to be sequestered into cyclin D-Cdk complexes (described below), while G1-arrest was shown to require both Ink4- and Cip/Kip-proteins (Sherr & Roberts, 2004; Sherr & Roberts, 1999).

In addition to phosphorylating pRb, cyclin D/Cdk4/6 has a function in sequestration of the cyclin dependent kinase inhibitors p21Cip1 and p27Kip1 from cyclin E/Cdk2. Once relieved and activated, cyclin E/Cdk2 phosphorylates p27Kip1 leading to its recognition by the SCF^{Skp2} E3 ubiquitin ligase and subsequent degradation via the ubiquitin-proteasome pathway (see chapter 1.1.6 and 1.5.2), thereby creating an autostimulatory feedback loop (Vlach *et al.*, 1997; Sherr & Roberts, 1995; Polyak *et al.*, 1994). Interestingly, sequestration of p21 and p27 seems to be a prerequisite for cyclin D/Cdk complexes to function.

First, it was shown that p27Kip1 immunoprecipitates exhibited specific kinase activity towards pRb (Soos *et al.*, 1996) followed by the findings that cyclin D/Cdk/p27/p21 complexes showed no kinase inhibition in contrast to cyclin E/Cdk2/p27/p21 complexes (Blain *et al.*, 1997; LaBaer *et al.*, 1997). Actually, the study by LaBaer et al. suggested that p21/p27 promoted cyclin D/Cdk kinase activity. This was further supported by a study a couple of years later showing that mouse embryo fibroblasts lacking either p21 or p27 had no detectable cyclin D/Cdk4/Rb kinase activity. Re-

introduction of the Cip/Kip proteins both increased the stability of cyclin D and redirected it to the nucleus (Cheng *et al.*, 1999).

These kinase-promoting functions of Cip/Kip proteins make the presence of the other Cdk-inhibitor family (the Ink4-family) even more important for keeping G1-S transition under control. p15Ink4b is induced by TGF-β signaling (see chapter 1.3.6), p16Ink4a promotes cell cycle arrest and its expression is increased when cells undergo cellular senescence. p18Ink4c and p19Ink4d has been suggested to have roles in differentiation (Phelps *et al.*, 1998; Serrano *et al.*, 1997; Zindy *et al.*, 1997; Reynisdottir *et al.*, 1995). Ink4 proteins will bind directly to Cdk4/6 and trap them in stable but inactive dimers, preventing nuclear translocation of Cdk4/6. It seems that the Cip/Kip and Ink4 cyclin-dependent kinase inhibitor families depend on each other to arrest cells in G1-phase. First, Ink4 proteins need to compete with Cip/Kip proteins in their binding for Cdk4/6. In contrast to Cip/Kip proteins which assemble the cyclin D/Cdk4/6 complex together, Ink4 proteins will only bind to the kinase protein leaving cyclin D free and destabilized. This relieves Cip/Kip proteins free to bind and inactivate cyclin E/Cdk2 complexes. With both Cdk4/6 and Cdk2 inactivated, the G1-phase arrest is accomplished (McConnell *et al.*, 1999; Parry *et al.*, 1999; Sherr & Roberts, 1999).

1.1.3 Rb and E2F

The Retinoblastoma protein family, pRb, consists of three members: Rb (p105), p107 and p130. Their binding to the E2F-family of transcription factors keeps cells restricted from entering S-phase. This is accomplished by an active recruitment of Histone Deacetylases (HDACs) and other chromatin remodeling factors to E2F-responsive promoters (Sherr & McCormick, 2002).

There are seven E2F family members. The first six act as heterodimers with DP family members in order to activate or repress transcription of target genes. E2F7 is able to perform its actions in a DP-independent way. The activity of E2F1-5 is controlled by the pRb-family of proteins whereas E2F6 and 7 act in a pRb-independent way. The E2Fs 1-3 are considered as activator E2Fs while E2F4-6 represses transcription. This is however not always the case and repressor E2Fs may activate transcription when for instance over-expressed (Frolov & Dyson, 2004).

The distinction between E2F1-3 and 4-6 is perhaps more accurate when it comes to interaction patterns with the pRb family members. The E2Fs 1-3 interact strictly with Rb while E2F4 interact with all pRb family members and E2F5 with p130. Repressor E2Fs seem to be dominant in G0/G1 phase. Phosphorylation of pRb family members by cyclin/Cdk complexes will relieve these complexes from E2F responsive promoters and facilitate the entrance of activator E2Fs important for S-phase transition. The exchange of these E2F members at the promoters coincides with a loss of HDACs and recruitment of Histone Acetyl Transferases (HATs) (Frolov & Dyson, 2004; Cam & Dynlacht, 2003).

Rb, which interacts specifically with activator E2Fs, blocks the action of these in several ways and thereby abrogate S-phase transition; first by binding directly to the activation domain of activator E2Fs (Flemington *et al.*, 1993) and secondly by recruitment and binding to E2F-responsive promoters thereby inhibiting pre-initiation complex formation and recruitment of necessary cofactors (Ross *et al.*, 1999). As a third mechanism, Rb recruits chromatin-modification enzymes to E2F-responsive promoters (Zhang *et al.*, 2000; Brehm *et al.*, 1998; Luo *et al.*, 1998). This last action of Rb showed different outcomes on different E2F target genes thereby regulating both G1 and S-phase exit (Zhang *et al.*, 2000).

1.1.4 G2 and M-phase

Like cyclin E, cyclin A is synthesized by E2F dependent transcription in the beginning of S-phase. Here cyclin A plays a role in DNA replication, for instance by phosphorylating Cdc6 as discussed above (Coverley *et al.*, 2002). Even if cyclin A can also activate Cdk2, its major function is to serve as the partner for Cdk1. Together with cyclin B/Cdk1 it regulates G2 and M-phase progression (Pagano *et al.*, 1992). The activity of cyclin A/Cdk1 is of specific importance for the initiation of prophase in G2-M transition while its degradation is necessary for cells to enter anaphase (den Elzen & Pines, 2001; Furuno *et al.*, 1999; Draetta *et al.*, 1989). As for cyclin E, cyclin A exists as two variants, cyclin A1 and cyclin A2. Cyclin A1 has been suggested to govern meiosis while cyclin A2 controls mitotic divisions (Sweeney *et al.*, 1996). This makes sense since mice knockout experiments show that cyclin A1-null mice are viable in contrast to cyclin A2-null mice which die after implantation (Liu *et al.*, 1998; Murphy *et al.*, 1997).

B-type cyclins are the major regulators of M-phase progression. Three mammalian B-type cyclins have been identified (Gallant & Nigg, 1994; Chapman & Wolgemuth, 1993; Pines & Hunter, 1989). Cyclin B1 and B2 interact with Cdk1 while cyclin B3 showed to interact with Cdk2 (Nguyen *et al.*, 2002; Draetta *et al.*, 1989). Cyclin B1, which associates with microtubuli and translocates to the nucleus in the end of G2-phase, has been shown to be important for condensation of chromosomes and assembly of the mitotic spindle (Ookata *et al.*, 1993; Pines & Hunter, 1991). Its deletion is lethal for embryonic development. In contrast to cyclin B1-null mice, cyclin B2-null mice develop normally. Cyclin B2 does not translocate to the nucleus during M-phase but plays its role in organization of the Golgi (Draviam *et al.*, 2001; Brandeis *et al.*, 1998), (for review see (Ciemerych & Sicinski, 2005)).

Recent work by Kaldis and colleagues show that knock in of Cdk2 at the Cdk1 locus leads to embryonic lethality if both copies of Cdk1 are replaced. In a Cdk2 negative background the phenotypes of Cdk1/Cdk2KI were also analyzed showing cell cycle properties of KI-Cdk2 comparable to endogenous Cdk2 except for a meiotic defect of Cdk2. This highlights the unique properties of Cdk1 that cannot be replaced by Cdk2 (Satyanarayana *et al.*, 2008).

1.1.5 CAK and CDC25

The proper function of Cdks does not only depend on the present cyclin partner, but also on the presence of a Cyclin-Dependent Activating Kinase (CAK) and in some cases also on the presence of a member of the phosphatase-family, Cdc25. The CAK-complex performs the activating phosphorylation of Cdk1, 2, 4 and 6. As indicated by its name, phosphorylation by the CAK-complex requires prior binding of the cyclin to the Cdk (Lolli & Johnson, 2005).

As mentioned previously the CAK-complex consists of Cdk7 and cyclin H. Phosphorylation of Cdk7 itself in the activation segment will lead to enhanced cyclin binding and CAK-activity even if it is not essential for CAK-activity (Fisher *et al.*, 1995). Cdk1 and Cdk2 have been identified in vitro as Cdk7 activating kinases indicating a positive feedback loop between these Cdks (Garrett *et al.*, 2001).

Apart from phosphorylation in their activity segment, Cdk1 and 2 are also phosphorylated at two residues in the ATP-binding domain, Threonine 14 and Tyrosine 15. These phosphorylations, which are mediated by two kinases named Wee1 and Myt1, will inhibit the activity of Cdk1 and 2. This is of importance for keeping these kinases on a leash and thereby preventing phase transition in the cell cycle. When the cell is ready to move on, the Cdc25 phosphatases will enter and remove the inhibitory phosphorylations from Cdk1 and 2, thereby allowing phase transition (Ray & Kiyokawa, 2008).

The Cdc25 family consist of three proteins, Cdc25A-C, where Cdc25A has been shown to regulate both G1-S as well as G2-M transition in contrast to Cdc25B-C which only are involved in G2-M transition. When phase transition should be prohibited, e.g. upon induction of DNA damage, the pathway starting with the ATM/ATR kinases will phosphorylate the Cdc25 proteins at several Ser/Thr sites through the checkpoint transducer kinases, Chk1 and Chk2, thereby inactivating them, (for review see(Ray & Kiyokawa, 2008)).

1.1.6 Cyclins and Cdks - role in cancer

The findings during the last years regarding knockout mice experiments showing that most of the cyclins and Cdks are dispensable for embryo development opened the possibility that inhibition of specific cyclins or Cdks might counteract cancer cells while sparing the normal cells (Lee & Sicinski, 2006).

Specifically, there are positive reports that cyclin D/Cdk4 could be a potential target in certain tumors. For instance mice deficient for cyclin D1 were shown to be resistant to ErbB-2-induced breast cancer. The same outcome was later shown for Cdk4-null mice (Yu *et al.*, 2006; Yu *et al.*, 2001). The first of these reports was further supported by a report showing knock in mice which express a kinase deficient mutant of cyclin D1 to be fully resistant to ErbB-2 tumors, while the mice developed normal mammary glands (Landis *et al.*, 2006). These elegant findings highlight the specific roles of cyclin D/Cdk4 in tumor development and potentiate their role as therapeutic targets (Ciemerych & Sicinski, 2005).

Results from knockout mice have demonstrated that Cdk2 and cyclin E are not essential on their own for the mitotic cell cycle (double knockout

cyclin E1/E2 mice died due to placenta abnormalities) (Berthet *et al.*, 2003; Geng *et al.*, 2003; Ortega *et al.*, 2003; Parisi *et al.*, 2003). Further, no proliferative effects were detected on certain colon cancer cell lines despite inhibition of Cdk2 (Tetsu & McCormick, 2003). These findings questioned the relevance of Cdk2 as a clinical target for cancer treatment (Ciemerych & Sicinski, 2005). Also the differences between Cdk2 and cyclin E-deficient mice raised speculations that another Cdk could replace the function of Cdk2 or that cyclin E harbored Cdk-independent functions (Yu & Sicinski, 2004). As mentioned earlier, the latter was shown to be true for MCM-loading during S-phase (Geng *et al.*, 2007).

However, Cdk2 was shown to have a critical role in melanoma growth (Du *et al.*, 2004), arguing for specific relevance in some tumors. Further, deregulated expression of cyclin E has been observed in many cancers and correlate with poor outcome in for instance breast cancer (Geng *et al.*, 2001). Previously it has also been shown that cells with deregulated E2F could be killed by short peptide motifs targeting the phosphorylation-activity of cyclin A/E-Cdk2 complexes (Chen *et al.*, 1999). To these Cdk2-findings one should add the results by the Bishop-lab showing that inhibition of Cdk1 could lead to apoptosis in tumors over-expressing Myc (Goga *et al.*, 2007). This potentiates that targeting of Cdk2 alone or in combination with Cdk1 could be of clinical relevance, at least for a subset of tumors.

If Cdk1 and 2 are potential targets in cancer treatment, the importance of targeting the Cdk1/2 activating phosphatase Cdc25A should be considered as well. If deregulated, Cdc25A will lead to increased genomic instability due to constitutive activated Cdk2 and Cdk1 with subsequent loss of checkpoint control in the G1-S and G2-M transitions.

Indeed, Cdc25A over-expression is reported in several cancer forms and Cdc25A has been shown to work together with Ras signaling in mammary tumors. In addition, heterozygous Cdc25A mouse embryo fibroblasts (MEFs) were less prone to undergo transformation together with activated Ras, (for review see (Ray & Kiyokawa, 2008)), indicating that Cdc25A, as an upstream activator of Cdk1/Cdk2, may have a role in promoting specific types of transformation events. Several compounds that inhibit Cdc25A have been developed and may be highly efficient in targeting tumors that are dependent on active Cdk1 and/or Cdk2. In support of this, heterozygous

Cdc25A+/- mice showed to be resistant to Ras-activated tumours (Ray *et al.*, 2007).

p27Kip1 has been demonstrated to play dual roles in tumorigenesis, although it is most known for its tumor suppressive functions. In support of the latter, reduced p27Kip1 levels have been shown to correlate with an increased frequency of lymphomas and leukemias, and p27 knockout mice show increased body size and hyperplasia (Nakayama *et al.*, 1996). Further, a loss of p27 decreased the survival rate of mice with Myc-driven lymphomas (Martins & Berns, 2002). These tumors also displayed an increase in Cdk2 activity substantiating the importance of Cdk2 in specific Myc-driven tumors. In contrast, a more recent report demonstrated that knockout of Cdk2 was dispensable for tumor suppression by p27 (Martin *et al.*, 2005). This could however be explained by a scenario where Cdk1 compensates for Cdk2 function, as shown by the Kaldis-laboratory around the same time (Aleem *et al.*, 2005).

In addition, a recent report revealed that p27 may serve as an oncogene in the cytoplasm. This novel role of p27 showed to be Cdk-independent using knock in mice expressing a mutant p27 deficient for Cdk-inhibition. The mutant p27-protein localized to the cytoplasm and was suggested to promote cell migration. These mice also developed lung adenocarcinomas and an increased pool of lung stem cells was reported, although no increased migration was reported for these particular cells. If this mutated form of p27 exerts oncogenic effects generally needs further examination (Besson *et al.*, 2007; Sicinski *et al.*, 2007).

In contrast to p27 knockout mice, S-phase kinase associated protein 2 (Skp2) knockout mice display hypoplasia. Skp2 is an F-box protein and subunit of the SCF E3 ubiquitin ligase that among several other substrates targets p27 for degradation, which ubiquitylation and degradation requires a prior phosphorylation at Thr-187 by Cdk2. Skp2 overexpression is observed in many human tumors leading to genetic instability due to increased activity of Cdk2 and 1, as a consequence of p27 degradation (Haglund & Dikic, 2005; Pagano, 2004; Latres *et al.*, 2001). The function of Skp2 as an oncogene was further substantiated by its cooperation with the oncoprotein c-Myc in Myc-dependent transcriptional activation (von der Lehr *et al.*, 2003) (see chapter 1.5.2).

1.2 Eukaryotic transcription

There are three eukaryotic RNA polymerases (Pol I-III) synthesizing rRNA, mRNA and tRNA respectively. RNA polymerase III also synthesizes a few additional small RNAs (e.g. 5S rRNA) that are important for RNA processing. Of the Pol I-III transcribed genes, only Pol II-genes encode proteins. However, Pol I and Pol III synthesized RNA constitutes together more than 80% of all RNA in growing cells. Pol I synthesizes rDNA in the nucleolus where also the subsequent processing into ribosomes occurs. Pol II- and Pol III-dependent transcription occurs in the nucleoplasm but parts of the processing of Pol III transcripts has been shown to take place in the nucleoli as well. Although the basic transcriptional machinery works in a similar way for all three polymerases, the specific mechanisms differs where the regulation of Pol II-transcribed genes in general is more complex and demands a higher number of actors (Paule & White, 2000). The rest of this chapter will be focused on the more extensively studied Pol II-dependent transcription.

