Control of growth and differentiation of human neuronal and hematopoietic tumour cells via Myc/Max/Mad-network proteins

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To my family with all my love

and

In memory of Emel

Bi hevîya aştî û azadî yê

Abstract

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The Myc/Max/Mad transcription factor network regulates a large number of genes involved in cell growth, differentiation and apoptosis. Myc activates and Mad represses and overlapping set of growth-promoting genes, and the network therefore seems to act as a molecular switch between growth and differentiation. Deregulation of *myc*–family genes occurs frequently in human cancers and is often associated with poor prognosis.

One aim of this thesis was to identify negative growth signals affecting the expression and/or activity of N-Myc- and Mad-family in neuroblastomas. Our results show that Mad1 was upregulated during phorbol ester (TPA)-induced neuronal differentiation of SH-SY5Y human neuroblastoma cells lacking N-myc amplification, whereas this response was largely inhibited in N-*myc*-amplified neuroblastoma cells. However, interferon-γ (IFN-γ) in combination with retinoic acid (RA) or TPA strongly enhanced differentiation and growth arrest in the latter. This was accompanied by reduced N-Myc and/or upregulated Mad1 expression, resulting in changed occupancy at the ODC target gene promoter in vivo towards Mad1/Max predominance, and repression of N-Myc/Mad1 target genes. This suggests that combined RA+IFN-γ "differentiation therapy" may potentially be of clinical interest in neuroblastoma treatment.

Addressing the role of Mad1 during hematopoietic differentiation, we show that enforced Mad1 expression in U-937 monoblasts by an inducible expression system led to inhibited cell growth and proliferation. However, Mad1 did not induce or enhance differentiation in the absence or presence of differentiation signals. Surprisingly, Mad1 stimulated TNF-α-, but not Fas-induced apoptosis in U-937 cells.

Finally, addressing the still unclear mechanism by which c-Myc represses transcription, our results showed that differentiation-induced transcription of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ gene was repressed by c-Myc through interaction with the Zn-finger protein Miz-1 in vivo at the p21 core promoter. c-Myc seems to switch Miz-1 function from an activator to a repressor, thereby inhibiting cell cycle exit and differentiation.

Keywords: Myc, Mad1, Miz-1, $p21^{Cip1}$, Interferon-γ, retinoic acid, phorbol ester, differentiation, neuroblastoma, hematopoiesis.

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This thesis is based on the following papers which will be referred to as their Roman numerals:

I Guzhova, I., Hultquist, A., Cetinkaya, C., Nilsson, K., Påhlman, S & Larsson, L.G. Interferon-γ cooperates with retinoic acid and phorbol ester to induce differentiation and growth inhibition in human neuroblastoma cells. Int. J. Cancer 94, 97-108, 2001.

II Cetinkaya, C., Hultquist, A., Wu, S., Guzhova, I., Påhlman, S. & Larsson, L.G. IFN-γ-stimulated terminal neuronal differentiation and growth arrest of N-*myc* amplified neuroblastoma cells involves repression of N-Myc target genes via upregulated Mad1. Manuscript.

III Hultquist, A., Cetinkaya C., Wu, S., Castell, A., Erlandsson, A. & Larsson, L.-G. Mad1 inhibits cell growth and proliferation but does not promote differentiation or overall survival in human U-937 monoblasts. Mol. Cancer Res. 2, 464-476, 2004.

IV Wu S, Cetinkaya C, Munoz MJ, von der Lehr N, Bahram F, Beuger V, Eilers M, Leon J and Larsson LG. Myc represses differentiation-induced p21Cip1 expression via Miz-1-dependent interaction with the p21 core promoter. Oncogene 22, 351-360, 2003

Abbreviations

bHLHZip	basic/helix-loop-helix/leucine zipper
Cdk	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CKI	cyclin dependent kinase inhibitor
CoIP	coimmunoprecipitation
CTD	carboxy terminal domain
GTF	General transcription factors
HDAC	histone deacetylase
HAT	histone acetyltransferase
IFNγ	interferon-gamma
Inr	initiator
MB	Myc box
MIZ-1	Myc interacting zinc-finger protein
NB	Neuroblastoma
ODC	ornithine decarboxylase
PKC	protein kinase C
pRb	Retinoblastoma protein
RA	retinoic acid
SID	Sin3 interaction domain
TAD	transactivation domain
TAF	TBP associated factor
TBP	TATA binding protein
TERT	Telomerase reverse transcriptase
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRRAP	Transformation/Transcription domain-associated protein
VITD3	Vitamin D3

Background

Development of Cancer

Cancer is a genetic disease that evolves through multiple genetic alterations (for review see (Hanahan and Weinberg, 2000)). Extensive research during last decades has broadened our knowledge of molecular mechanisms involved in the process of tumour formation. But a complete picture of the cancer development is still missing.

Alterations in three types of gene families are believed to be responsible for the generation of cancer: proto-oncogenes, tumour-suppressor genes and stability genes (for review see (Vogelstein and Kinzler, 2004)). Proto-oncogenes, which normally function to promote cell growth or survival, can contribute to tumour formation when mutated or expressed at abnormal high levels. Tumour suppressor genes, on the other hand are genes whose functional absence can lead to cancer. Carcinogenic mutations in such genes may result in inactive gene or gene product, which are then unable to inhibit cell growth. The stability genes (or caretakers), is a class of genes that include genes responsible for DNA repair. When stability genes are inactivated, mutations in other genes occur at a higher rate and genetic changes can not be kept to a minimum.