The activity of eukaryotic transcription depends on the state of the nucleosome and histones. The nucleosome is a protein octamer, consisting of eight histones, embedded in coiled DNA. The histone octamer is composed by two histones each of H2A, H2B, H3 and H4 (Kornberg & Lorch, 1999; Kornberg, 1974).

The nucleosome was later discovered to have a negative impact on initiation of transcription (Knezetic & Luse, 1986) followed by the observation that nucleosome loss increases transcriptional initiation (Han & Grunstein, 1988). A clearer picture on how this was accomplished emerged when chromatin remodelling complexes and histone modifying complexes were observed a few years later (Brownell *et al.*, 1996; Cote *et al.*, 1994) (for review, see(Li *et al.*, 2007)).

1.2.1 Transcription initiation and histone modifications

For transcriptional initiation to occur, a transcriptional activator binds upstream of the transcription start site through its DNA Binding Domain (DBD). These so called transcription factors (TF), have been suggested to have access to both nucleosome-free DNA, and DNA within nucleosomes. Transcription factor binding is followed by recruitment of cofactor complexes, containing chromatin modifying activity, via the activation domain (AD) of the TF. One such recruited complex is the Mediator, a

subunit of the RNA Pol II holoenzyme. The mediator contains 25-30 subunits and only supports activated transcription. For example, the Med23 subunit of the mediator was specifically demonstrated to interact with the activation domains of the E1A and Elk-1 transcription factors (Li *et al.*, 2007; Green, 2005). The recruited complexes serve as signals for General Transcription Factors (GTFs), which enter and bind the transcription initiation site (Thomas & Chiang, 2006). Recruitment of Pol II together with TFIIH forms the pre-initiation complex (PIC) at the core promoter together with the GTFs TFIIA, TFIIB, TFIID, TFIIE and TFIIF. A subunit of the TFIID-complex, the TATA-box binding protein (TBP), binds the core promoter sequence (TATA-box) which is located just a few basepairs upstream of the transcription start site. Before initiation of transcription, TFIIH will denature a few base pairs of DNA to provide Pol II a single strand of DNA as template to start the transcription process. A simplified model of an active transcription initiation site is depicted below.

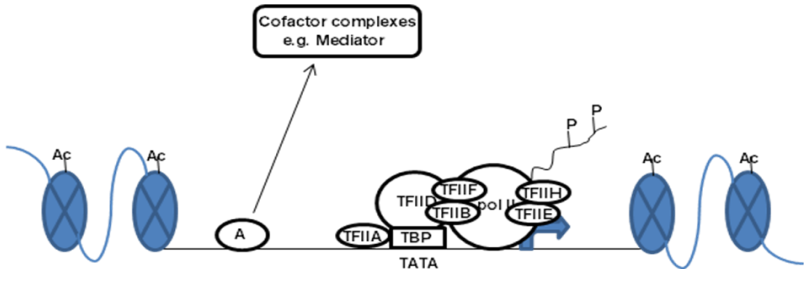

Figure 3. The basic components involved in transcription initiation. A; Activator, Ac; Acetylation, P; Phosphorylation

The initial phase of transcription will be further facilitated by the phosphorylation of the Pol II Carboxy Terminal Domain (CTD) tail governed by TFIIH/Cdk7/cyclin H, subsequently followed by other factors controlling the elongation phase of transcription. The CTD tail contains a number of tandem repeats of the sequence YSPTSPS where the sites Ser5 and Ser2 are the targets of phosphorylation. Ser5 is phosphorylated by TFIIH/Cdk7/cyclin H and is negatively regulated by Cdk8/cyclin C. Cdk8/cyclin C is a component of the complete and operative form of Pol II, the holoenzyme, and regulates phosphorylation of the CTD-tail negatively by direct phosphorylation of cyclin H, thereby inhibiting the CTD kinase activity of TFIIH (Hahn, 2004; Buratowski, 2003; Akoulitchev *et al.*, 2000).

Whether a specific gene will be transcribed or not further depends on the state of the chromatin surrounding that particular gene. This is controlled by chromatin remodelling factors and histone modifications. Histone tails are targets for a number of different posttranslational modifications. The most common ones are acetylations and methylations, which are carried out by Histone Acetyl Transferase (HAT)-complexes and Histone Methyl Transferase complexes, respectively. Modifications resulting in active chromatin are acetylations of Histone 3 and 4 as well as methylations of Histone 3, Lysine 4 (H3K4) and Lysine 79 (H3K79). In contrast, repressed chromatin often exhibits methylations at Histone 3, Lysine 9 or 27 (H3K9; H3K27). In addition histone phosphorylation at Histone 3, Serine 10 plays a role in activation while histone ubiquitination of Histone H2B and H2A, activates and represses chromatin, respectively. Apart from being of high importance for the decision to initiate transcription of a gene or not, histone modifications also play major roles during active transcription, for instance to keep the elongation process from pausing (for review, see (Li *et al.*, 2007)).

1.2.2 Transcription elongation

After Ser-5 phosphorylation of the CTD-tail and transcriptional initiation, other factors will take over to keep up the elongation process. The Positive Transcription Elongation Factor (P-TEFb), which consists of Cdk9/cyclin T1/T2/cyclin K, phosphorylates Ser-2 which in turn recruits RNA processing and termination/polyadenylation factors to the elongating Pol II-complex. The activity of Cdk9/cyclin T is also regulated positively by p300-mediated acetylation and negatively by HDAC3-mediated deacetylation (Fu *et al.*, 2007; Hahn, 2004). A doubly phosphorylated CTD-tail has further been shown to bind to the Pin1 peptidylproline isomerase indicating a flexibility of the CTD-tail making it possible to interact with several structurally unrelated partners (Verdecia *et al.*, 2000).

Before P-TEFb is engaged to phosphorylate Ser-2 of the CTD-tail and initiate elongation, the RNA 5′-transcript needs to be capped. This is achieved by recruitment of human capping enzymes (HCE) and the negative transcription elongation factors (N-TEF), identified as DSIF and NELF. After proper capping, recruited P-TEFb will not only phosphorylate Ser-2 but also DSIF and NELF. This will lead to the release of NELF and a

phosphorylated DSIF, which will switch and function as a positive elongation factor (Peterlin & Price, 2006).

So how is P-TEFb recruited to the transcription unit? The chromatin remodeling factor and Bromodomain protein Brd4 was shown to be one factor responsible for this as well as phosphorylation of Ser 10 at Histone 3. Further, the transcription activators NF-κB, c-Myc, MyoD, VP16, steroid hormone receptors and the class II transactivator (CIITA), have all been shown to interact with P-TEFb and affect RNA Pol II-elongation. These interactions most likely reflect specific P-TEFb recruitments to particular target genes, while recruitment via the Brd4 chromatin remodeling factor seems to be a more general mechanism (Peterlin & Price, 2006). Brd4, which only associates with the active form of P-TEFb, interacts directly with the cyclin T1 subunit in mammalian cells and thereby recruit P-TEFb to target genes. An increase in the interaction between P-TEFb and Brd4 is detected when cells is about to leave mitosis and progress into early G1-phase. Consistent with this, knockdown of Brd4 will lead to apoptosis and G1-arrest (Bres *et al.*, 2008).

Another important player in transcription elongation is the human RNA polymerase II-associated factor complex (hPAF). This complex interacts with both the non-phosphorylated and phosphorylated Ser-5 and 2 forms of the Pol II enzyme, suggesting roles in both transcription initiation and elongation. hPAF was shown to be required for Histone H2B monoubiquitination, the first step in a process to get rid of H2A/H2B dimers and to traverse nucleosomes for efficient elongation of RNA Pol II-transcription (Chaudhary *et al.*, 2007; Rozenblatt-Rosen *et al.*, 2005).

In summary, transcription elongation is dependent on the recruitment of P-TEFb, which occurs through chromatin remodeling factors or transcription activator factors, but also on the ability to traverse nucleosomes. Nucleosome traversion is only achieved if at least one of the two dimers of H2A/H2B is displaced and depends on the hPAF-complex.

1.2.3 HATs and HDACs

The presence of acetyl groups on histones was discovered 45 years ago (Phillips, 1963) subsequently followed by a report suggesting a link between histone modifications, particularly acetylations and RNA synthesis (Allfrey *et al.*, 1964). The identification of the first histone acetyl transferases (HATs)

and the first histone deacetylases (HDACs) was however not reported until 30 years later. These findings were highly important since they linked histone acetylation directly to transcriptional regulation (Bannister & Kouzarides, 1996; Brownell *et al.*, 1996; Mizzen *et al.*, 1996; Rundlett *et al.*, 1996; Brownell & Allis, 1995; Kleff *et al.*, 1995).

Today, it is well known that HATs and HDACs stand for much more than being histone modifiers. With their ability to modify lysine residues, on histones as well as other proteins, they regulate a number of cellular processes like transcription, RNA processing, translation, cytoskeleton, signaling, apoptosis, DNA repair and replication, (for review see(Yang & Seto, 2007)).

There are three families of HATs: GNATs, p300/CBP and MYST proteins. The General control non-depressable 5 (GCN5) related N-acetyl transferase (GNATs) family contains GCN5 and PCAF (p300/CBP Associated Factor). An integral component of the PCAF complex is TRRAP (transformation/transcription domain associated protein) which was initially described as a cofactor for c-Myc and E2F (McMahon *et al.*, 1998). The TRRAP-related protein 1, Tra1 in yeast, is a subunit of the SAGA-complex and TRRAP is also a subunit of the human TIP60 complex which in turn belongs to the MYST family (Doyon & Cote, 2004).

Due to differences in sequence and what cofactors are needed for function, HDACs are grouped into four classes and two families, the classical family and the sirtuin family. The classical family contains HDAC class I (HDAC I, II, III and VIII), HDAC class II (HDAC IV, V, VI, VII, IX, X) and HDAC class IV (HDAC XI) while seven members constitute the sirtuin family (SIRT I-VII, class III). Of these members, SIRT I, II, III and V are involved in deacetylation. The rest of the family members play a part in ADP ribosylation. The sirtuin family is also distinct from the other families by the requirement of NAD^+ as cofactor (Yang & Seto, 2007) and has been implicated both as tumor suppressors and tumor promoters.

For unknown reasons, HATs have not been seriously examined as therapeutic targets. In contrast to this, the first HDAC inhibitor was discovered over 30 years ago when butyrate, an ester of butyric acid, was found to exert this function. This HDAC inhibitor led to cell cycle arrest and induction of differentiation. This was followed by the discovery of trichostatin A (TSA) 12 years later (Yang & Seto, 2007; Yoshida *et al.*,

1990). Later, a hybrid polar compound now known and marketed as vorinostat, was identified as an HDAC inhibitor similar in structure to TSA (Richon *et al.*, 1998). Today this compound is used to treat cutaneous T-cell lymphoma (CTCL) (Yang & Seto, 2007).

In contrast to the above, activators of the HDAC class III family of inhibitors (the sirtuin family) have demonstrated promising effects in targeting of both cancer and ageing (Yang & Seto, 2007). However, as shown in Paper IV later in this thesis, specific Sirtuins may play promoting roles in some tumors and should therefore be re-evaluated when considering targeting in different types of cancer.

1.3 Cell signaling

There are seven major signaling pathways in the cells; Wnt, Hedgehog (Hh), Notch, Receptor Tyrosine Kinase (RTK), nuclear receptor, TGF-β and Jak/STAT. These pathways are carried out by distinct players to finally exert transcriptional regulation (Barolo & Posakony, 2002). The basic mechanisms of these signaling pathways are outlined briefly below.

1.3.1 Wnt-signaling

In Wnt-signaling, Wnt-proteins serve as ligands binding to a receptor complex consisting of Frizzled (a seven transmembrane protein) and low-density lipoprotein receptor-related protein, LRP. There are nineteen coding Wnt-genes in mammals. Wnt-signaling can be divided up in β-catenin dependent (canonical Wnt-signaling) and β-catenin independent signaling. In the canonical Wnt-signaling pathway, β-catenin serves as an important component of the transcription factor TCF. Upon Wnt-binding to its receptor, β-catenin accumulates in the cytoplasm followed subsequently by its nuclear translocation and activation of targets. During low Wnt-signaling, cytoplasmic β-catenin levels are kept under control by the Axin/Adenomatous polyposis coli/Glycogen Synthase Kinase 3β (Axin/APC/GSK3β)-complex. GSK3β-mediated phosphorylation of β-catenin leads to its proteasomal degradation via the β-TRCP E3 ligase. Upon Wnt-binding binding to its receptor, the APC/GSK3β-complex becomes inhibited, and hypophosphorylated. β-catenin accumulates in the cytoplasm, followed by translocation to the nucleus and regulation of its target genes. To activate target genes, β-Catenin replaces the transcriptional

repressor Groucho in binding to the T-cell specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF), (for review see (Gordon & Nusse, 2006)).

In β-catenin independent Wnt-signaling, Wnt may bind to an RTK-receptor called Ror2. Downstream signaling from Ror2 inhibits β-catenin dependent transcriptional activation. Further, Wnt has been shown to bind to another RTK-receptor, Ryk, and regulate axon repulsion. In addition, Wnt controls the planar cell polarity (PCP)-pathway though the Frizzled-receptor but independent of β-Catenin (Gordon & Nusse, 2006).

1.3.2 Hedgehog signaling

Three *hedgehog* genes, *Desert hedgehog*, *Indian hedgehog* and *Sonic hedgehog*, were identified in the mouse 15 years ago, followed by the identification of human homologs. The hedgehog (Hh) family gene product is synthesized as a precursor that is cleaved into two fragments, a reaction catalyzed by the C-terminus of the precursor. The outcome is a non-functional C-terminal part and a cholesterol-modified N-terminal part named Hh-N which will carry out all downstream signaling. This form associates with membranes facilitated by the transferase Skinny hedgehog (Ski), which transfers palmitic acid to the most N-terminal cysteine in Hh, a modification necessary for the activity of Hh-N. Hh is then released from the membrane of secreting cells by another transmembrane protein, Dispatched (Disp). After release and transport, Hh binds to target cells via its receptor Patched (Ptc). When Hh signaling is low, Ptc will inactivate another transmembrane protein in the target cell, Smoothened (Smo). Hh-binding to Ptc abrogates this activity of Ptc and the Hh signal is transmitted into the cell via Smo. This leads to activation of Ci/GLI transcription factors, nuclear translocation and activation of target genes. Hh signaling shares many similarities with the Wnt-signaling pathway. During low Hh-signaling, Ci/GLI factors are under control by GSK3β/β-TRCP also leading to proteasomal degradation, (for reviews see (Varjosalo & Taipale, 2007; Ingham & McMahon, 2001)).

1.3.3 Notch signaling

There are four mammalian Notch receptors called Notch 1-4. Notch precursors are proteolytically cleaved in the Golgi and transported to the cell membrane where the mature Notch receptor, consisting of two subunits is incorporated. The extracellular subunit contains the ligand-binding domain which is built up of epidermal growth factor-like repeats. The

transmembrane subunit consists of the transmembrane domain and an intracellular domain where the nuclear localization sequences are located. Additionally, the Notch 1-3 receptors contain cytokine response sequences and Notch 1-2 also possesses a transcription activation domain (TAD). After binding to its ligands; NotchL, Delta or Jagged on target cells, the intracellular domain of Notch is cleaved off by two successive proteolytic cleavages. This results in activation and translocation of Notch to the nucleus where it will interact with the transcription factor CSL (CBF1/RBP-Jk, Suppressor of Hairless, LAG-1). The interaction between intracellular Notch (ICN) and CSL replaces co-repressors in the nucleus and recruit co-activators. Target gene activation further requires the Mastermind-like polypeptides (MAML), which interact with ICN through specific repeats in the ICN-subunit. This transcription complex activates a number of different target genes, such as the cell-cycle regulators cyclin D1 and p21Cip1, (for review see (Allenspach *et al.*, 2002)).

1.3.4 RTK signaling

The human genome encodes 58 different receptor tyrosine kinases (RTKs). Well known members of the this family are epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR). RTKs are generally activated by ligand-induced dimerization. This leads to autophosphorylation of tyrosine residues and a conformational change of the receptor, which in turn result in recruitment of factors transmitting the downstream signal, (for review see (Hubbard & Miller, 2007)). Examples of downstream pathways activated by RTKs are the MAP kinase and phosphatidylinositol-3 kinase (PI3-K) pathways (see section 1.4.1).

1.3.5 Nuclear receptor signaling

Nuclear receptors are activated by membrane-permeable ligands entering the cell or synthesized and/or processed within the cell. Typical nuclear receptors are the retinoic acid receptor (RAR), vitamin D3 receptor (VDR) and estrogen receptor (ER). Nuclear receptors are either translocated from the cytoplasm to the nucleus upon ligand binding (e.g. ER) or already present at DNA in the absence of ligand (e.g. RAR). In the latter case ligand binding will switch the nuclear receptor from a repressor into an activator of transcription. Nuclear receptors may also act in a ligand

independent fashion. In this case, the nuclear receptor is activated by post-translational modifications, such as phosphorylations, mediated by growth factors and hormones stimulating different signal transduction pathways. Nuclear receptors can bind as monomers, homodimers or heterodimers. After ligand binding, the properties of nuclear receptors are very broad and include crosstalk with other cell signaling pathways as well as recruitment of co-activators for transcription, (for review see (Aranda & Pascual, 2001)).

1.3.6 TGF-β signaling

The human TGF-β family consist of more than 30 members. The TGF-β ligand (e.g. TGF-β1) will signal by bringing the type-I and type-II receptors together. These receptors exhibit serine/threonine kinase activity. After ligand binding, the type-II receptors activate the type I-receptors through phosphorylation. The type I-receptors then transmit the signal by phosphorylating Smad transcription factors. Depending on subfamily ligands and receptors, different Smads will be activated. The TGF-β branch phosphorylates Smad 2 and 3. The activated Smads then translocate to the nucleus and form complexes with Smad4 in order to activate or repress transcription of target genes, such as *p21Cip1*, *p15Ink4b* (activation) and *c-Myc* (repression) with the outcome of Cdk inhibition and G1 cell cycle arrest (*Figure 4*).

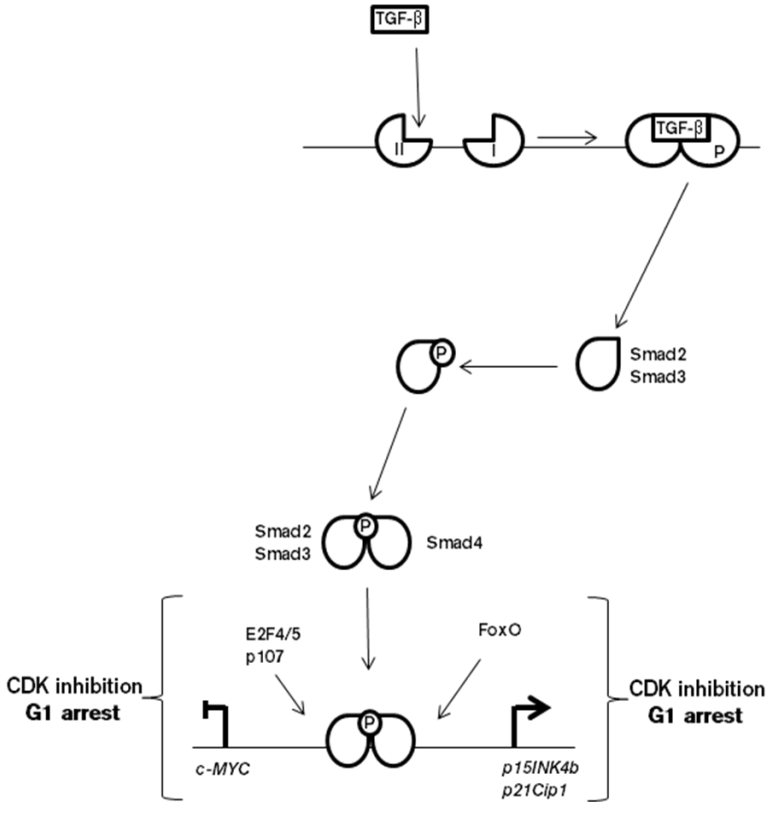
TGF-β
II
I
TGF-β
P
P
Smad2
Smad3
Smad2
Smad3
P
Smad4
E2F4/5
p107
FoxO
CDK inhibition
G1 arrest
P
CDK inhibition
G1 arrest
c-MYC
p15INK4b
p21Cip1

Figure 4. Canonical TGF-β signaling involving activation and repression of certain target genes leading to G1 cell cycle arrest

In addition to the canonical pathway, there are several Smad4-independent pathways as well as type-I receptor independent pathways. The relevance of these additional pathways in cancer needs however to be clarified (for review see (Massague, 2008)).

TGF-β plays dual roles in cancer. In normal and premalignant cells, TGF-β serves as a tumor suppressor regulating proliferation and differentiation among others. In malignant cells however, these tumor suppressive effects of TGF-β are often abrogated by deregulation of different components in the TGF-β pathway, thereby switching TGF-β to a tumor promoter. This includes mutation of the receptors and/or Smads and downregulation of Smads. For instance, a recent report showed that deletion of the type-II TGF-β receptor in mammary carcinoma lead to increased tumor invasion. This was due to recruitment of immune suppressive myeloid cells which produced large amounts of TGF-β1 in the invasive front of the tumor. The increased TGF-β1 production suppressed the immune system thereby promoting tumor invasion (Massague, 2008; Yang *et al.*, 2008). TGF-β inhibits both the innate and adapted immune system by performing inhibitory actions on Macrophages, Natural Killer (NK) cells, Cytotoxic T Lymphocytes (CTL) and T helper (Th) cells (Massague, 2008; Becker *et al.*, 2006).

TGF-β is also a potent inducer of epithelial-mesenchymal transition (EMT), thereby contributing to tumor invasion (see section 1.4.3). The Smads induce expression of the high-mobility group A2 (HMGA2) factor, which in turn induces expression of Snail, Slug and Twist, transcription factors which are all involved in EMT. TGF-β induced EMT has also been shown to be promoted by Ras-signaling (Derynck & Akhurst, 2007; Thuault *et al.*, 2006).

In summary, the complex outcome of TGF-β signaling acting both as a tumor suppressor and tumor promoter is context dependent. This highlights the importance of evaluating the properties of specific tumors before considering targeting via TGF-β signaling.

1.3.7 IFN-γ and Jak/STAT signaling

The cytokine IFN-γ is generally expressed from T cells, both cytotoxic and helper cells, and NK cells with the primary function connected to antiviral and antibacterial activity (Young, 2006). For IFN-γ to stimulate gene expression, the activation of a signal transduction pathway involving the Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) is required. There are seven members of the STAT-family and four members of JAKs in mammals (Murray, 2007). IFN-γ signaling starts by IFN-γ binding to the receptor complex. This receptor complex is built up by a heterotetramer consisting of the R1 and R2 subunits. The assembly of this complex will stimulate the associated Jak1 and Jak2 tyrosine kinases which lead to phosphorylation at tyrosine residues of the R1 subunit of the IFN-γ receptor. The phosphorylated R1 subunit serves as a docking platform for STAT-1 which becomes phosphorylated upon binding, homodimerize and translocate to the nucleus to activate transcription of genes containing a Gamma Activated Sequence (GAS) within their promoter. IFN-γ signaling may also result in STAT-3 phosphorylation, although to a much lesser degree, with STAT-3 binding to GAS-elements, either as homodimers or as heterodimers together with STAT-1 (Rose *et al.*, 2007).

STAT-1 dependent transcriptional regulation involves a large set of genes. Among activated genes is the Cdk-inhibitor p21. IFN-γ/STAT-1 also represses a number of different target genes such as c-Myc, Cyclin D, Cyclin A and Cdc25A explaining its inhibitory effect on cell proliferation. Consistent with this, STAT-1 depletion leads to increased cell proliferation upon IFN-γ treatment, presumably due to enhanced STAT-3 occupation at these target genes and opposite regulation. The tumor suppressive roles of IFN-γ further involves synergistic effects with tumor necrosis factor-α (TNF-α), and constitutive activation of caspases via STAT-1 complexes, suggesting IFN-γ as an inducer of apoptosis (for reviews see (Ramana *et al.*, 2002; Ramana *et al.*, 2000)).

1.4 Cancer

At the molecular level, tumor development is initiated by accumulations of genetic changes targeting oncogenes (genes able to transform cells) or tumor-suppressor genes (genes which inactivation leads to increased risk of cancer development), leading to a gain of function or loss of function,

respectively. Multiple genetic lesions targeting oncogenes and/or tumor suppressor genes lead to cellular transformation, a process where a normal cell is converted to a cell displaying properties of cancer cells (Sherr, 2004; Hunter, 1997).

At the physiological level, not less than six alterations are shared by most malignant tumors (Hanahan & Weinberg, 2000). These alterations represent self-sufficiency in growth-signals, insensitivity to antigrowth signals, sustained angiogenesis, metastasis, evasion of apoptosis and loss of cellular senescence. The processes of apoptosis and cellular senescence also represent the major intrinsic tumor suppressive mechanisms, existing latently in many tumors, thereby suitable for therapeutic reactivation (Lowe *et al.*, 2004; Hanahan & Weinberg, 2000).

1.4.1 Self-generation of growth signals

For a normal cell to enter an active proliferating state it requires mitogenic growth signals. These usually originate from other cells in the body in the form of peptide growth factors or cytokines and signal to cells via transmembrane growth factor receptors, thereby regulating development and behavior of different tissues. A clear difference in growth signaling dependency between normal cells and cancer cells becomes apparent when culturing cells *in vitro*. While normal cells are dependent on supplied mitogenic factors, cancer cells can become more or less independent of exogenous stimulation. Cancer cells have different ways on how to generate their own growth signals. This includes alterations of transmembrane transmitters, or intracellular signaling molecules amplifying the signaling cascade. The first is achieved by synthesizing their own growth factors while the other alterations, involving ligand independent signaling, are due to mutations in the downstream components leading to structurally altered or over-expressed receptors and signal translators (Hanahan & Weinberg, 2000; Di Fiore *et al.*, 1987). Deregulation of signal translators is the most complex mechanism of acquiring self-sufficiency to growth signals. A major player in this branch is the Ras protein which initiates the Ras-signaling pathway. Ras is found to be deregulated in 25% of human tumors (Hanahan & Weinberg, 2000).

The *ras* oncogene encodes a G-protein (guanidine nucleotide-binding) membrane-bound GTPase that cycle between inactive GDP- and active GTP-bound states. Ras is activated by a guanine nucleotide exchange factor

(GEF) switching GDP to GTP-binding of the Ras protein. Cells harboring an activated oncogenic mutation in one of the Ras proteins (H-Ras, K-Ras, N-Ras) (Der & Cooper, 1983; Taparowsky *et al.*, 1983; Taparowsky *et al.*, 1982) fires mitogenic signals constantly since the oncogenic mutation traps the Ras molecule in the active GTP-bound state. This leads to recruitment and activation of the Raf-kinase and subsequent activation of the mitogen-activated protein kinase (MAPK) pathway which regulates transcription through phosphorylation of transcription factors (TFs). Ras-transformed cells have also been found to produce growth-factors like tumor growth factor-α (TGF-α), which leads to an autocrine loop activating tyrosine kinase receptors like the epidermal growth factor (EGF)-receptor. This in turn activates adaptor proteins that retain Ras in its active conformation. The subsequent activation of the MAPK pathway leads to induction of for instance AP1 and Ets transcription factors affecting cell proliferation. Here, Ras activates the Raf-kinase which in turn phosphorylates and activates the MEK1/2 kinases. These phosphorylates serine/threonine/tyrosine residues to activate the extracellular signal regulated kinases 1 and 2 (Erk1/2). Subsequent phosphorylation of substrates via Erks leads to the cellular outcomes stated in figure 5. Activated Ras also stimulates additional kinase pathways like the phosphatidylinositol 3-kinase (PI3K) pathway. The PI3K pathway plays an important role in the control of cell survival, proliferation and growth. Of special importance here is the Akt/PKB kinase, which phosphorylates a number of substrates leading to increased survival, proliferation and cell growth. A simplified illustration of Ras-signaling including the MAPK and PI3K-pathways is shown below. The summarized effects of Ras highlight the potency of deregulated Ras-signaling in cancer development (for reviews, see (Ramjaun & Downward, 2007; Downward, 1997)).

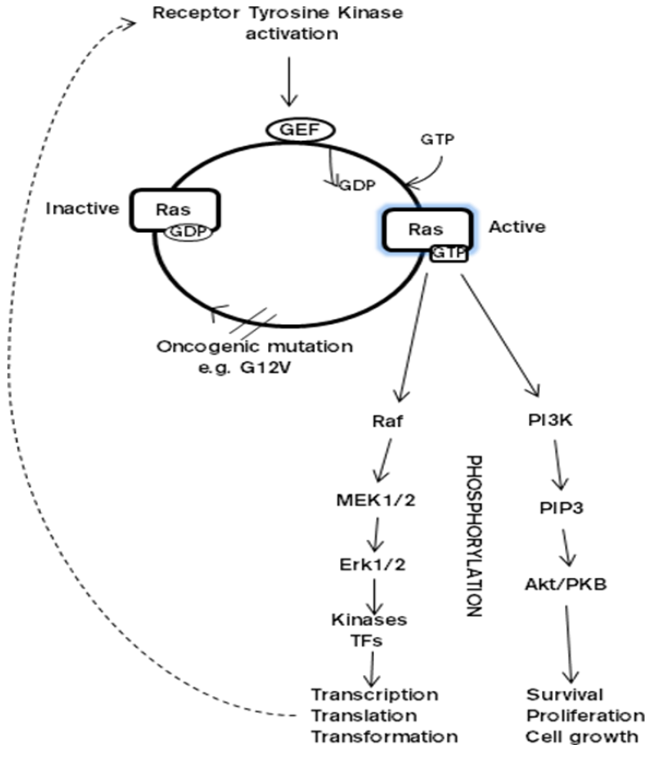

Figure 5. Activated Ras-signaling exerts multiple tumor-promoting effects through the downstream Raf- and PI3K-pathways. Phosphorylation cascades starting from the Raf and PI3K proteins lead to the stated cellular outcomes.

1.4.2 Insensitivity to anti-growth signals

Like growth-signals, anti-growth signals are transmitted by transmembrane receptors connected to intracellular recipients. The outcome of this is either a cell-cycle arrest, from which the arrested cells my relapse at a later stage, senescence or induction of differentiation (Hanahan & Weinberg, 2000).

The transforming growth factor-β (TGF-β) family of cytokines are one of the most studied antigrowth factor families. TGF-β signaling leads to G1 arrest by upregulation of CKIs and downregulation of proliferation-promoting genes such as *myc*, thereby preventing inactivation of pRb with the outcome of an arrest in the G1 phase of the cell cycle (Massague & Gomis, 2006; Hanahan & Weinberg, 2000; Weinberg, 1995).

There are numerous ways by which tumors escape the TGF-β/pRb-pathway. Tumors have been shown to have mutated or downregulated Smad3 or 4 and silenced CKIs in both acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (Lin *et al.*, 2005). Further, pRb-phosphorylating kinases may be mutated leading to increased phosphorylation of pRb (Zuo *et al.*, 1996). pRb itself may be non-functional due to mutation or sequestration by viral oncoproteins as in cervical carcinomas (Dyson *et al.*, 1989). All this will relieve the E2F transcription factors from pRb leading to induction of several genes important for transition into S-phase (Hanahan & Weinberg, 2000).