A single mutation is not sufficient to transform a normal mammalian cell to a cancer cell since there are safeguard mechanisms existing. The ability to transform differs fundamentally between cells of different mammalian species. For example, cultured rodent cells require at least two genetic alterations while human cells require at least three (Hahn et al., 1999). But which genetic changes are needed for a normal cell to become a malignant cell? A cell possesses multiple control mechanisms. Circumvention or disruption of these control systems, leading to growth advantages, is always involved in tumour development. These genetic changes include self-sufficiency in growth signals, insensitivity to antigrowth signals, block of differentiation, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis and genomic instability (Hanahan and Weinberg, 2000). These processes will be described briefly below (summarised in figure 1).

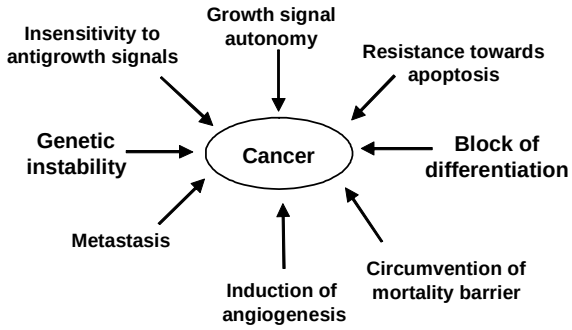

Figure 1: Outline of cellular processes affected by genetic alterations in cancer cells

Normal cells need mitogenic signals to proliferate, signals that are usually supplied by neighbouring tissues. Transmission of these signals into the cell occurs through signalling molecules that bind to specific transmembrane receptors. Diffusible growth factors, extracellular matrix molecules, and cell-to cell adhesion/interaction molecules are the signalling molecules. In contrast to normal cells, tumour cells grow in absence of mitogenic signals. Many of the oncogenes involved in cancer mimic normal growth signalling and thereby reduce their dependence on stimulation from their microenvironment. Independence of mitogenic signals can be achieved in different ways. One way is the autocrine stimulation there cancer cells produce their own growth factors. Mutations that activate transmembrane receptors leading to signalling in the absence of ligand or making the receptors hypersensitive, is another way (Slamon et al., 1987). A possible mechanism is also the ability of cancer cells to switch the types of extracellular matrix receptors (integrins) they express, a switch that favours receptors that transmit progrowth signals (Giancotti and Ruoslahti, 1999, Lukashev and Werb, 1998). Both growth factors and matrix components can activate SOS-Ras-Raf_MAP kinase pathways (Aplin et al., 1998, Giancotti and Ruoslahti, 1999). A more complicated mechanism of acquired growth signal autonomy derives from changes in components of the downstream circuitry that receives signals from ligand-activated growth receptors and integrins. The SOS-Ras-Raf_MAP kinase pathway plays a central and important role here. In many cancers, approximately in 25% of all human cancers, Ras proteins are altered structurally (Medema and Bos, 1993) and activated Ras contributes to several aspects of malignant phenotype (Shields et al., 2000). Tumors can also influence adjacent cells, like fibroblasts or immune cells, to produce growth signals.

In a normal cell, multiple antiproliferative signals operate together to limit cell proliferation and maintain tissue homeostatis. These signals can block proliferation either by forcing cells out of the active proliferative cycle into the quiescent (G_0) state or by inducing cells into a postmitotic state, usually differentiation. Insensitivity to such antigrowth signals is a common genetic alteration for cancer cells. Examples of such antigrowth signals are the cytokines interferons (IFN-α, -β and -γ) and transforming growth factor- β (TGF-β). IFNs comprise a family of secreted proteins that bind to specific receptors on the surface of target cells, and they induce activation of gene transcription that negatively controls cell growth. IFNs signal through the Jak-Stat pathway, involving activation of members of the Janus family of kinases (JAKs) and the signal transducers and activators of transcription (STATs), (Brivanlou and Darnell, 2002). TGF-β blocks progression through G1-phase of the cell cycle. Components of the TGF-β signalling pathway, such as Smads (Smad2, Smad4), are frequently mutated in several forms of cancer, (for review see, (Shi and Massague, 2003, ten Dijke and Hill, 2004).
Many antiproliferative signals are associated with the cell cycle clock, specifically the components involved in G1 phase transition (for a recent review see (Massague, 2004)). Many, if not all, antigrowth signals are canalized through the tumour suppressor protein and cell cycle regulatory factor retinoblastoma (pRb). Most human tumours have mutations that deregulate or inactivate the function of pRb or other proteins involved in the regulation of Rb activity (Sherr, 2004).

Another tumour suppressor gene whose inactivation is implicated in a high percentage of human cancer is *p53*. In response to various types of stress, p53 becomes activated. As a consequence of this activation, cells can undergo increased DNA repair, senescence and apoptosis (Sherr, 2004).

Cell proliferation depends on more than avoidance of antiproliferative signals. Normal tissues also restrict cell proliferation by instructing cells to enter differentiated states by diverse mechanisms. Tumour cells use various strategies to avoid normal differentiation (for review see (Tenen, 2003)). Block of cellular differentiation is particularly evident among hematopoietic tumours. One strategy to avoid differentiation involves disruption of specific transcription factors that have crucial roles in the differentiation process. Translocations and deregulation of these transcription factors are seen, for instance, in leukaemia. Additionally, overexpression of many oncogenes can also block differentiation. For example, the c-Myc oncoprotein can induce a shift from differentiation to proliferation when overexpressed, (for review see (Oster et al., 2002))