1.4.3 Angiogenesis and metastasis

Angiogenesis, or the process of new blood vessels formation, is tightly regulated by the balance between proangiogenic growth factors and anti-angiogenic factors. Typical proangiogenic growth factors represent vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) which bind to tyrosine kinase receptors in endothelial cells. Typical inhibitors are thrombospondin-1 (TBP-1) and type-1 interferons (IFNα+β) (Hanahan & Weinberg, 2000; Hanahan & Folkman, 1996).

There is a delicate balance between angiogenic inducers and inhibitors that is tilted towards the inducers in malignant tumors. This could be due to hypoxic conditions leading to increased production of VEGF or loss of tumor suppressors like p53, which normally acts as positive regulators for angiogenic inhibitors such as TBP-1 (Hanahan & Folkman, 1996). Also the *VEGF* gene may be regulated at the molecular level by activation of the *ras* oncogene or loss of the von hippel landau tumor suppressor gene, *VHL*, which degrades the transcription factor hypoxia-inducible factor (HIF) under normoxic conditions. It is apparent that different tumors use different mechanisms to tilt the balance towards induction of angiogenesis making this field very challenging for therapeutic treatments (Kaelin, 2008; Hanahan & Weinberg, 2000).

While the molecular mechanisms that generate primary tumors are relatively consistent, the understanding of the pathways leading to the following tissue invasion is much vaguer. What determines if a primary cancer cell will become invasive or not and when is this ability acquired? Either most of the cells in a primary tumor have the ability to metastasize or this feature is specific for a subpopulation acquiring additional metastasis-specific genetic changes at a later stage. Arguing for the first case, sequencing studies have revealed that primary tumors generally exhibit the same genetic lesions as their derived metastasis (Weinberg, 2008).

Epithelial-mesenchymal transitions (EMT) have been suggested to be necessary for a primary epithelial tumor to acquire the additional steps to become invasive. This process is controlled by transiently induced transcription factors, activated by signaling pathways like Wnt, Hedgehog, TGF-β and other receptor tyrosine kinase pathways, that normally play their role in embryonic development and wound healing (Savagner *et al.*, 2005).

The loss of E-Cadherin, which is a target for several EMT-inducible transcription factors, is of particularly importance for the maintenance of the mesenchymal state in EMT and thereby the ability of the cell to invade its surrounding tissue. E-Cadherin is a transmembrane protein that forms cell-cell adherence junctions. The intracellular part of E-Cadherin is linked to the actin cytoskeleton via linker proteins such as β-Catenin. When not associated to E-Cadherin, β-Catenin is the executioner of Wnt-signaling (see chapter 1.4.1) serving as a transcription factor activating target genes involved in cell growth and proliferation. Downregulation of E-Cadherin by EMT-specific transcription factors will relieve β-Catenin. Free β-Catenin can then translocate into the nucleus and activate a number of target genes like the EMT-specific transcription factor Twist. Twist will subsequently repress expression of E-Cadherin to maintain the mesenchymal state. Tumor progression due to loss of E-Cadherin in certain cells may also occur independently of β-Catenin indicating that other signals are induced upon E-Cadherin loss (Onder *et al.*, 2008; Herzig *et al.*, 2007). The molecular mechanisms of the complete invasion-metastasis cascade are however still poorly understood and await further description in the future (Weinberg, 2008).

1.4.4 Escape of apoptosis

Programmed cell death – apoptosis - is defined as a genetically controlled response for cells to commit suicide when deregulated. When apoptosis occurs, the cellular membranes are disrupted, the nuclei fragmented, the DNA degraded and surface signals exposed to ensure phagocytosis, all within a timeframe of a couple of hours (Lowe *et al.*, 2004; Hanahan & Weinberg, 2000; Wyllie, 1994). Apoptosis may be triggered by two distinct pathways, an intrinsic and an extrinsic cell death pathway (see below).

Cysteine aspartyl-specific proteases (Caspases) are the executioners of both intrinsic and extrinsic apoptotic stimuli. They are divided into two families: initiator- and the effector-caspases. Upstream apoptotic stimuli will activate the initiator caspases, which will cleave and activate effector caspases. An example is caspase-9 that will activate caspase-3 and 7. These effectors caspases then degrade a number of proteins ultimately leading to cell death (Shi, 2004). The intrinsic pathway acts through release of pro-apoptotic factors from the mitochondrion, such as cytochrome c which triggers activation of caspase-pathways completing the apoptosis process. What eventually leads to apoptosis is controlled upstream by a balance between pro-apoptotic Bax/Bak proteins and their anti-apoptotic counterparts Bcl2/$Bclx_L$. Bcl2/$Bclx_L$ acts to keep the mitochondrion channels releasing cytochrome c closed while Bax/Bak proteins work in the opposite manner. These proteins are structurally related, sharing several domains and resides in the mitochondrial membrane, where they compete to determine the fate of cytochrome c. If the Bax/Bak proteins are in excess, the mitochondria will be permeabilized to release cytochrome c. The extrinsic program utilizes cell surface receptors such as Fas and tumor necrosis factor receptor (TNF-R) leading to caspase-8 activation to trigger the rest of the caspase cascade and apoptosis process (Lowe *et al.*, 2004).

It is known that several oncogenes, including E1A, Myc and E2F induce p53 which is one of the main regulators of apoptosis (Vogelstein *et al.*, 2000). p53 is a transcription factor and a major regulator of cellular processes in response to cellular stresses. p53 induces several pro-apoptotic proteins, such as *puma, noxa, bid* and *bax* as well as members from the death-receptor family (Fridman & Lowe, 2003). Inactivation of p53 itself or interference of the downstream machinery dramatically accelerates the ability of Myc, E2F and T antigen to induce tumorigenesis in transgenic mice (Lowe *et al.*, 2004). Mutated *p53* gene is observed in a majority of human tumors. In addition to this, activation of the PI3-kinase pathway through Ras plays a

role in escaping apoptosis as well as loss of the PTEN tumor suppressor protein (Hanahan & Weinberg, 2000).

1.4.5 Reactivating apoptosis

The linkage between proliferation and cell death was discovered when the c-Myc oncoprotein was shown to induce apoptosis in fibroblasts and myeloid cells (Evan *et al.*, 1992; Askew *et al.*, 1991). The mechanisms behind oncogene-induced apoptosis was later shown to be rather complex but could roughly be divided up in p53-dependent and p53-independent mechanisms. More specifically, apoptosis-promoting oncoproteins like c-Myc, E2F and E1A regulate a balance between pro-apoptotic targets and anti-apoptotic targets. If no additional genetic lesions occur, this balance will be shifted in favour for the pro-apoptotic proteins and the death threshold will be reached (Lowe *et al.*, 2004).

In tumors, several genetic deregulations will suppress these apoptosis-promoting signals from individual oncoproteins. However, if the pathways driving apoptosis are still intact, they may be reactivated, shown by several in vivo studies. For instance, Akt-signaling promotes tumor progression by escaping apoptosis, thereby leading to drug resistance. However, when targeted, drug sensitivity was restored in a Myc lymphoma mice model (Wendel *et al.*, 2004).

1.4.6 Loss of replicative senescence

Cancer cells are distinguished from normal cells by their capacity to proliferate indefinitely. Until mid-20th century, many scientists believed that normal mammalian cells possessed intrinsic immortality. The reason for this was that repeated attempts to isolate cells from tissues and propagate them *in vitro* lead to cells that proliferated indefinitely. This immortalization was later shown to be due to accumulation of genetic and epigenetic changes that arose during long-term culturing, and did not reflect the initial properties of normal cells. The mortality of normal cells was demonstrated by Hayflick and Moorhead in 1961. They showed that normal human fibroblasts under controlled growth conditions were shown to have a limited replicative life span before entering a stage of replicative senescence. At this time, several immortal cancer cell lines already existed, which made the authors to propose that immortal cell lines had acquired properties of cancer cells. A few years later, in 1965, Hayflick defined two classes of cultured cells, based

on their mortality, with their respective relationship to *in vivo* conditions. Here, immortal cells corresponded to transplantable tumors while mortal cells were found in normal somatic tissue. (Hahn, 2002; Hayflick, 2000).

Senescence is defined as irreversible cell cycle arrest. At each progression through S-phase, telomeric DNA loses 50-100bp of DNA eventually leading to fused chromosomes, crisis and cell death (Schmitt, 2007; Counter *et al.*, 1992). Telomere shortening is shown to be important as a tumor suppressive mechanism. Telomerase-deficient mice expressing both Myc and Bcl2, thereby apoptosis-resistant, showed a decreased incidence of tumor-development correlating with increased signs of senescence (Feldser & Greider, 2007).

For cancer cells to avoid this senescent-fate and maintain functional telomeres, they upregulate telomerase (*TERT*), leading to immortality. *TERT* encodes the reverse transcriptase catalytic subunit, one of two subunits that constitute the enzyme telomerase. Together with the RNA subunit, this enzyme extends telomeric DNA, thereby preventing telomere shortening and replicative senescence. However, *TERT* has shown not to be sufficient to transform human fibroblasts, not even in combination with Ras and human papillomavirus-16 E6/E7 oncoproteins, which inactivate pRb and p53, demonstrating that human cells require many deregulations to become tumorigenic (Morales *et al.*, 1999).

1.4.7 Oncogene induced senescence

Premature cellular senescence, or cellular senescence acutely induced by oncogenes, was first observed in virally transformed human fibroblasts (O'Brien *et al.*, 1986). Later it became apparent that the major oncogenic pathway leading to premature cellular senescence was the Ras/Raf pathway (Zhu *et al.*, 1998; Serrano *et al.*, 1997). Subsequent studies highlighted the mitogen-activated protein kinase (MAPK) cascade, which is initiated by oncogenic Ras, in the role of induction of the important senescence markers p16 and p53 (Lin *et al.*, 1998).

Senescent cells are characterized by a larger flattened out morphology. Apart from growth arrested with a lack of DNA synthesis, they exhibit a specific gene expression pattern. Senescence-Associated β-Galactosidase activity (SA-β-Gal) is widely used as a visual marker of senescent cells with upregulated SA-β-Gal in their lysosomal compartment. As mentioned

above, p16 and p53 are classical markers of cellular senescence but also p21, Arf and markers of DNA damage have been reported. Further, a direct involvement of p53 and p16 in cellular senescence have been demonstrated where deletion of either the *p53* or *Ink4a* gene was shown to abrogate cellular senescence *in vivo* (Schmitt *et al.*, 2002). A direct involvement of p21 and Arf in cellular senescence is less supported and needs further evaluation (Schmitt *et al.*, 2002; Pantoja & Serrano, 1999). More recently, repressed chromatin displayed as an increase in trimethylated lysine 9 of histone 3 was reported as a marker of cellular senescence (for review see (Schmitt, 2007)).

The most reasonable explanation to Ras-induced senescence is that mitogenic oncogenes will promote growth until a certain limit. At this limit a threshold is passed activating the intrinsic tumor suppressive properties leading to either apoptosis or senescence. For Ras, passing of this threshold will change the directions downstream of the MAPK signaling pathway with the induction of the *p16INK4a* gene via Ets-induced transcription factors and cellular senescence as outcome (Schmitt, 2007). Further supporting this, p16INK4a deficient human diploid fibroblasts were shown to be resistant to Ras-induced senescence (Brookes *et al.*, 2002). Oncogenes like Ras and Myc also produce reactive oxygen species, leading to DNA damage and premature cellular senescence (Schmitt, 2007; Vafa *et al.*, 2002).

Senescence has emerged as a potent tumor suppressive mechanism during the last years. First, it was reported that Ras-transgenic mice harboring lesions of Suv39h1, a methyltransferase involved in cellular senescence, or p53 developed invasive T-cell lymphomas in contrast to control animals. At the same time, two other reports showed roles of both Ras and Raf in cellular senescence in vivo. Premalignant mouse lung adenomas, initiated by a conditional K-Ras-V12 allele displayed senescent cells and Raf-induced senescence was shown in human naevi (Braig *et al.*, 2005; Collado *et al.*, 2005; Michaloglou *et al.*, 2005).

Is apoptosis and cellular senescence of equal importance for therapeutic treatments? Scientists favoring apoptosis usually claim that tumor-cells need to be eliminated in order for a tumor to be permanently regressed. However, these two safe-guard mechanisms seem to complement each other with specific importance of reactivating cellular senescence in tumors with disabled apoptotic pathways (Lowe *et al.*, 2004; Schmitt *et al.*, 2002).

1.4.8 Cancer Stem Cells

During recent years, much focus has been drawn to the term "Cancer Stem Cells" (CSCs). Cancer Stem Cells are suggested to be rare cells that drive tumorigenesis with indefinite potential for self-renewal (Reya *et al.*, 2001). Normal stem cells may turn into cancer stem cells after acquiring an oncogenic mutation. The reason for Cancer Stem Cells to be resistant to therapy is the fact that they are quiescent and hence do not proliferate. As for stem cells, CSCs will give rise to proliferating cells that eventually may turn into differentiated resting cells. However, the number of proliferating cells originating from a CSC will be higher due to the initially acquired oncogenic mutation (Blagosklonny, 2007; Barnes & Melo, 2006). It has recently been debated if targeting of CSCs is a good approach at all considering the potential side effects that may arise due to elimination of normal stem cells (for review see (Blagosklonny, 2007)).

Proliferating cells, generated from a CSC, may acquire additional oncogenic mutations abrogating their path into differentiated resting cells and eventually giving rise to cancer. In Chronic Myelogenous Leukemia (CML), a stem cell disorder which eventually may turn into Acute Myelogenous Leukemia (AML), the initial *BCR/ABL* translocation has shown a lack of consistent expression in Hematopoietic Stem Cells (HSCs). To test the importance of malignant HSCs in ALL and also the impact of additional oncogenic events, Irving Weissman with colleagues generated mice where the *BCR/ABL* translocation was absent in HSCs but present in the progenitor cells. By utilizing these mice they were able to investigate if both chronic and acute leukemia could develop without deregulated HSCs. Indeed, almost one third of the founder mice developed chronic leukemia. These mice were then crossed with apoptosis-deficient *BCL2* mice. Of the confirmed double-transgenic mice, 50% developed AML. These results suggest that cells downstream of the HSC may be better targets of leukemic transformation (Jaiswal *et al.*, 2003).

If the progenitor cells of a cancer stem cell are both proliferating and self-renewing, they are referred to as "cancer stemloids". These cancer stemloids may indeed be better therapeutic targets since they both proliferate and are stem-cell like. Stem cells and cancer stemloids often express ATP-binding cassette (ABC) transporter-proteins making them resistant to drug-treatment due to an active efflux of many drugs. However, by using specific combinations of drugs it seems to be possible to selectively target proliferating cells with stem cell markers, such as cancer stemloids. There are

several examples of such drug combinations. In principle, drug 1 will be pumped out from cancer stem-like cells while drug 2 will target proliferating cells no matter of cell type. To avoid the problem with drug 2, drug 1 is used in a low concentrations leading to arrest of normal healthy cells. This combination render normal cells resistant to drug 2 and the combined treatment specific for proliferating stem-like cells (Blagosklonny, 2007; Patrawala *et al.*, 2005; Hirschmann-Jax *et al.*, 2004).

1.5 The Myc oncoprotein

Since its discovery as a viral oncogene in avian myelocytomatosis virus and the subsequent mapping of the cellular counterparts in various species in the early 1980s, there has been an extensive amount of Myc research performed. The *myc* family of proto-oncogenes encodes a number of transcription factors where the most studied are c-Myc, N-Myc and L-Myc. Myc binds DNA together with its cofactor, Myc-associated protein X (Max), and regulates a significant amount (10-15%) of all genes in mammals. By controlling these genes, Myc affects a diverse set of cellular processes including proliferation, growth, apoptosis, metabolism and differentiation. Myc/Max complexes are counteracted by the Mad/Mnt family of transcriptional repressor which can form alternative heterodimers with Max. More recently, a role of Myc in generation and maintenance of stem cells has also emerged (for review see (Eilers & Eisenman, 2008)).