Another safe-guarding mechanism that normal cells have and that tumour cells need to block is apoptosis, the programmed cell death. An imbalance in normal cells, such as DNA damage, overexpression of oncogene, hypoxia or absence of survival factors, can trigger apoptosis. Many tumours suffer massive apoptotic cell death at early stages of the development due to these reasons. Therefore it is crucial for tumour cells to develop resistance against apoptosis (for recent review see (Lowe et al., 2004)). The most frequent strategy is the loss of pro-apoptotic tumour suppressor gene p53. Enhancement of survival molecules is another possible mechanism. Apoptosis, which is an energy dependent process, is performed and regulated by a large group of molecules, including surface receptors (such as the death receptor Fas), mitochondria associated molecules of the bcl-2 family, and caspases. All these molecules can be targeted when cancer cells develop strategies to avoid apoptosis. The Bcl-2 family proteins exhibit both pro-apoptotic (Bcl-2, Bcl-xl and Bcl-x) and anti-apoptotic (Bax, Bak, Bid and Bin) characteristics. Overexpression of Bcl-2 occurs in many tumours (e.g B-cell lymphoma, B-cell leukemia) and leads to inhibition of apoptosis. Upregulation of Bax by p53 activation, on the other hand, can elicit apoptosis. Caspases, that are a group of cysteine proteases and one of the main effectors of apoptosis, can be activated by death receptors such as Fas, (so called extrinsic pathway) or directly by cytocrome C release from mitochondria (so called intrinsic pathway). These proteins initiate and amplify various death signals and breakdown or cleave key cellular substrates that are required for normal cellular function, (Igney and Krammer, 2002, Okada and Mak, 2004). Despite the key role of caspases in programmed cell death, caspase mutations have been identified at low frequency in some tumors (e.g. colorectal cancer or head and neck carcinoma). Instead, caspases are frequently inactivated by epigenetic mechanisms (Hopkins-Donaldson et al., 2003).

Maintaining the stability of the genome is critical to normal cell growth and development. DNA monitoring systems, check points (especially in mitosis), and repair enzymes ensure that mutations are rare. Even if mutation of a specific gene is not enough to cause cancer, cancers do yet appear. Genomes of tumour cells

must acquire increased mutation rates for tumours to progress (Loeb, 1991). Malfunction of the existing ¨caretaker¨ systems is believed to be involved in this increased mutability. Genetic alterations accumulated in tumour cells that have impact on genetic instability can include (Lengauer et al., 1998): chromosome losses, gains and translocations, such as 8:14 translocation in Burkitt's lymphomas involving c-myc oncogene, amplification of certain genes, e.g *myc* gene amplification in neuroblastomas (Oster et al., 2002), inactivation of DNA mismatch repair genes, such as *msh2* or *mlhl* (Cahill et al., 1999), loss of normal mitotic checkpoint pathways (Cahill et al., 1998) and disabled p53 pathways (Zhivotovsky and Kroemer, 2004).

Normal mammalian cells have limited replicative capacity (Hayflick, 1997), that enforce them stop dividing after a certain number of divisions (approximately 50) and enter a specific quiescent state called senescence. This is due to shortening of the telomeres, the repetitive DNA-sequences and associated proteins protecting the ends of chromosomes. Telomerase, the enzyme maintaining the length of telomeres, is upregulated in malignant cells which add telomeres to the end of chromosomes after each division and makes cells avoid senescence. Most cancer cells maintain their telomere length by high expression of telomerase (85%–90% of cases), but there is also another mechanism, called alternative lengthening of telomeres (ALT) existing (Cech, 2004).

Both normal and tumour cells require oxygen and nutrients to survive and grow. Cells can only survive within a certain distance from a capillary. Normally, cells do not have the ability to initiate and induce blood vessel formation, a process called angiogenesis. Therefore a tumour cell has to develop angiogenic abilities in order for tumour to grow and expand (Bouck et al., 1996, Hanahan and Folkman, 1996). Activation of angiogenic inducers, such as vascular endothelial growth factor (VEGF) is common in many tumours (Ferrara, 2002).

Another hallmark of cancer is the ability of tumour cells to expand to neighbouring tissues or invade distant organs, a process named metastasis. Majority (90%) of human cancer deaths are caused by metastasis (Sporn, 1996).The mechanism underlying invasion and metastasis is complex and poorly understood. Proteins that regulate cell-cell and cell-matrix interactions seem to play a central role. For instance, the expression pattern of integrin can be switched and the function of E-cadherin is lost in many invasive epithelial cancers (Cavallaro and Christofori, 2004). Downregulation or removal of proteins acting as suppressor of invasion and metastasis are also among observed processes (Hanahan and Weinberg, 2000).

The Eukaryotic Cell Cycle

The most fundamental property of a living organism is its ability to reproduce itself in order to grow and develop. This property is based upon the aspect that cells are able to duplicate by a highly ordered process known as the cell cycle. The cell cycle traditionally is divided into four phases, namely gap 1 (G1), DNA-synthesis (S), gap 2 and mitosis (M), (see fig 2) (Johnson and Walker, 1999).

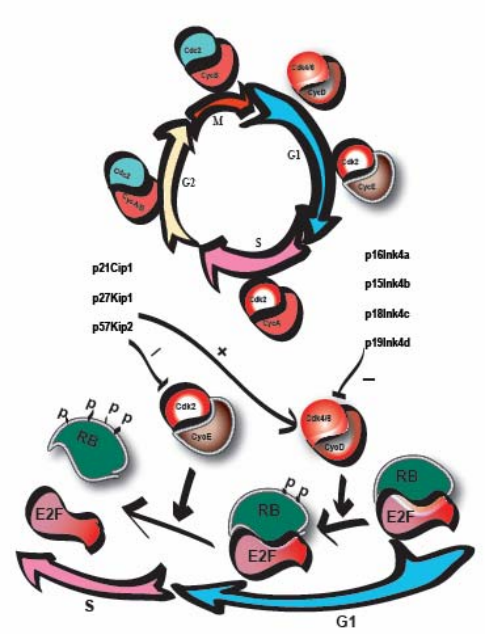

Fig.2: Overview of the mammalian cell cycle with emphasis on G1/S transition

During G1 phase, cells grow in size, prepare for the process of DNA replication and are receptive to extracellular signals, which may trigger a commitment to entering the next phase of the cell cycle or exiting it. It has been shown, both in yeast and mammalian cells, that there are several points in the cell cycle called checkpoints, at which the cell cycle can be arrested if previous events have not been completed. Late in G1, at the G1 checkpoint, cells become committed to DNA replication and entering the next phase. Previous to the checkpoint, a cell will stop growing if soluble growth factors are removed from the surrounding fluid. However, subsequent the checkpoint cells will continue to progress through the remainder of the cell cycle regardless of presence of growth factors. DNA synthesis occurs in S phase while in G2 the cell prepares for the chromosome segregation and cell division. The checkpoint in G2 ensures that DNA is completely replicated, the cell is big enough and the environment is favourable. At this stage, mitosis is prevented until the cell complete all of the steps required for division. For instance, if DNA is damaged DNA repair system can be activated during this time of period. During M phase the cell physically divides into two separate daughter cells and an additional checkpoint here makes sure that the chromosomes are attached correctly to the spindle. After going through mitosis, a normal cell that re-enters G1 can either start cycling again or exit the cell cycle and enter a resting state called G0. Cells may stay in this quiescent state for days, weeks, or years, until they get signals indicating that it is time to divide again (Johnson and Walker, 1999).

Cell cycle progression is regulated by two families of proteins, the cyclin dependent kinases (Cdk:s) and cyclins. In higher eukaryotes nine Cdks and at least 16 cyclins have been identified (Johnson and Walker, 1999). Cdks are a family of

serine/threonine kinases that control phosphorylation of several substrates, including the tumour suppressor protein Rb (pRb). Association with a cyclin at specific regulatory subunit called the cyclin box is required for the Cdk to be active. All cyclins contain this common homologous region. For full Cdk activation phosphorylation of a conserved Cdk threonine residue and dephosphorylations on some tyrosine residues are also needed. This phosphorylation is carried out by a Cdk-activating kinase (CAK) and the phosphate is removed by the Cdk-associated phosphatase (cdc25). Each cell cycle phase has its own distinct cyclin-Cdk combination. For instance in mammalians, cyclins D and E in complex with Cdk 4/6 and Cdk2 are active during G1 whereas cyclins A and B in association with Cdk1 in G2/M phases (Arellano and Moreno, 1997, Nigg, 1995).

One of the most important tasks for cyclin-Cdk complexes in G1 is ensuring G1/S transition by inactivating the retinoblastoma protein (pRb). The active unphosphorylated form of pRb arrests cells at the check point in late G1 phase of the cell cycle. This function of pRb, at least partly, depends on interactions with E2F family transcription factors (Harbour and Dean, 2000). It is suggested that the ability of pRb to arrest cells in G1 is mainly due to repression of E2F target genes important for cell cycle entry. The repression by Rb/E2F complex is partly dependent on the requirement of histone deacetylases (HDACs) (Magnaghi-Jaulin et al., 2000). Phoshorylation of Rb by Cyclin-Cdk complexes disrupt the interaction between Rb and E2F and transforms E2F from a repressor to an activator that allows transcription of E2F target genes necessary for completion of the cell cycle.

The first cyclin-Cdk complex, that is induced in response to mitogenic signals and activated during the G1-phase, is composed of a D-type cyclin (CycD1, D2 and D3) in association with Cdk4 or Cdk6 depending on cell type (Sherr, 1995). The cyclin D dependent kinases phoshorylate pRb and thereafter the cyclinE/Cdk2 complexes become active and complete the phoshorylation. E2F is released from pRb which in turn allows activation of the transcription of E2F target genes involved in DNA replication (Sherr and Roberts, 1999). Cyclin E and A, that are necessary for the G1/S transition are among these genes. CyclinE/Cdk2 is involved in maintaining pRb in the hyperphosphorylated state and as a consequence participates in a positive feedback loop. An additional function of CyclinD/Cdk4 complexes is to sequester Cdk inbitors (CKI) p21 and p27. By doing this CyclinD/Cdk2 makes it possible for CylinE/Cdk2 to reduce restriction by CKIs and facilitate its activation. When cells enter S phase, cyclin E is degraded and Cdk2 then associates with cyclin A. Cyclin A associated kinase activity (Cdk2 and in late S phase Cdk1) is necessary for entry into S phase, completion of S phase and entry into M phase. Some members of E2F are negatively regulated by cyclin A creating a negative feedback loop. Eventually, cyclin A and the B-type cyclins associate with Cdk1 to promote entry into mitosis. To exit mitosis, cyclin B destruction is required.

There are several different ways to regulate Cdks. Beside the positive regulation by cyclins, and multiple phosphorylations and dephosphorylations, Cdks are regulated by Cdk inhibitors (CKIs). The CKIs are divided into two protein

families based on their structure and Cdk targets. The first family, consisting of $p16^{INK4A}$, $p15^{INKB}$, $p18^{INK4C}$ and $p19^{INK4D}$, is called INK4 family since they target the catalytic subunit of Cdk4 and Cdk6 and therefore, is specific only for early G1 phase. The second family, named the Cip/Kip family, consist of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and inhibit most Cdks and is not specific for a particular phase. Cip/Kip proteins can both interact with cyclin and Cdk in the complex while INK4 proteins only bind to Cdk.
The INK4 family proteins prevent association with D-type Cyclins by inhibiting Cdk4 and Cdk6. The Cip/Kip family members, on the other hand, interfere with the activities of cyclin E-, and A-dependent kinases. The Cip/Kip proteins are potent inhibitors of cyclin E- and A-dependent Cdk2, but positive regulators of cyclin D-dependent kinases Cdk4 and Cdk6 (Sherr and Roberts, 1999).