In this chapter, the broad functions of Myc will be briefly summarized with some emphasis on the specific parts studied in this thesis.

1.5.1 The *c-myc* promoter

The *c-myc* locus spans a region of 160 kb containing both hetero and euchromatin. The euchromatin region of the *c-myc* promoter displays hyperacetylated histone H3 and H4. This euchromatin is flanked by heterochromatin in contrast displaying hypoacetylated histone H3 and H4 as well as methylated histone H3, lysine 9 (Farris *et al.*, 2005; Gombert *et al.*, 2003). The *c-myc* gene has been shown to exhibit hypermethylation when silenced in for instance differentiated human K562 cells. The opposite outcome was displayed in different tumors, like gastric cancer (Fang *et al.*, 2004; Baker *et al.*, 1994).

There are four different start sites for initiation of transcription in the *c-myc* promoter, named P0, P1, P2 and P3. The majority of transcription starts from the P2 site, mainly because of the TATA-box sequence present here as well as the two initiator (Inr) elements. The second most common start site, P1, has a less optimal TATA-box sequence and no initiator elements present while P0 and P3 are deficient of TATA-sequences (Wierstra & Alves, 2008a; Battey *et al.*, 1983).

Several signaling pathways are known to regulate the *c-myc* promoter. Over a decade ago, the Wnt pathway was shown to regulate the *c-myc* promoter through Tcf4-binding sites (He *et al.*, 1998). More recently, conserved Tcf4 binding sites within two enhancer regions were reported *in vivo* and shown to play tissue-specific roles in organ development (Hallikas *et al.*, 2006). Further, two additional enhancer elements have been identified downstream of the 3rd exon using different tumor cell-lines and primary lymphocytes (Mautner *et al.*, 1995). Examples of factors that are reported to bind and either activate or repress the *c-myc* promoter are E2F 1-6, DP-1, Smad 2-4, STAT1, STAT3, NF-κB, ETS-1/2 and c-Jun/c-Fos, (for review see (Wierstra & Alves, 2008a)). In addition to this, Myc and Max are reported to auto-repress the *c-myc* promoter (Mao *et al.*, 2003).

In a recent report showing that P/CAF and E1A regulate the *c-myc* promoter in a negative way through FoxB1, cyclin E/Cdk2 was shown to activate the promoter through FoxM1 binding to the TATA-box, indicating a tighter relationship between cyclin E/Cdk2 and Myc than believed earlier (Wierstra & Alves, 2008b).

1.5.2 The transcription factor Myc

The c-Myc protein consists of three regions; an N-terminal transactivation domain (TAD), a central region and the carboxy-terminal domain (CTD). The TAD contains the conserved Myc boxes 1 and 2, which are important for interactions with cofactors (through Myc box 2) and for biological processes exerted by the Myc protein (Myc box 1). This domain is required for Myc's transactivation activity, while Myc dimerizes with its partner Max and bind DNA through the CTD-domain, where the basic motif is required for DNA-binding and the helix-loop-helix leucine zipper motif for dimerization with Max. The central region contains the nuclear localization signal and has been shown to be important for some protein-interactions (for

review see(Pelengaris *et al.*, 2002)). The functional domains of c-Myc are depicted below.

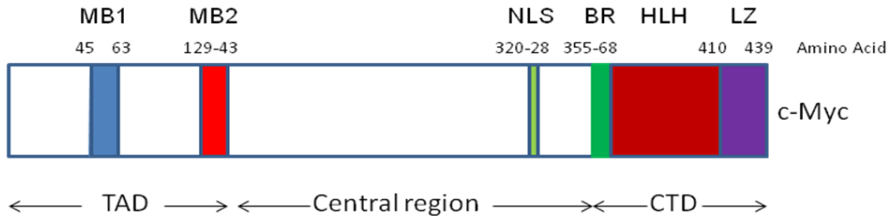

Figure 6. c-Myc protein domains. *MB1*: Myc box 1, *MB2*: Myc box 2, *NLS*: nuclear localization signal, *BR*: basic region, *HLH*: helix-loop-helix motif, *LZ*: leucine zipper motif.

Myc is a so called basic helix-loop-helix leucine zipper (bHLHLZ) transcription factor that will activate its target genes by binding to E-box elements (CACGTG or similar sequences) together with the bHLHLZ partner Max. Myc further recruits a number of transcriptional cofactors, including histone acetyl transferases, chromatin remodelling complexes, kinases, demethylases, ubiquitin ligases and histone deubiquitinating enzymes. Myc was initially associated with transcriptional activation only, but later it became apparent that Myc is also involved in transcriptional repression. As mentioned earlier, Myc regulates a significant proportion of all genes in mammals. Generally, Myc activates genes important for protein synthesis, metabolism and cell cycle progression while repressing cell growth inhibitors, thereby serving a very powerful role as an oncoprotein when deregulated (Eilers & Eisenman, 2008). In contrast, Myc also induces apoptosis-promoting genes and represses apoptosis-inhibiting genes as a safeguard-mechanism described earlier.

The repression mechanisms of Myc are not as well studied as the ones causing activation, but at least one mechanism involves repression through interaction with the zinc finger transcription factor Miz-1. The transcription activator Miz-1 normally activates the Cdk-inhibitor coding genes *p21Cip1* and *p15Ink4b* via initiator sequences (INR), thereby promoting cell cycle arrest. Myc was shown to repress both of these genes by binding to Miz-1 and by recruiting the histone methyltransferase DNMT3a (Brenner *et al.*, 2005; Wu *et al.*, 2003; Herold *et al.*, 2002; Seoane *et al.*, 2002; Staller *et al.*, 2001).

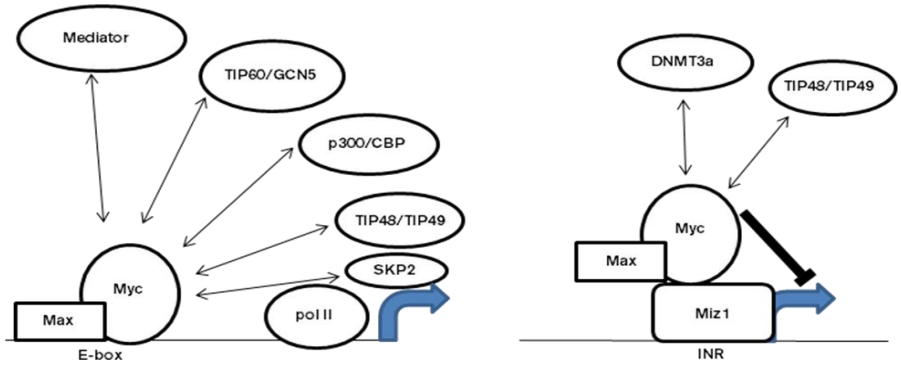

Figure 7. Transcriptional activation (left) and repression (right) by the Myc transcription factor. The cofactor complexes recruited by Myc to facilitate its functions mainly involves histone acetyl transferases (e.g. TIP60/GCN5) for activation and a DNA methyltransferase, DNMT3a, for repression.

The majority of Myc-regulated genes are Pol II-dependent. However, work during the last years has revealed that Myc also regulates Pol I (Steiger *et al.*, 2008; Gomez-Roman *et al.*, 2003) and Pol III-dependent transcription (Arabi *et al.*, 2005; Grandori *et al.*, 2005; Grewal *et al.*, 2005). Unexpectedly, the work of Steiger et al showed that Drosophila Myc activated Pol I-transcription independent of Max.

Another important function of Myc is the regulation of energy metabolism. Myc was first shown to activate the lactate dehydrogenase-A gene (*LDH-A*) which product converts pyruvate to lactate in normal cells under anaerobic conditions. In tumor cells, this occurs also under aerobic conditions, referred to as the Warburg effect. LDH-A was further shown to be important for Myc-dependent transformation under hypoxic conditions (Shim *et al.*, 1997). After demonstrating that cytochrome c was a direct target gene of Myc, the Hockenbery-lab was able to show that Myc induced a number of genes important for mitochondrial gene transcription, translation and assembly when cells re-entered the cell cycle (Morrish *et al.*, 2008). In contrast, Myc repressed expression of the pyruvate dehydrogenase complex (PDHC) but induced expression of the histone acetyl transferase, GCN5, as well as the NAD^+-dependent histone deacetylase SIRT3, all indicating a role of Myc in increasing the pace of cell cycle entry (Morrish *et al.*, 2008).

Myc is known to induce apoptosis through the Arf-p53 pathway. Myc can however induce apoptosis in a p53-independent manner as well, through the pro-apoptotic Bcl-2-interacting mediator of cell death, Bim (Adhikary & Eilers, 2005; Egle *et al.*, 2004; Zindy *et al.*, 1998). During the last decade there have been a number of reports suggesting the potential of targeting Myc in tumors, due to its ability to sensitize cells to apoptosis. Dean Felsher with colleagues showed that a brief inactivation of Myc lead to tumor regression. Reactivation of Myc in these tumors did not lead to relapse but instead to apoptosis (Jain *et al.*, 2002; Felsher & Bishop, 1999). Recently, it was further shown that levels of the Myc protein determines whether it will induce proliferation or apoptosis with the outcome that only overexpressed Myc induced apoptosis (Murphy *et al.*, 2008).

Apart from the processes mentioned above, Myc is a major regulator of proliferation, inducing targets like E2F and cyclins while inhibiting cyclin dependent kinase inhibitors. Also, more recently Myc has been shown to regulate the expression of microRNA (miRNA) both in a positive and negative manner. Regulation of miRNA expression by Myc will serve as a new mechanism via which Myc will repress certain target genes, (for review see (Eilers & Eisenman, 2008)).

10-15% of all loci are estimated to have associated Myc, which would correspond to around 60% of all E-box containing promoters. When expressed at high levels, Myc has also been found to bind non-optimal E-box sequences and recent ChIP-work demonstrates that almost 40% of bound loci do not contain E-box sequences (Zeller *et al.*, 2006). This should at least partly be explained by Myc's additional function as a transcriptional repressor when bound to initiator sequences through Miz-1.

Myc associates with euchromatic promoters with specific histone marks, such as di- and tri-methylation of histone 3 lysine 4, and 79 as well as acetylation of lysines 9 and 18. While at promoters, Myc will recruit different histone acetyl transferases, e.g. TIP60, GCN5/PCAF and CBP/p300. The TIP60 and GCN5/PCAF complexes are recruited to the N-terminus of Myc by their core subunit TRRAP while CBP/p300 interacts with the C-terminal part of Myc. TRRAP has also been shown to recruit the H3S10 kinase Pim1 to promoters in order to induce transcription and transformation. The TIP60-complex also contains the TIP48 and TIP49 proteins, two ATPases shown to be necessary for oncogenic transformation by Myc and for Myc/Miz-1 dependent repression (Eilers & Eisenman, 2008;

Adhikary & Eilers, 2005; Etard *et al.*, 2005; Vervoorts *et al.*, 2003; Wood *et al.*, 2000).

Further, recruitment of the SCF^{SKP2} E3 ubiquitin ligase was shown to be required for transactivation of several Myc target genes. Skp2 was also the first identified E3 ligase to degrade Myc via the ubiquitin-proteasome pathway. The authors demonstrate that Skp2 and the proteasome are recruited to Myc target promoters where they exert coactivator functions on transcriptional activation of unknown nature (von der Lehr *et al.*, 2003). Later, lysine 63 (K63)-linked ubiquitylation by the E3 ligase HectH9 was shown to regulate transcriptional activation by Myc. K63-linked ubiquitylation has also been implicated in error-free DNA-repair and activation of protein kinases. The effect on Myc did not lead to proteasomal degradation of the Myc protein, but rather stabilized it and induced cell proliferation. HectH9-mediated ubiquitylation also involved recruitment of p300-supported transcriptional activation, and was counteracted by Miz-1 (Adhikary *et al.*, 2005; Haglund & Dikic, 2005). The results demonstrated in this report regarding acetylation and ubiquitylation connects to the results presented in paper IV later in this thesis.

In addition to Skp2 and HectH9, Myc is also regulated by the Fbw7 E3 ligase. This involves subsequent phosphorylations of Ser-62 and Thr-58 in the Myc box 1 followed by degradation via the ubiquitin proteasome pathway. In contrast to Skp2 and HectH9, Fbw7 has not been suggested to exert any transcriptional promoting function for Myc and seems to be a strictly negative regulator of Myc (Welcker *et al.*, 2004; Yada *et al.*, 2004).

In a recent report, Myc was found to induce acetylation of several additional sites on histone H3 and H4, apart from the ones mentioned previously. These include acetylations at histone 3, lysine 14 and 18 and at histone 4, lysine 5 and 12 followed by acetylation of lysine 8 and 91 (Eilers & Eisenman, 2008; Martinato *et al.*, 2008). This indicates that Myc also promotes ongoing transcriptional processes. Indeed, Myc was recently found to both promote phosphorylation of the RNA Pol II-tail through TFIIH in the P-TEFb complex as well as 5'-mRNA cap methylation, leading to increased transcriptional and post-transcriptional mechanisms (Cowling & Cole, 2007a). The c-Myc activation domain interacts with the P-TEFb complex via cyclin T1. Further, overexpression of this domain leads to enhanced levels of both Ser-5 and Ser-2 phosphorylated RNA Pol II due to c-Myc binding to CDK7. c-Myc also recruits TFIIH to target promoters and increases mRNA cap methylation, thereby contributing to increased

transcription in an activation domain-dependent manner (Bres *et al.*, 2008; Cowling & Cole, 2007b).

The authors claim that these actions of Myc are independent on the strict function of Myc as a transcription factor and do not require the DNA-binding domain. This could be a further explanation to the surprisingly low percentage of Myc bound to E-box containing loci. However, the function of Myc as a transcriptional repressor through Miz-1 binding probably represents the major fraction of these Myc-bound loci.

The function of Myc as a transcription factor can also be antagonized by competition of binding to E-box elements with the Mad/Mnt family of transcriptional repressors. The members included in this family are Mad1, Mxi1, Mad3, Mad4, Mnt and Mga (Rottmann & Luscher, 2006). These are also bHLHLZ proteins that heterodimerize with Max and in sufficient amounts competes out Myc/Max binding, leading to transcriptional repression through recruitment of histone deacetylases via the adaptor protein Sin3 (Eilers & Eisenman, 2008; Adhikary & Eilers, 2005). The interplay between Myc and the Mad/Mxd family has shown to be of high importance in cell cycle progression and differentiation. A recent report showed that the PI3K/Akt and MAPK-pathways phosphorylate a member of the Mxd-family, Mad1, leading to its proteasomal degradation, thereby promoting Myc-dependent transcription (Zhu *et al.*, 2008). Further, Mad1 has been shown to cooperate with the cell cycle inhibitor p27 in inducing terminal differentiation through inhibition of Myc expression and cyclin E/Cdk2 activity (McArthur *et al.*, 2002). This points out how closely regulated processes in the Myc network are, and also connects to the findings in Paper I in this thesis.

1.5.3 Myc as a regulator of stem cell function and differentiation

The ability to reverse somatic cells back to pluripotent stem cells has recently been shown possible by introduction of certain transcription factors, referred to as the "magic quartet", (for review, see(Eilers & Eisenman, 2008)). The involved transcription factors are Oct3, Sox2, Klf4 and c-Myc. Ectopic expression of these genes have been shown to reprogram murine differentiated B-cells as well as murine and human fibroblasts (Hanna *et al.*, 2008; Okita *et al.*, 2007; Takahashi *et al.*, 2007; Wernig *et al.*, 2007). Since the ectopic c-Myc was shown to be silenced after cells had reached the pluripotent state, Myc only seems to be important in the establishment of pluripotency.

c-Myc is essential for embryo development as demonstrated by the finding that embryos lacking the *c-myc* gene die before midgestation. In a recent report, mice placental rescue showed that c-Myc is necessary for hematopoietic stem cells and progenitor cells (Dubois *et al.*, 2008). However, ectopic expression of Myc inhibits the differentiation of murine embryonic stem (ES) cells. To self-renew, ES cells need leukemia-inhibitory factor (LIF) which after binding to its receptor activates STAT3 with subsequent activation of *c-myc*. If STAT3 is constitutively expressed or if a T58A mutant allele of *c-myc* is used, ES cells remains in a self-renewing or pluripotent state and do not differentiate (Cartwright *et al.*, 2005). The c-Myc T58A protein is stabilized due to abrogated degradation by Fbw7 (Welcker *et al.*, 2004; Yada *et al.*, 2004). Taken together, these reports suggest that Myc, at least when overexpressed, controls exit from the stem cell niche.

In vitro, Myc has been shown to promote differentiation of keratinocytes after long-term culture (Watt *et al.*, 2008). In addition, overexpression of Myc in hematopoietic stem cells leads to depletion of stem cells, while deletion of Myc expands the amount of stem cells due to disabled ability to differentiate into progenitor cells (Wilson *et al.*, 2004). Myc promotes differentiation by reducing the cell-cell contacts between the stem cells and the stem cell niche. In keratinocytes, Myc represses many genes involved in cell-cell contacts through binding to Miz-1. Among these genes are several integrins (Gebhardt *et al.*, 2006).

In a recent report, immature HSCs were shown to express both *c-myc* and *N-myc* mRNA at equal levels. Further, the proliferation of HSCs was dependent on both *c-myc* and *N-myc* and in a double knock-out situation (dKO) a majority of hematopoietic cell types stopped cycle and underwent apoptosis (Laurenti *et al.*, 2008). This demonstrates that Myc activity is necessary for proliferation, differentiation and apoptosis in HSCs.

Also, it has been speculated if Myc is able to promote formation of cancer-initiating cells. This was strengthened in a recent report showing that c-Myc in contrast to other oncogenes was sufficient to reactivate ES cell programs in both normal and tumor cells. Further, c-Myc was shown to induce cancer-initiating cells in transformed primary keratinocytes, suggesting that Myc may influence cancer stem cell function in different tumors (Wong *et al.*, 2008).

The findings that Myc promote exit from the stem cell niche in favor for a premature differentiation but not terminal differentiation are interesting

from a cancer point of view. Deregulated Myc promotes tumor formation but if targeted, these tumors regain their ability to terminally differentiate and since they are out of the stem cell niche, Myc inhibition should have no effect on normal development (for review see (Eilers & Eisenman, 2008)).

1.5.4 Myc & Ras and cellular transformation

As mentioned previously, the cooperation of at least two oncogenes are needed for primary rodent cells to transform into cancer cell. A classic example of this, are the *myc* and *ras* oncogenes which are sufficient to transform primary fibroblasts from rat (Land *et al.*, 1983). Human cells are however less susceptible to oncogenic transformation and require at least three cooperating oncogenses (*tert*, *ras* and *T-antigen*) for transformation (Boehm *et al.*, 2005; Drayton *et al.*, 2003; Hahn *et al.*, 1999).

At the molecular level, Ras has been demonstrated to regulate Myc-stability by controlling the phosphorylation of two amino acid residues in the N-terminal domain, Ser62 and Thr58. Ras-signaling affects Ser62 phosphorylation through the MAPK-pathway (Sears *et al.*, 2000). Ser62 is also targeted by several additional kinases. Phosphorylation of Thr58 is carried out by glycogen synthase kinase 3 (GSK3) (Gregory *et al.*, 2003) which is still the only kinase suggested for this site. Ser62-phosphorylation primes GSK3 to phosphorylate Thr58, subsequently followed by recruitment of the E3 ubiquitin ligase Fbw7, and proteasomal degradation of the Myc protein. Ras-signaling also inhibits GSK3-activity through an inhibitory phosphorylation via the PI3K/Akt pathway, thereby abrogating Thr58 phosphorylation (Welcker *et al.*, 2004; Yada *et al.*, 2004). This could potentially be one of the explanations how Ras inhibits Myc-induced apoptosis (Kauffmann-Zeh *et al.*, 1997). A Thr58 mutated Myc is deficient in apoptosis and for induction of apoptosis specific targets, like the BH3-only protein, Bim (Hemann *et al.*, 2005). Whether the phosphorylation of Thr58 is necessary for the induction of Myc-target genes promoting apoptosis needs however further examination.

In addition, the Akt pathway was demonstrated to phosphorylate the transcription factors FoxO1 and FoxO3a, which normally bind many Myc-target genes, and target them for destruction via the ubiquitin-proteasome system (Adhikary & Eilers, 2005; Bouchard *et al.*, 2004; Plas & Thompson, 2003).

As a third mechanism, Ras-signaling leads to phosphorylation of Ser71 in the Myc-protein by Rho-dependent kinase acting downstream of PI3K.

This phosphorylation exerts repression of Thrombospondin-1 resulting in increased angiogenesis. Further, Akt phosphorylates Miz-1, thereby abrogating the ability of Miz-1 to bind to its target p21, resulting in increased cell proliferation (Adhikary & Eilers, 2005).

All together, these mechanisms provide support for the role of Ras in suppressing Myc-induced apoptosis in Myc+Ras transformed cells. The role of Myc in this cooperation is less studied but will be discussed in chapter 3 as well as in Paper II.

2 Aims of this study

The content of this thesis work is divided into three parts. In the first part we examine the molecular relationship between Myc and TGF-β signaling, a classic tumor suppressor pathway, in hematopoietic cells with deregulated Myc. The aim of the second part was to elucidate the mechanism(s) by which Myc cooperates with Ras in cellular transformation and investigate a possible role of the Myc target, cyclin dependent kinase 2, in Myc-driven cellular processes. Finally, we elucidated a potential relationship between Myc and the deacetylase SIRT1.

The general aim of this thesis is therefore to aquire better insight into some of the basic tumor-promoting mechanisms by Myc and their regulation. Such increased knowledge may uncover new pathways suitable for therapeutic interventions in Myc-driven tumors.

2.1 Specific aims

- To review the impact of TGF-β signaling with regard of Myc-activity, Myc/Max/Mad network regulation and cell cycle progression in hematopoietic cells with deregulated Myc expression (paper I).

- To address the specific role of Myc in the oncogenic cooperativity between Myc and Ras during malignant transformation (paper II).

- To elucidate the mechanism(s) by which IFN-γ overrides the activity of Myc in cells with deregulated Myc (paper II).

- To evaluate the role of Cdk2 in Myc-driven proliferation and other Myc-regulated cellular processes by utilizing cells depleted of this kinase (paper III).

- To dissect a possible connection between the histone deacetylase SIRT1 and Myc, with regard to proliferation, apoptosis and transcriptional activation (paper IV).

3 Results and discussion

3.1 TGF-β induces senescence in Myc-transformed hematopoietic cells (paper I)

TGF-β exerts growth inhibitory functions in many different cell types, although after deregulation it may function as a tumor-promoter in some cancers (Massague, 2008). The general mechanism underlying the anti-proliferative effects of TGF-β involves upregulation of the cyclin dependent kinase inhibitors *p15Ink4B*, *p21Cip1*, *p27Kip1* and *p57Kip2* (Scandura *et al.*, 2004; Alexandrow & Moses, 1995) and downregulation of growth-promoting genes like *myc* (Gomis *et al.*, 2006). Further, overexpression of c-Myc has been shown to block the growth-inhibitory functions of TGF-β, demonstrating that Myc and TGF-β signaling counteract each other (Alexandrow *et al.*, 1995).

TGF-β has been shown to inhibit proliferation of cells in the hematopoietic system (Fortunel *et al.*, 2000) and also exerts negative effects on the *c-myc* gene by directly repressing its promoter (Kim & Letterio, 2003). However, the effect of TGF-β in the context of deregulated Myc expression in hematopoietic cells has not been extensively studied. We have previously utilized the U937 monoblast system to study the impact of Myc on cell growth and differentiation. Transformation of these cells with a viral *myc* (v-*myc*) gene makes them resistant to G1 arrest and differentiation induced by agents such as vitamin D3, retinoic acid and the phorbol ester TPA. However, TGF-β treatment was shown to induce growth arrest in these cells despite the transduced *v-myc* gene (Oberg *et al.*, 2001).

In our present study we show that TGF-β forces these cells into cellular senescence, correlating with potent induction of the Myc-antagonist Mad1. Parental U937 and v-Myc expressing U937 cells were first analyzed for cell cycle distribution by fluorescence activated cell sorting (FACS). The results strengthened our previous findings, i.e. that v-Myc expressing cells underwent G1-arrest after TGF-β treatment, but not after treatment with the phorbol ester TPA, a classical inducer of differentiation in this system, in contrast to parental cells where both agents caused G1-arrest. After 6 days of treatment, the v-Myc expressing cells also exhibited increased staining for senescence associated β-galactosidase (SA-β-Gal), a well-established marker of cellular senescence (Dimri *et al.*, 1995). TGF-β signaling has previously been reported to promote cellular senescence (Debacq-Chainiaux *et al.*, 2005; Tremain *et al.*, 2000). However, the effects seen here are under conditions of enforced Myc-expression. This is unexpected since Myc previously was shown to block TGF-β induced cell cycle arrest in mouse keratinocytes (Alexandrow *et al.*, 1995), and indicated that TGF-β may target Myc through other mechanisms than transcriptional repression.

3.2 TGF-β shifts the balance of Myc/Max/Mad resulting in transcriptional repression (paper I)

In paper I, we further found that TGF-β induced expression of the Myc antagonist Mad1. Using radioactive labelled cell extracts from TGF-β treated v-Myc expressing U937 cells, we detected increased Mad1 protein synthesis while synthesis of endogenous Myc was decreased. We also observed a slight decrease in v-Myc expression. The elevated Mad1 protein level correlated with increased mRNA expression. In addition, we detected increased mRNA-expression of two other *mad*-family members, *mxi1* and *mad4*. The increase in Mad1 synthesis was not unique for the U937-cells but also apparent in HL-60 and ML-1 myeloid cells as well as in HaCaT keratinocytes after TGF-β treatment.

Induction of Mad1 after TGF-β treatment was also reported recently in keratinocytes (Descargues *et al.*, 2008). This is consistent with our detection of Mad1 up-regulation in HaCaT keratinocytes. Our results further show that the induction of Mad1 induced a switch from Myc to Mad1 dominance at an E-box containing region of the *cyclin D2*-gene. Cyclin D2 is involved in the p16Ink4a-Rb pathway and may therefore play important roles in cellular senescence, strengthening our finding that TGF-β/Mad1 forces Myc-transformed cells into cellular senescence. However, other genes

implicated in cellular senescence need to be investigated as well, such as *p16Ink4a* and *Bmi-1*.

Finally, we were able to demonstrate that this shift after TGF-β treatment correlated with transcriptional repression of Myc-target genes. The expression of Myc-responsive promoter/reporter systems decreased by 10-fold after TGF-β treatment. This effect could be partially reversed by treating cells with trichostatin A (TSA), an inhibitor of histone deacetylases (HDACs), suggesting that TGF-β induced repression of Myc-dependent transcription is executed through recruitment of HDAC-complexes, probably to the Sin3-interacting domain of Mad1.

3.3 Mad1 increases TGF-β responsiveness in Myc-transformed cells (paper I)

To investigate if responsiveness to TGF-β could be enhanced by increased Mad1 expressions, v-Myc expressing U937 cells with inducible expression of *mad1* were generated using the Lac repressor system. Expressing *mad1* together with TGF-β treatment shortened the time of accomplishing G1 arrest from 72 hours to 48 hours and the percentage of cells in S-phase decreased by 2-fold. Interestingly, induction of *mad1* alone failed to arrest cells in G1 phase, indicating that additional TGF-β targets play a role. One such target could be p27Kip1, which was induced upon TGF-β treatment. Further, Mad1 was able to restore TPA-mediated G1 arrest but not differentiation in these cells suggesting that induction of differentiation may work independently of Mad1 in these cells.

3.4 Myc versus TGF-β in cancer: competition for biological outcome? (paper I)

Our results thus demonstrate that TGF-β activates cellular senescence in Myc-transformed hematopoietic cells. Interestingly, this did not seem to be the result of downregulation of v-Myc in these cells. Instead, TGF-β treatment switched the occupancy at Myc target promoters from Myc/Max to Mad/Max complexes, unravelling an alternative function of TGF-β in combating Myc. TGF-β thus seems to be a potent antagonizer of Myc in this system. TGF-β was previously shown to induce G1 arrest in murine pre-B cells expressing Eμ-*c-myc*. The expression of a second oncogene, in this case *abl* or *ras*, was required to block this effect (Letterio *et al.*, 2006).

Our results however differ from results in murine leukemic cells overexpressing Myc, where TGF-β induced apoptosis (Selvakumaran *et al.*, 1994). This indicates that TGF-β signaling favors induction of either apoptosis or cellular senescence depending on the cellular context. In cells with deregulated Myc, this may further depends on the amount of Myc levels present (Massague, 2008; Murphy *et al.*, 2008). It should also be mentioned that our Myc-transformed U937 cells carries a Thr-58 mutation which potentially could make them less susceptible to apoptosis (Hemann *et al.*, 2005), thereby favoring induction of senescence upon TGF-β treatment.

Enforced Myc expression has previously been shown to block the tumor-suppressive role of TGF-β in epithelial cells (Alexandrow *et al.*, 1995), thus the potency of TGF-β in U937-cells despite continuous Myc expression was unexpected. Additionally, Myc is a known repressor of TGF-β target genes. We find it likely that the TGF-β induced expression of Mad1 and possibly other Mad-family members contribute to overriding Myc in the U-937 system. The combined induction of Mad-family proteins by TGF-β in this system may therefore exceed a threshold that is difficult to overcome in epithelial cells. It is also possible that the effect of TGF-β as a derepressor of these genes is stronger than the suppressive effect mediated by Myc in our cell system. Also, we cannot rule out that TGF-β might induce a senescence pathway that acts independently of Myc-network pathways that acts in concert with the induced Mad1 expression.

In conclusion, our finding that TGF-β can override deregulated Myc, despite a stabilizing cancer mutation may be of therapeutic interest for tumors with deregulated Myc in the future, with particular interest for tumors exhibiting deregulated apoptotic pathways.

3.5 Myc represses Ras-induced senescence (paper II)

Combinations of oncogenes like *myc* and *ras* are known to transform rodent cells since over 2 decades (Land *et al.*, 1983), but the molecular mechanism behind this cooperative transformation has remained unsolved. The finding that oncogenes may induce intrinsic tumor-suppressive properties of cells emerged partly from the reports that overexpressed Myc is inducing apoptosis (Evan *et al.*, 1992; Askew *et al.*, 1991) and that oncogenic Ras forces cells into cellular senescence (Serrano *et al.*, 1997). However, Ras may overcome c-Myc induced apoptosis, an action probably promoting the tumorigenic properties of Ras in Myc+Ras transformed cells (Kauffmann-Zeh *et al.*, 1997).

With the knowledge that Ras suppresses Myc-induced apoptosis, we addressed whether Myc will be able to suppress Ras-induced senescence. Using primary rat embryo fibroblasts (REFs), we first confirmed that overexpressed Myc induced apoptosis in this system and that this was inhibited by co-expressing oncogenic H-Ras. Further, by staining for SA-β-Gal, we confirmed that overexpressed Ras alone forced these cells into cellular senescence. This senescence entry was abrogated by co-expressed Myc, confirming our theory that Myc represses Ras-induced senescence in order to transform cells.

As mentioned previously, two conserved phospho-acceptor sites in the N-terminus of Myc, Ser-62 and Thr-58 (see 1.5.4), have been implicated in Myc-dependent processes like apoptosis and proliferation (for reviews, see (Hann, 2006; Vervoorts *et al.*, 2006)). Interestingly, using mutants for these sites, we found that Myc repressed senescence only if Myc Ser-62 was intact. Thr-58, which was reported to be necessary for Myc-induced apoptosis (Hemann *et al.*, 2005), did not seem to play a role in repression of cellular senescence, indicating that these sites play distinct roles in these two processes.