p21, which is particularly relevant to this thesis, is a potent inhibitor of Cdk2 activity and its levels normally increase in early G1 to prevent further cell cycle progression. Increased p21 levels can also occur because of transcriptional activation caused by the p53 transcription factor, as a response to DNA damage. In addition to Cdks, p21 can also interact with PCNA that is an elongation factor for DNA polymerase δ, as well as a component of the DNA repair machinery. This binding inhibits the ability of PCNA to function in DNA replication but not DNA repair (Li et al., 1994). As cyclin D-cdk4/6 increases, it titrate the level of p21 by sequestering it as part of a complex containing PCNA. Later, this titration allows CyclinE/Cdk2 to become activated, which is required for cell cycle progression through G1/S.

Numerous growth inhibitory signals such as IFNs and TGF-β, upregulate members of both CKI families. Upregulation of the INK4 proteins resulting in inactivation of Cdk4/6 leads to disruption of CyclinD/Cdk/Cip/Kip complexes. Cip/Kip inhibitors are released from the complex which in turn can bind to and inhibit CyclinE/Cdk2 complexes leading to cell cycle arrest in G1/G0.

The cell cycle regulatory factors are responsible for key events in the cell cycle progress. Any failure of the regulatory system can result in cancer (for review see (Kastan and Bartek, 2004, Massague, 2004)).

Apoptosis

Apoptosis, or programmed cell death, is a naturally occurring process in the body that is necessary during development, for the immune defence and for homeostatis.
Apoptosis is induced by a variety of death signals, both extrinsic and intrinsic, including withdrawal of growth factors, damages caused by chemical compounds, irradiation or endogenous molecules such as oxidants. Morphologically, apoptosis is characterized by chromatin condensation, cell shrinkage (in early stage), nucleus and cytoplasm fragmentation and formation of membrane-bound apoptotic bodies which can be taken up and destroyed by phagocytes. These fragments are enclosed in membranes so they can not harm nearby cells and cause an inflammatory reaction. In contrast to apoptosis, cells undergo another form of cell death called necrosis which is an uncontrolled cell death and is usually a consequence of a

pathophysiological condition, such as infection, inflammation or ischaemia. In necrosis cells swell, rupture and the released intracellular contents can damage surrounding cells and often cause inflammation (for review see (Adams, 2003, Jiang and Wang, 2004)).

Several human diseases, such as cancer, neurodegenerative disorders, and autoimmune diseases, have been directly linked to genetic defects in the apoptotic pathways. Failure of the death machinery results from the mutation of genes coding for factors involved in the initiation, mediation, or execution of apoptosis (Green, 1998, Mullauer et al., 2001). It is therefore crucial that these mechanisms are tightly regulated. The mechanisms involved in the regulation of cell death involve many factors. There are two major initiating pathways, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. These two pathways activate caspases that are the main effectors of the apoptosis. The caspases are a group of cysteine proteases that exist within the cell as inactive pro-forms (zymogens) which can be cleaved to form active enzymes following the induction of apoptosis. The extrinsic pathway is mediated by the activation of so called death receptors which are cell surface receptors that transmit apoptotic signals after ligation with their specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas (CD95), and the TRAIL receptors (Ashkenazi, 2002). When death receptors bind their ligand a signal is transmitted to the cytoplasm that leads to activation of caspase 8 which in turn activates downstream effector caspases leading to phagocytosis of the cell. The intrinsic pathway involves Bcl-2 family proteins which are both anti- (Bcl-2, Bcl-xl and Bcl-x) and pro-apoptotic (Bax, Bak, Bid and Bin). This family of proteins are regulated by factors including the PI-3K/Akt pathway inducing survival and the p53 tumour suppressor protein inducing apoptosis (due to DNA damage or overexpression of genes). Regulation of apoptosis by Bcl-2 proteins mainly seems to be depending on release of cytochrome c from mitochondria. Cytochrome *c* is normally sequestered between the inner and outer membranes of the mitochondria and is released into the cytosol in response to a variety of proapoptotic stimuli (Jiang and Wang, 2004). The released cytochrome c and Apaf-1 bind to molecules of procaspase 9 and ATP and form a complex (an apoptosome) that activates other downstream caspases (caspase 3). The extrinsic pathway can also induce release of cytochrome c through a Bcl-2 family member, Bid. Cross-talk between the two pathways exists and enhancement of apoptosis through Bid protein is such an example. The Bcl-2 family members with anti-apoptotic abilities stabilize the mitochondria by preventing cytochrome c release and in that way protect the cell from apoptosis.

Signaling Pathways for Growth and Differentiation

A cell needs to communicate with its environment to be able to generate appropriate cellular responses. The responsiveness of a cell relies on detection systems that sense changes outside the cell and are coupled to signal transduction pathways. There are several different signal transduction pathways existing which involve different signal molecules (such as growth factors, hormones, cytokines, ions and adhesion molecules on neighbouring cells) and different receptors (such

as tyrosine kinases, G-protein coupled receptor, nuclear receptors). Here I will only focus on the signals and signal pathways that have direct relevance to this thesis.