Due to the important role of Ser-62 as a phospho-acceptor site in the Myc protein, we hypothesized that the phosphorylation of Ser-62 might be important for the observed repression of cellular senescence. Indeed, a phospho-mimicking S62D mutant, where the serine site is exchanged for aspartic acid, abrogated Ras-induced senescence as efficiently as wildtype Myc.

These results suggest that Myc represses Ras-induced senescence, and that this function is dependent on the Ser-62 site in the N-terminus of the c-Myc protein.

3.6 Myc-repressed cellular senescence requires phosphorylation of Serine 62 by Cdk2 (paper II)

The Ser-62-site in Myc has been suggested as a target of several kinases (Sjostrom *et al.*, 2005; Sears *et al.*, 2000). To identify Ser-62 kinases responsible for senescence regulation, the effect of a panel of inhibitors on Ser-62 phosphorylation was investigated. Since roscovitine inhibited Ser-62 phosphorylation most efficiently, Cdk1 or Cdk2 were good candidates.

Since a more specific inhibitor of Cdk1 did not show any effect while Cdk2-selective inhibitors were still efficient, Cdk2 was the most likely kinase. Ras-signaling was previously shown to affect Ser-62 phosphorylation via Erk (Sears *et al.*, 2000). However, inhibition of Erk showed quite weak effects under our experimental conditions. The novel implications of Cdk2 as a Ser-62 kinase was further strengthened by siRNA-experiments, where Cdk2 and cyclin E-knockdown decreased Myc Ser-62 phosphorylation in contrast to knockdown of Cdk1. This indicates that Cdk2 acts as a major Ser-62 kinase for Myc.

A possible involvement of Cdk2 in Myc-repressed cellular senescence was tested in Myc+Ras expressing REFs. We found that Cdk2-inhibition by pharmacological inhibitors reversed the senescence-inhibitory effect of Myc and forced these cells into cellular senescence. This result was unique for Cdk2-inhibition and was not observed using inhibitors against Cdk1, Cdk9 or Mek1/2. Further, co-expression of p27Kip1, a physiological Cdk2-inhibitor, was even more efficient in abrogating the effect of Myc. Importantly, these effects were only seen if Myc and Ras were co-expressed and no effect of treating cells with the pharmacological inhibitors alone could be detected for either cellular senescence (scored by SA-β-Gal activity) or cell proliferation.

This suggests that Cdk2 has a unique function in Myc/Ras cellular transformation by phosphorylating Myc at a specific site, Ser-62, thereby repressing Ras-induced senescence. In this respect Cdk2 is also unique among Ser-62 kinases, since neither Cdk1 nor Erk1, the latter being activated by Ras-signaling (Sears *et al.*, 2000), seemed to play a role in the anti-senescence function of Myc, although we cannot rule out that Ras per se could contribute to the senescence-repressing properties of Myc. These findings are highly important since they highlight a unique function of Cdk2 that cannot be replaced by other cyclin dependent kinases. Although the clinical relevance of Cdk2-targeting has been questioned previously, we suggest that Cdk2-targeting may be of importance in tumors with deregulated Myc and/or Ras.

3.7 IFN-γ causes Cdk2-inhibition and senescence in Myc-transformed human hematopoietic cells (paper II)

To address the question whether Cdk2-inhibition could be used to restore senescence in Myc-driven human tumor cells, *v-myc* transduced monocytic U937-cells were used. These cells are sensitive to IFN-γ treatment, which abrogates the effect of v-Myc with the outcome of restored differentiation and growth arrest. This occurs although v-Myc is continuously expressed indicating that the effect of IFN-γ in this system is independent of transcriptional shut-off of the endogenous *c-myc* gene through STAT1 (Bahram *et al.*, 1999).

In the light of the previously observed growth arrest in this system after IFN-γ treatment, we addressed whether IFN-γ activates cellular senescence. Indeed, after 6 days of treatment with IFN-γ, a substantial percentage of cells stained positive for SA-β-Gal, indicating a state of cellular senescence. This correlated with a reduction of Myc Ser-62 phosphorylation, inhibition of Cdk2-activity and upregulation of the Cdk2-inhibitor p27Kip1, consistent with reports saying that IFN-γ signaling up-regulates certain Cdk-inhibitors and has a negative effect on Cdk2 (Mandal *et al.*, 1998; Harvat *et al.*, 1997). Since we also detected an increased complex formation between p27 and Cdk2 upon IFN-γ signaling, we speculated that p27 may play a role as the physiological inhibitor of Cdk2, in particular for activation of senescence in this system. To check this, v-Myc expressing U937 cells were transduced with retroviral vectors expressing either wt p27 or p27 mutated at Thr-187 (T187A), generating a more stable protein due to impaired degradation. p27-transduced cells were sorted and stained for SA-β-Gal. All p27-transduced cells stained positive for SA-β-Gal. The SA-β-Gal staining was more pronounced in cells expressing the T187A-mutant construct. This suggests that p27 mediates the effect of IFN-γ in this system, presumably through inhibition of Cdk2 and highlights p27 as a potent inducer of cellular senescence in cells with deregulated Myc. In support of this, treatment with two specific Cdk2-inhibitors also resulted in an increase of SA-β-Gal positive cells and strong growth inhibition.

These results demonstrate that IFN-γ may force tumor cells into cellular senescence, in addition to its role in apoptosis (Ramana *et al.*, 2000; Ohmori *et al.*, 1997). With the knowledge of how c-Myc is regulated by IFN-γ at the transcriptional level together with the results presented above, a role of IFN-γ signaling in inducing cellular senescence should be considered at least in tumors with deregulated Myc.

3.8 Cyclin E/Cdk2/p27 associates with Myc at target promoters (paper II).

The results described above indicated that there might be a closer relationship between Myc and cyclin E/Cdk2/p27 than previously anticipated. To address this, we first looked for physical interactions between these proteins in cell lysates. We found that Myc co-immunoprecipitated with both cyclin E and p27. The Myc/p27 interaction was further increased when cells were treated with IFN-γ.

Since Myc exerts its function as a transcriptional activator we wanted to investigate if the impact of cyclin E/Cdk2/p27 could actually occur at Myc target promoters. Interestingly, using chromatin immunoprecipitation (ChIP), we found that all these proteins associated with the Myc target *cyclin D2*. As mentioned previously, cyclin D2 is a protein regulating the p16Ink4a/Rb-pathway, and thereby a target with potential impact of cellular senescence. Using re-ChIP experiments, we could further show that Myc interacted with both Cdk2 and p27 at the *cyclin D2*-promoter.

Examining other Myc-target genes involved in regulating cellular senescence, we found that *p16Ink4a, p21Cip1, Bmi-1* and *hTert* were all targets for cyclin E/Cdk2 and p27 using ChIP experiments as above. The amount of bound cyclin E/Cdk and/or p27 was further regulated by pharmacologically inhibition of Cdk2 or by IFN-γ. Both these treatments lead to reduced levels of cyclin E/Cdk2 at all these targets, but increased p27-levels. This together with the observed reduction of Ser-62-phosphorylated Myc and acetylated histone 4 at these targets indicated that cyclin E/Cdk2 may function as a positive cofactor for Myc. In contrast, p27 could abrogate this mechanism via cyclin E/Cdk2 inhibition. This would likely affect the Myc-dependent transcriptional activation, or repression of Myc-targets involved in cellular senescence.

Ser-62 phosphorylation by Erk was previously shown to be important for Myc-dependent transcriptional activation of the γ-GCS gene, encoding an enzyme catalyzing glutathione biosynthesis, in response to oxidative stress (Benassi *et al.*, 2006). Ser-62 phosphorylation might therefore be exerted by different kinases phosphorylating Myc target genes in response to different stimuli.

3.9 Cyclin E/Cdk2 functions as a Myc cofactor (paper II).

These observations suggested a cofactor function of cyclin E/Cdk2 in Myc-driven transcription. Overexpressing cyclin E/Cdk2 together with Myc enhanced the activity of a Myc-responsive reporter-construct much further than Myc alone, thus supporting this view. Further, since an S62A-mutant did not activate an hTERT reporter-construct, in contrast to wt Myc, T58A-Myc or S62D-Myc, further strengthened the Ser-62 dependency of Myc in repressing Ras-induced senescence. IFN-γ treatment instead resulted in repression of Myc-driven promoter/reporter construct, which is consistent with our ChIP-results showing that IFN-γ reduced cyclin E/Cdk2 levels but increased p27-levels at Myc target promoters.

We next analyzed mRNA expression for the Myc-targets *hTert, Bmi-1, p16Ink4a* and *p21Cip1*. Pharmacological inhibition of Cdk2 or IFN-γ treatment reduced the mRNA levels of *hTert* and *Bmi-1*, well in line with the fact that these both are normally activated by Myc. The mRNA-levels of *p16Ink4a* and *p21Cip1* increased upon these treatments. *p21Cip1* is known to be repressed by Myc, while the effect on *p16Ink4a* could possibly be indirect through Bmi-1 (Guney *et al.*, 2006). These results are consistent with the ChIP-results and strengthen the unique role of cyclin E/Cdk2 as a mediator of Myc-repressed senescence.

In paper II, we provide evidence for a novel function of Myc in Myc+Ras transformation. We demonstrate that Myc represses Ras-induced senescence, thereby contributing to active suppression of failsafe tumor-suppressive mechanisms in Myc+Ras induced transformation. This function was also revealed elsewhere in a recent report showing that overexpression of Myc suppressed Raf and Ras-induced senescence in melanoma cells (Zhuang *et al.*, 2008), which is consistent with our own findings. We further provide a mechanism for the repressed senescence by Myc with the finding that phosphorylation of Ser-62 is required for this function and with the identification of the kinase, Cdk2, phosphorylating this residue. Our findings uncover a novel and unique role of cyclin E/Cdk2 as a Myc cofactor and mediator of repression of senescence. Importantly it seems like other kinases cannot compensate for this role of cyclin E/Cdk2, highlighting the function of this kinase in cells with deregulated Myc+Ras.

3.10 Myc induces senescence in *Cdk2-/-* cells (paper III)

Activated oncogenes like Myc and Ras promote cell proliferation in part by inducing cyclins and cyclin dependent kinases. If deregulated in non-transformed cells, they may however also induce safeguard mechanisms, where the primary effect of Myc is to induce apoptosis but also cellular senescence under certain conditions. To avoid such fate, oncogenes like Myc and Ras are dependent on additional genetic lesions or mediators suppressing the safeguard mechanisms (Drayton *et al.*, 2003; Grandori *et al.*, 2003). Cyclin dependent kinases have previously been implicated in Myc dependent tumorigenesis (Goga *et al.*, 2007; Miliani de Marval *et al.*, 2004). We investigated the role of Cdk2 in Myc-driven proliferation and tumorigenesis by using primary wt and *Cdk2-/-* MEFs transduced with a retrovirus expressing the 4-hydroxy-tamoxifen (4-OHT) inducible Myc-estrogen receptor (MycER) fusion protein. Upon treatment, 4-OHT binds to the ER-receptor part of the MycER fusion protein with subsequent translocation of MycER to the nucleus. Using this system, we discovered an unexpected difference between *Cdk2+/+* and *Cdk2-/-* MEF cells. While both cells showed similar growth-rates upon Myc-activation initially, mutant MEFs eventually entered cellular senescence as determined by positive staining for SA-β-Gal activity. The senescence entry could be prevented if Cdk2 was re-expressed and no arrest occurred in *Cdk6-/-* or *Cdk4-/-*;*Cdk6-/-* MEFs showing that senescence entry upon Myc-activation was specific for the depletion of Cdk2. To address whether the observed phenotype was dependent on the Arf-p53-p21 and/or the p16-pRb pathway, double knockout MEFs for Cdk2 and each of the individual components in these pathways were utilized, showing a requirement of all these players in Myc-induced senescence.

To rule out that the senescence seen in *Cdk2-/-* cells was not just a consequence of impaired apoptosis (Deb-Basu *et al.*, 2006), apoptosis-deficient MycER T58A constructs were used. *Cdk2-/-* cells expressing this construct still underwent cellular senescence while no senescence induction could be observed in *Cdk2+/+* cells, ruling out senescence induction as a consequence of abrogated apoptosis. All this suggested that Cdk2 also serves to suppress Myc-induced senescence, in addition to its function as a mediator of Myc-repressed senescence shown in paper II.

3.11 Cdk2 depletion delays lymphoma onset *in vivo* (paper III)

Due to its ability to suppress Myc-induced senescence, we hypothesized that Cdk2 might promote tumor progression. Eμ-*myc* mice transgenic for a *c-myc* gene driven by the IgH enhancer, an established model for Burkitt's lymphoma, were bred with *Cdk2*+/- mice to obtain mice depleted, heterozygous, or wt for Cdk2. When comparing these animals we could observe a significant delay in lymphoma onset in the Eμ-*myc Cdk2*-/- mice. Up-regulation of p16Ink4a was also observed in the *Cdk2*-/- mice, although a direct involvement of this Cdk inhibitor in the delayed lymphoma development remains to be addressed. These data suggests that Cdk2 favours lymphoma onset by inhibiting Myc-induced senescence. This is also consistent with the finding that short telomeres suppressed tumor progression in the Eμ-*myc* mice model (Feldser & Greider, 2007).

During Myc-driven tumor progression in this system, there is mainly a selective pressure for bypassing apoptosis. This can be achieved by different mechanisms such as loss of p53, mutation of Myc Thr-58 or upregulation of anti-apoptotic proteins like Bcl-2 and Bcl-x_L. Targeting of Cdk2 may be particularly suitable for tumors that have managed to bypass apoptosis through mechanisms not impinging on senescence, such as Burkitt's lymphoma that harbor Thr-58 mutations. Such tumors are likely to be more resistant to reactivation of apoptotic mechanisms but may still be very sensitive to activation of cellular senescence. Myc has recently been reported as a suitable therapeutic target. This is based on mouse tumor models with inducible Myc systems. Previous findings have shown that inactivation of Myc in a Myc-driven tumor can induce tumor regression by apoptosis (Murphy *et al.*, 2008; Goga *et al.*, 2007). However, the Felsher-lab demonstrated recently that inactivation of Myc in many tumor models cause tumor regression through senescence (Wu *et al.*, 2007), findings also compatible with the results in this paper. Further, since Cdk1-inhibition has been reported to sensitize Myc-driven tumors to apoptosis (Goga *et al.*, 2007), combined targeting of Cdk1 and Cdk2 may be more efficient for Myc-deregulated tumors and should be considered for reactivation of intrinsic tumor suppressive mechanisms in the future (*Figure 8*).

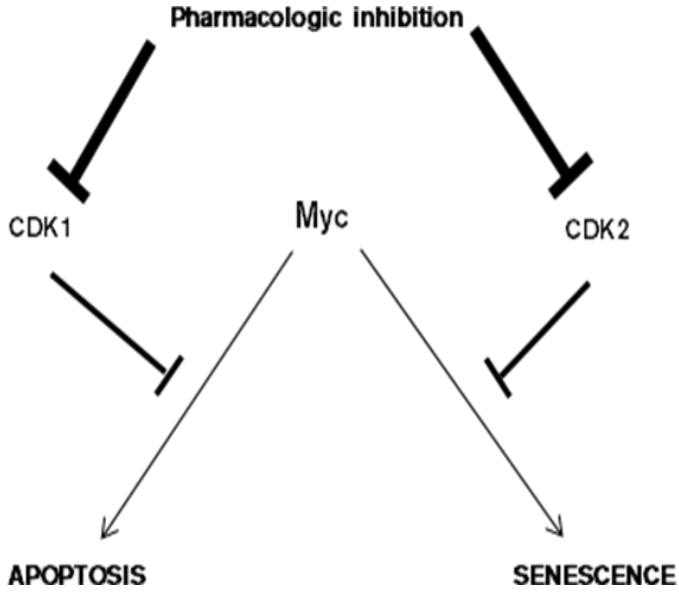

Figure 8. Reactivating intrinsic tumor suppressive pathways induced by Myc via inhibition of Cdk1 and/or Cdk2. Depending on the type of Myc-driven tumor, single or combined Cdk-inhibition may differ in efficiency.