IFN-γ Signaling Pathway

Interferons (IFNs) were originally described as agents capable of protecting cells from viral infections by interfering with viral replication. They belong to the large class of glycoproteins known as cytokines and are involved in antiviral defence, the regulation of cell growth and immune response. IFNs are subdivided into two classes on the basis of structural and functional criteria as well as the stimuli that induce their expression. The type I IFNs consist predominantly of IFN-α and –β and type II only of IFNγ. The type I IFNs are induced in most cells by viruses and dsRNA while IFNγ is mainly secreted by T helper type 1 (Th1) lymphocytes and natural killer (NK) cells. It has been shown that IFNγ is also secreted by B cells, natural killer T cells, and professional antigen-presenting cells (APCs). IFNγ production is controlled by cytokines secreted by APC, especially interleukin (IL)-12 and IL-18. One way to regulate IFNγ action is to control its production. IFNγ activity can also be controlled through modulation of IFNγ signalling**.** These two classes of IFNs activate transduction pathways via different cell surface receptors (Stark et al., 1998). Binding of both type I and II IFNs result in the activation of Janus kinases (JAK) that phosphorylate cytoplasmic transcription factors called signal transducer and activator of transcription (STATs). Phosphorylated STATs translocate to the nucleus, bind specific DNA sites and direct transcription of IFN responsive genes. There are four mammalian JAKs (JAK1, JAK2, JAK3 and Tyk2) and seven STATs (STAT1, 2, 3, 4, 5a, 5b and 6) identified (for review see (Aaronson and Horvath, 2002)).

IFNγ exerts its functions by binding to a specific receptor composed of two subunits, IFNGR1 and IFNGR2 which in unstimulated cells are preassociated with JAK1 and JAK2, respectively. IFNγ bind to two IFNGR1 subunits and causes a conformational change generating binding sites for IFNGR2 subunits. The recruitment of the IFNGR2 into the complex brings JAK1 and JAK2 closer to each other. The inactive JAK2 kinase undergoes autophosphorylation and activation, which in turn allows JAK1 to be transphosphorylated by JAK2. The activated JAK1 then phosphorylates functionally essential tyrosines on residue 440 near the C-terminus of each IFNGR1, creating paired docking sites for STAT1. Afterwards two latent phoshorylated STAT1 molecules bind these sites through their Src homology (SH-2) domain and get phosphorylated by the receptor kinases at tyrosine 701 in their C- terminal. The activated STAT1 proteins dissociate from the receptor complex, form a homodimer which subsequently translocates into the nucleus and bind the gamma activated site (GAS) to activate transcription (for review see(Schroder et al., 2004)). To a lesser extend, the STAT1 homodimers are also able to bind to IFN-stimulated response element (ISRE) promoter regions in target genes to regulate transcription. Pathways other than JAK/STAT are also involved in IFN signaling, but their mechanisms are less clear. The best studied ones are the MAPK cascade, the components of the T cell receptor signaling cascade and the PI3 kinase pathway (Kalvakolanu, 2003).

Transcriptional regulation by the JAK/STAT pathway is involved in most of the biological functions of interferons such as antiviral response, cell growth inhibition, differentiation, tumour suppression and regulation of apoptosis. STAT1 has shown to be required for the antiproliferative effects of IFNs. For instance, expression of the Cdk inhibitor *p21* is upregulated by IFNγ whereas *c-myc, cyclin D3* and *cdc25a* expression is decreased in response to IFNα (Chin et al., 1996, Kominsky et al., 1998, Ramana et al., 2000, Tiefenbrun et al., 1996). It has been shown that STAT1 and 2 interact with transcriptional coactivators such as Nmi (N-Myc-interacting protein) and CBP/p300 (Bhattacharya et al., 1996, Zhu et al., 1999). These interactions are important since antibodies directed against CBP/p300 blocks transcription induced by IFNγ.

Retionic Acid (RA)

Vitamin A (retinol) and its derivates (retinoids) are essential for life and have important roles in embryonic development and cellular differentiation. In the diet, Vitamin A is obtained as retinyl ester from meat or from β-carotene found in a variety of plants. Vitamin A is absorbed by intestinal enterocytes, esterified to retinyl esters and stored in the lever. The retinyl esters are cleaved to form retinol which is released into the circulation bound to retinol binding protein (RBP) Retinol enters the target cell and is oxidised to retinal and retinoic acid (RA). Biologically active metabolities of RA include all-trans RA (atRA), 9-cis RA, 13-cis RA and 3,4-didehydro RA. Once RA has been synthesised in the cell, it enters the nucleus and binds specific nuclear receptors that regulate gene transcription. There are two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Three subtypes of both RARs (RARα, -β, -γ) and RXRs (RXRα, -β, -γ) have been cloned, all belonging to steroid/thyroid hormone family of transcription factors. These ligand-induced transcription factors possess specific DNA binding sites and RA binding sites, namely retinoic acid response elements (RAREs) (Chambon, 1996, Marill et al., 2003). Different forms of active RA have been found to bind different receptors. For instance, atRA binds mainly the RAR receptor while 9-cis RA interacts with both RAR and RXR receptors. These receptors form hetero- or homodimers to exert their function. RAR homodimers or RAR/RXR heterodimers can signal through RAREs upon ligand binding whereas RXR/RXR binds RXREs in response to 9-cis RA stimulation. In the absence of ligand, RAR and RXR receptors bind DNA and repress genes by interacting with HDACs via coreppressors (CoR) and Sin3 (Glass and Rosenfeld, 2000) (See the section about transcriptional repression). Upon ligand binding, a conformational change in ligand-binding domain occurs which disrupts CoR binding and allows interaction with coactivators (CoAs) instead. CoAs recruit histone acetyltransferases (HATs) complexes which leads to chromatin decondensation and activation of target genes. This will be discussed further in the section concerning transcription.

It is well known that RA is important for development and for regulation of differentiation and proliferation of numerous cell types throughout adult life. Already in 1925 a link between vitamin A deficiency and tumour development in epithelial cells was reported (Wolbach and Howe, 1978). It is well documented that RA can promote differentiation in various cells *in vitro* including

neuroblastoma, myelogenous leukemia and hematopoetic cells (Breitman et al., 1980, Sidell, 1982). This important function has made RA a potential drug for cancer treatment (Altucci and Gronemeyer, 2001, Soprano and Soprano, 2002). RA is already used successfully to treat various types of cancers, including acute promyelocytic leukemia, breast cancer, and precancerous lesions such as oral leukoplakia and cervical dysplasia (Dragnev et al., 2000). In addition, retinoids have been shown to delay the development of skin cancer. 13-cis RA is used to treat neuroblastoma patients and good effects of this treatment is reported (for review see (Matthay et al., 1999, Reynolds et al., 2003)).

Vitamin D3

Vitamin D3 is involved in calcium homeostatis, bone formation and modulation of cell growth and differentiation. Vitamin D3 is a secosteroid that is produced in the skin upon exposure to ultraviolet light or obtained from the diet. The biologically active form of vitamin D3 is 1,25-dihydroxyvitamin D3 (VitD3). Two successive hydroxylations are required to form the active metabolite of vitamin D3. The biological effects of VitD3 are mediated through vitamin D receptor (VDR) which is a nuclear receptor. Upon binding its ligand (VitD3), VDR heterodimerize with RXR and bind vitamin D responsive elements (VDREs) in the promoters of target genes, genes that are usually involved in regulation of VitD3 metabolism and bone formation (for review see (Lin and White, 2004)). Growth arrest and differentiation of various cell types *in vitro* such as U937 myelomonocytic cells and keratinocytes is induced by VitD3(Olsson et al., 1983, Smith et al., 1986) and p21 is upregulated in these differentiated cells (Liu et al., 1996). In hematopoetic cells, VitD3 treatment leads to downregulation of the *c-myc* protooncogene (Reitsma et al., 1983).

TPA

Phorbol esters and their derivatives are potent tumour promoting agents but it has also been shown that they are able to induce growth arrest/inhibition and differentiation of various cell types such as neuronal and myeloid cells (Harris and Ralph, 1985, Pahlman et al., 1981). They can also promote proliferation of certain cell types. The three most widely used active phorbols are 4*ß*-12-*O*-tetradecanoylphorbol-13-acetate (TPA), phorbol 12-myristate-13-acetate (PMA) and 4*ß*-phorbol-12,13-dibutyrate (PDBu). The best known receptors for these substances are the novel and classical isotypes of protein kinase C (PKC), which are members of the large family of Serine/Threonine protein-kinases (for review see (Parker and Murray-Rust, 2004)). PKC is activated by the physiological second messenger diacylglycerol (DAG). Phorbol esters can mimic the effect of DAG and activate PKC which in turn can activate other kinases such as ERK.

Activation of classical and novel types of PKC by TPA shortcuts the normal activation pathway of PKC. It has been reported that inhibitors of PKC restrain differentiation of neuroblastoma cells (Fagerstrom et al., 1996). The antiproliferative effect of TPA has been characterized by c-myc downregulation and p21 upregulation for instance in hematopoetic, neuroblastoma and breast

cancer cell lines ((Hammerling et al., 1987, Mitchell and El-Deiry, 1999, Todd and Reynolds, 1998)).

Transcriptional Regulation

Transcription of the different classes of RNAs in eukaryotes is carried out by three different polymerases. RNA pol I synthesize the large rRNAs, RNA pol II synthesizes the mRNAs and some small nuclear RNAs (snRNAs, which is involved in splicing) and RNA pol III synthesizes a variety of small stable RNAs, including the 5S rRNA and the tRNAs.
Regulation of gene expression is a central step in the maintenance of cellular homeostasis. Gene expression can be regulated at multiple steps. These steps can include: initiation of transcription, chromatin structure, processing of the transcript, transport to the cytoplasm, translation of mRNA, stability of mRNA and protein activity. Even though there are multiple stages where control of gene expression can occur, it is well established that the majority of regulatory events take place at the initiation of gene transcription. Various components are involved in every step of the regulation. In this chapter the steps and components involved in RNA PolII mediated transcriptional regulation will be briefly discussed.

Initiation of transcription

RNA polymerase II (RNA pol II) is responsible for the transcription of protein-coding genes in eukaryotes, but the polymerase can not initiate transcription on its own. Additional proteins, so called general transcription factors (GTFs) that directly or indirectly interacts with the RNA pol II, are required. These proteins have to assemble with RNA polymerase II into a complex on the promoter DNA. The promoter region in a gene contains different sequence elements, including TATA-box, the initiator (Inr) and the downstream promoter element (DPE), which allow regulatory components to bind and exert their activity. The start of transcription by RNA pol II is directed by these core promoter elements.

GTFs include five basal factors, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. TFIIA is the sixth factor and it potentiates the magnitude of transcription (Reese, 2003). TFIID binding is thought to be the first step in transcription initiation. TFIID is a multi-component transcription factor consisting of a DNA binding subunit, TATA-binding protein (TBP), and several, at least 14, TBP-associated factors (TAFs). The binding of TBP causes a bend in the DNA which creates a platform for the interaction of the remaining factors. The next GTF to bind the promoter is TFIIB, which interacts with TBP and recruits RNA pol II partly through an interaction with the small subunit of TFIIF. TFIIB stabilizes TFIID at the promoter by contacting TFIID and sequences next to the TATA box. TFIIB, in turn, recruits TFIIH which contains helicase and kinase activities. TFIIH catalyzes ATP-dependent melting of the promoter (separation of the two DNA strands) at the transcriptional start site. The kinase of TFIIH (cyclinH/Cdk7) phosphorylates the C-terminal domain of the RNA pol II largest subunit (Rbp1). TFIIH appears to be dependent on TFIIE for incorporation into the pre-initiation complex (PIC).