3.12 Pharmacological inhibition of Cdk2 triggers Myc-induced cellular senescence (paper III)

The relevance of Cdk2 as a therapeutic target was questioned after the finding that Cdk2 is dispensable for cell cycle progression (Berthet *et al.*, 2003) and for growth of certain cancer cells (Tetsu & McCormick, 2003).

By using two specific Cdk2-inhibitors we show that Myc-expressing but not control MEFs and REFs are forced into cellular senescence after Cdk2-inhibition. Further, Myc-transformed human U937-cells also display a senescent phenotype after Cdk2-inhibition in contrast to the parental line. In Myc-expressing MEFs, the senescent phenotype was demonstrated by up-regulation of p21Cip1 and p16Ink4a in addition to increased SA-β-Gal staining. In Myc-expressing U937 cells, a marked increase of p16Ink4a as well as increased SA-β-Gal staining was observed after Cdk2-inhibition.

Some of the experiments were performed in both 3% and 20% oxygen to rule out that senescence entry was simply due to a culture shock when growing cells in hyper-oxygen conditions (Parrinello *et al.*, 2003). The reduced proliferation observed in response to the pharmacological Cdk2 inhibitors was not a result of increased apoptosis, as determined by analysis of the fraction of cells in sub-G1 phase, indicating that these inhibitors did not target Cdk1 to any greater extent.

3.13 Myc plays a dual function in regulation of senescence (paper II-III)

From the results in paper II and III, it seems that Myc plays a dual role in the regulation of cellular senescence, apparently able both to repress and induce senescence depending on the context.

How is this possible? Myc is known to be a strong inducer of apoptosis via the Arf/p53 pathway (Zindy *et al.*, 1998). Apoptosis and senescence are both regulated by p53 via the p16 and p21 pathways (Schmitt, 2007). This tight switch between apoptosis and senescence may explain why Myc under certain conditions also induces senescence. This is probably linked to Myc's ability to cause DNA damage as a result of replication stress and generation of reactive oxygen species (ROS) (Vafa *et al.*, 2002).

Our results suggest that Myc suppresses Ras-induced senescence by regulating genes involved in the senescence process, such as *Bmi-1*, *p16*, *p21*, *hTert,* and *cyclin D2* (paper II). The p16 and p21 pathways are known to be activated during Ras-induced senescence (Serrano *et al.*, 1997). As we demonstrate in paper III, these pathways are also required for Myc-induced senescence. An important finding from both paper II and III is that Cdk2 function is required for both the inhibition of Myc-induced senescence and for the Myc-mediated inhibition of Ras-induced senescence. Interestingly, depletion of Cdk2 affects Myc- but not Ras-induced senescence, although both oncogenes seem to promote senescence through the same pathways. One possible interpretation of these results is that Myc acts as a general repressor of oncogene-induced senescence, including senescence generated by Myc itself, by engaging Cdk2 as a cofactor in the control of genes involved in this pathway.

Another important finding from paper II is that inhibition of Ras-induced senescence by Myc is dependent on phosphorylation of Ser-62 by Cdk2. However, while Cdk2 is also required to suppress Myc-induced senescence, it is still unclear whether this is connected to Myc Ser-62

phosphorylation by Cdk2 or not, or whether other substrates of Cdk2 mediate this function.

These unique functions of Cdk2 as a cofactor for Myc-repressed senescence and suppressor of Myc-induced senescence presented in paper II and III, respectively, highlight the importance of Cdk2 as a potent target in cancer therapy. Our findings may have potential for therapeutic intervention in both Myc and Ras-dependent tumors and argues for a re-evaluation of Cdk2 as a therapeutic target. If combined with Cdk1-inhibitors (Goga *et al.*, 2007), such therapy may be even more efficient reactivating both Ras-induced senescence and Myc-induced senescence/apoptosis.

3.14 c-Myc induces and interacts with the SIRT1 protein deacetylase (paper IV)

The silent information regulator 1-gene, *SIRT1*, encodes a NAD^+-dependent protein deacetylase belonging to the sirtuin family. Mammalian SIRT1 exerts its cellular functions by deacetylating histones and several transcription factors, including p53, thereby affecting survival of mammalian cells. SIRT1 also plays a role in regulation of metabolic pathways by promoting mitochondrial biogenesis and oxidative phosphorylation. c-Myc is known to induce expression of genes involved in both glycolysis, for instance lactate dehydrogenase A (*LDH-A*), mitochondrial biogenesis and oxidative phosphorylation (Morrish *et al.*, 2008; Zhang *et al.*, 2007), suggesting that SIRT1 and Myc activities may be connected. In addition, high expression of LDH-A may potentially affect the NAD^+/NADH ratio resulting in increased SIRT1 activity. This together with reports demonstrating that SIRT1 is up-regulated in several human tumors (Saunders & Verdin, 2007), suggested a possible cooperation between SIRT1 and c-Myc.

By utilizing MycER induction in serum-deprived cells we found that Myc induced SIRT1 expression. Further, upon c-Myc depletion by siRNA, SIRT1 protein expression decreased. Analysis of primary human colon cancer displayed increased levels of both proteins. The increase in SIRT1 protein levels after c-Myc activation was regulated post-transcriptionally since SIRT1 mRNA was not affected by the treatments. Using *c-myc* deficient RAT1 MycER cells, c-Myc activation led to a four-fold increase in the NAD^+/NADH ratios, likely mediating the activation of the SIRT1

protein. This is supported by a recent report showing that elevated activity of SIRT1 through NAD^+ coincides with higher protein levels (van der Veer *et al.*, 2007).

SIRT1 activity was recently shown to be inhibited by deleted in breast cancer 1 (DBC1) (Kim *et al.*, 2008; Zhao *et al.*, 2008), which also interacts with c-Myc (Koch *et al.*, 2007). We detected binding between endogenous SIRT1 and c-Myc in U2OS cells and the interaction was also detected *in vitro* using recombinant c-Myc, indicating that c-Myc and SIRT1 may interact directly. Further, c-Myc was shown to compete with DBC1 for binding to SIRT1, suggesting that c-Myc also increases SIRT1 activity by competing off DBC1. This may be of specific importance in tumors with elevated Myc levels where Myc promotes apoptosis.

3.15 SIRT1 protects against c-Myc induced apoptosis and senescence (paper IV)

Due to its ability to deacetylate p53, SIRT1 has been implicated in protecting cells against apoptosis. Therefore, it was relevant to check if SIRT1 also modulated c-Myc induced apoptosis. By using nicotinamide (NAM), a physiological SIRT1-inhibitor, we detected increased apoptosis after c-Myc activation. The same effect was observed when using a synthetic inhibitor of SIRT1 and 2, sirtinol. Further, using the U937 monoblast system, we observed inhibition of proliferation as well as a dramatic increase of apoptosis. This was seen in v-*myc* expressing cells after both sirtinol treatment and treatment with a SIRT1-specific inhibitor, EX527, but not in parental cells. Increase apoptosis was observed after sirtinol treatment also in c-Myc immortalized human diploid fibroblasts (HDFs), while primary HDFs or hTERT-immortalized HDFs were unaffected. The same effect was achieved when utilizing siRNA mediated knockdown of SIRT1 in the HDFs.

In addition to increased apoptosis, c-Myc immortalized HDFs also underwent cellular senescence after SIRT1 knock down, as visualized by increased cell size and an up-regulation of SA-β-Gal activity. These results suggest that SIRT1 suppresses apoptosis and cellular senescence in response to Myc, thereby contributing to c-Myc-induced immortalization and transformation.

The tumor-promoting effect of SIRT1 observed here conflicts with reports that SIRT1 functions as a tumor-suppressor in certain settings (for review see (Saunders & Verdin, 2007)). Clearly, SIRT1 seems to exert multiple roles depending on the cellular conditions or possibly SIRT1-levels. For instance, ectopic expression of SIRT1 reduced tumor formation in a colon cancer mouse model (Firestein *et al.*, 2008).

In summary, we propose that endogenous SIRT1 acts as a tumor promoter in c-Myc driven neoplasia, mainly due to its functions to repress both apoptosis and cellular senescence, two major barriers for oncogenic transformation.

3.16 SIRT1 feeds back to c-Myc (paper IV)

SIRT1 is known to deacetylate several nuclear transcription factors which raise the question if SIRT1 could also deacetylate c-Myc. After *in vitro* acetylation of c-Myc by CBP, we showed that recombinant SIRT1 deacetylated c-Myc. This was further demonstrated to be dependent on NAD^+ and blocked by nicotinamide. The next step was to examine whether deacetylation regulates c-Myc activity and stability. It has previously been shown that acetylation may regulate ubiquitination and degradation of c-Myc (Vervoorts *et al.*, 2003).

When depleting SIRT1 by shRNA or microRNA, the half-life of c-Myc decreased. Treatment with a SIRT1/SIRT2 inhibitor, tenovin-6 (Lain *et al.*, 2008), gave the same results as SIRT1 knockdown. These results are consistent with the higher Myc-abundance in *SIRT1+/+* MEFs compared to *SIRT1-/-* MEFs. Unexpectedly, we observed reduced poly-ubiquitination of Myc in several cell-lines with down-regulated SIRT1. This may indicate that SIRT1 promotes conjugation of non-K48 linked ubiquitin chains, such as K63-linked chains that will not support degradation. Expressing a K48R ubiquitin mutant, we observed that poly-ubiquitination still decreased upon SIRT1 downregulation, supporting this view. Indeed, using a K63-specific antibody we observed decreased amounts of K63-ubiquitin chains after SIRT1 knockdown. K63-ubiquitination has been linked to higher transcriptional activation by c-Myc (Adhikary *et al.*, 2005). This was indicated also in our systems since co-expression of SIRT1 increased c-Myc dependent activation of a reporter-construct 20-fold.

The SIRT1 mediated deacetylation of c-Myc likely makes target lysines available for K63-linked ubiquitylation. This may in turn lead to recruitment of cofactors, like p300, as shown for c-Myc previously (Adhikary *et al.*, 2005). This could explain the increased transcriptional activity demonstrated in this work as well the increased c-Myc stability. Since K63-chains do not promote proteasomal degradation, they may instead increase the stability of c-Myc by occupying lysine residues otherwise available for K48-chains.

These results demonstrate that SIRT1 deacetylates c-Myc, leading to increased c-Myc stability (shown by decreased half-life of c-Myc after SIRT1 depletion) and an increase in c-Myc dependent transcriptional activation. In contrast, we observed increased turnover of c-Myc when ectopically over-expressing SIRT1 (data not shown). This suggests that SIRT1 may have different impact on Myc turnover and possibly tumorigenesis depending on its expression level. This may also explain why SIRT1 has been reported to act both as a tumor promoter and tumor suppressor as mentioned above.

In summary, our results suggest that c-Myc and SIRT1 constitutes a positive feedback loop promoting tumor progression. Therefore, SIRT1 targeting may have therapeutic potential in tumors with deregulated Myc.

3.17 General discussion (paper I-IV)

Many tumors seem to harbor intrinsic tumour-suppressive mechanisms that exist latently but are kept in check by oncogenic proteins. Such anti-tumor activities can be reactivated if targeting the oncoprotein responsible for such suppression. Two major safeguard mechanisms against tumorigenesis are apoptosis and cellular senescence (Lowe *et al.*, 2004). Several reports during the last years from mouse models have demonstrated that turning off or targeting Myc lead to regression of Myc-driven tumors. This can for instance be due to apoptosis or terminal differentiation, but recently cellular senescence was shown to be one frequent mechanism of tumor regression in such tumors (Wu *et al.*, 2007). Further, depletion of one copy of *myc* induced cellular senescence in human fibroblasts (Guney *et al.*, 2006). In a recent report, inhibition of Myc by a dominant-negative construct also led to regressed Ras-driven tumors, with just a small impact on normal tissues

(Soucek *et al.*, 2008). These reports strengthen the suitability of Myc as a therapeutic target.

Here, we provide evidence that regulating specific cofactors or endogenous antagonists of Myc may be important for combating Myc-dependent tumors. TGF-β treatment forced cells with deregulated Myc into cellular senescence despite continuous Myc-expression. This correlated with induced expression of Mad1 and increased association of Mad1/Max complexes at Myc target promoters as well as induced p27. The mechanism by which TGF-β overrides Myc to induce cellular senescence show similarities with that of IFN-γ presented in paper II. Post-transcriptional regulation of Myc by anti-mitogenic signaling may therefore be of potential therapeutic interest in cells with constitutively expressed Myc, or cells that escaped apoptotic pathways. The results indicate that there may be cooperation between TGF-β-induced p27Kip1 and Mad1 in inhibition of Myc-dependent transcription in agreement with a previous report (McArthur *et al.*, 2002).

Paper II and III present a novel function of Cdk2 as a positive cofactor/collaboration partner for Myc in tumorigenesis, and highlight Cdk2-inhibition as a potential mechanism combating certain tumors. The outcome of targeting Cdk2 in tumors has shown both promising and discouraging results. Certain cancer cell lines were shown to proliferate despite Cdk2 inhibition, thereby questioning the relevance of Cdk2 as a therapeutic target. Later, Cdk2 was shown to play a role in melanoma (Du *et al.*, 2004; Tetsu & McCormick, 2003). Also, we cannot rule out that the relevance of Cdk2 in Myc-dependent tumors may depend upon the tumor-type (Macias *et al.*, 2007). Therefore, our results warrant more studies of Cdk2-inhibition in different mice models. To start addressing this, we are setting up Myc-induced leukemia mice models at the moment to investigate the effect of both our Myc-mutants as well as our specific Cdk2-inhibitors. In order to exclude apoptosis, this will be performed in the background of Bcl-2 or Bcl-x_L expression, to ensure a specific effect of Cdk2 in mediating repression of senescence when targeting tumors *in vivo*.

In paper IV, we present a novel interplay between c-Myc and SIRT1 necessary for both increased SIRT1 expression and c-Myc activation. Due to its function as a deacetylase of p53, SIRT1 may be a potent collaborator with Myc in tumorigenesis. By acetylating and inactivating p53, SIRT1 may abrogate both apoptosis and cellular senescence. This was also demonstrated in cells with deregulated Myc, where SIRT1 inhibition by either pharmacological compounds or knockdown increased both apoptosis and

cellular senescence. Further, HDFs immortalized for hTERT, and with MycER expression underwent apoptosis after SIRT1-inhibition even in the presence of serum. This puts SIRT1 as a novel and potential target in tumors with deregulated Myc and warrant studies of SIRT1 in murine Myc-driven tumors.

In summary, the results in this thesis demonstrate that cells with deregulated Myc may be targeted in several ways by hitting specific Myc cofactors of importance for Myc function. This might uncover safe-guard mechanisms against tumorigenesis induced or repressed by Myc, resulting in a more severe impact of inhibiting Myc function in cells expressing deregulated Myc compared to normal cells. Our work underscores the importance of not only targeting Myc itself for cancer treatment, but also further exploring therapeutic principles based on targeting cofactor or collaboration partners essential for Myc function.

4 Conclusions

- TGF-β induces senescence in Myc-transformed cells preceded by a shift from Myc/Max to Mad1/Max complexes at Myc target promoters
- TGF-β represses Myc-driven transcription and synergizes with Mad1 to induce growth arrest in Myc-transformed cells
- Myc represses Ras-induced senescence dependent on Serine 62 and mediated by cyclin E/Cdk2
- Cyclin E/Cdk2 functions as a Myc cofactor necessary for regulation of genes involved in cellular senescence
- Pharmacological inhibitors of Cdk2 and IFN-γ treatment via p27Kip1 forces Myc-transformed cells into cellular senescence
- Myc induces senescence in Cdk2 knockout cells
- Cdk2 depletion delays Myc-dependent lymphoma onset *in vivo*.
- c-Myc induces SIRT1 post-transcriptionally
- c-Myc activates SIRT1 expression and binds SIRT1 in competition with the SIRT1-inhibitor DBC1
- SIRT1 protects cells with deregulated Myc from apoptosis and cellular senescence
- SIRT1 feeds back and stabilizes c-Myc constituting a positive feedback loop

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