Both TFIIF and TFIIH are necessary for promoter clearance and the progression into the elongation phase (Reese, 2003, Sims et al., 2004b). Elongation factors involved in the elongation process are divided into two classes; negatively acting factors that inhibit transcription and positively acting factors that overcome the inhibition (Sims et al., 2004a). The positive transcription elongation factor b (P-TEFb) complex, which consists of a common catalytic subunit (Cdk9) and one of several regulatory cyclins T1, T2 and K, phoshorylates CTD of RNA pol II and allows the elongation to proceed. NELF and DSIF are examples of negatively acting factors. It has been suggested that NELF and DSIF function through interactions polymerase containing a hypophosphorylated CTD. Additionally, pausing of RNA pol II is suppressed by a various collection of proteins, including TFII, ELL and Elongin. It should be also mentioned that beside elongation process, several steps in mRNA maturation, including pre-mRNA capping, splicing, 3'-end processing and export, are modulated via interactions with the RNAP II transcript elongation complex (Sims et al., 2004a).

Transcription of most genes is regulated by specific regulatory transcription factors that affect the transcription rate positively (activotors) or negatively (repressors). These proteins interact with specific regulatory DNA sequences including enhancers/silencers, upstream promoter elements and initiators in eukaryotes. In order to activate or repress transcription these factors need to interact with the PIC. This interaction involves contacts with components of mediator complex and with TAFs. The DNA-binding transcription factors have at least two important domains: a DNA binding domain (DBD) that positions the protein on specific sequences and a transactivation domain (TAD) that activate transcription by for instance recruiting or accelerating the assembly of GTFs on the promoter. The negative regulating transcription factors contain a domain that is able to repress the recruitment of RNA pol II or is able to interact with corepressors. The DNA binding transcription factors are divided into different groups depending on the structural motifs they possess. The four well described motifs are the basic-helix-loop-helix (HLH) motif, the basic leucine zipper (LZ), the zinc finger (ZF) and the helix-turn-helix (HTH) domain.

Coactivators and coreppressors

Transcriptional regulation is not only dependent on TF activation or repression. Additional proteins called coregulators (coactivator/corepressor) are also reqired in transcriptional regulation. Studying the role of these coregulators is necessary to understand transcriptional regulation in eukaryotes.

Coactivators usually do not bind DNA and are recruited to target promoters through protein-protein interactions with transcription factors. They often contain enzymatic activities necessary for an alteration in chromatin structure. Coactivators can broadly be divided into three classes (Spiegelman and Heinrich, 2004). The first class consist of proteins that modify histones leading to a greater access of other proteins to DNA. For instance, p300, CBP and histone acetyltransferases (HATs) are examples of this class. Members of the TRAP/DRIP/Mediator/ARC complex constitute the second class of coactivators. These proteins bind transcription factors, recruits RNA pol II and interact with the

general transcription machinery. The last group is protein complexes of the yeast SWI/SNF family (mammalian homologs BRG1 or BRM) which have ATP-dependent chromatin remodelling activities.

Corepressors have the opposite effect on chromatin structure compared to the coactivators. These proteins in general coordinate the inactivation of transcriptionally active complexes. Their direct interaction with DNA binding transcription factors and recruitment of chromatin modifying enzymes leads to an inactive state of the nucleosome making the chromatin inaccessible to other proteins. Examples of such proteins are nuclear hormone receptor corepressor (NCoR) and thyroid hormone receptors (SMRT). Corepressors are often associated with histone deacetylase (HDAC) activity (see below).

Histone Modifications and Chromatin Remodeling

In eukaryotes, genomic DNA is packaged into chromatin which is composed of DNA and proteins. Two superhelical turns of DNA are wrapped around a histone octamer which forms the nucleosome core particle. The nucleosomes form the basic repeating unit in chromatin and consist of four histone partner, one H3-H4 tetramer and two H2-H2B dimers (for review see (Spiegelman and Heinrich, 2004)). The packaging of DNA into nucleosomes makes it less accessible to both sequence specific and general transcription factors. For transcription to take place this highly ordered and condensed chromatin structure must be altered. There are two elegant mechanisms that have evolved to modify chromatin structure: modification of chromatin by acetylation/deacetylation of histones and nucleosome remodelling.

Histones are targets for a variety of post-translational modifications (often occur on tail domains of these proteins) including acetylation, methylation, phosphorylation and ubiquitylation (Strahl and Allis, 2000). Acetylated histones have been associated with actively transcribed genes whereas deacetylated histones have preferentially been found in silenced genes. The HATs and HDACs regulate acetylation and deacetylation of the conserved lysine residues present in histones. Acetylation of histones by HATs leads to a less condensed chromatin structure and increase the accessibility to GTFs, the RNA pol II and to additional regulatory proteins. A quantity of transcriptional coactivators have been shown to have HAT activity, including CREB binding protein (CBP) and the related protein p300, p300/CPB-associated factor (P/CAF), and the p160 family (SRC-1, TIF2 and Pcip). There are proteins with intrinsic HAT activity, for instance $TAF_{II}250$ which is a TFIID subunit that has critical function in transcription initiation (Mizzen et al., 1996). Another example is Nut1 that is part of the yeast mediator complex, which is directly associated with RNA polymerase II (Lorch et al., 2000). Many of the HATs are components of multisubunit complexes. Two well-known example is the yeast SAGA and ADA complexes (Grant et al., 1997). hGCN5 and P/CAF are the mammalian homologs of GCN5. Both yeast and mammalian GCN5 type coactivators contain a Tra1/TRAPP polypeptide which has been shown to interact with specific coactivators, such as c-Myc (McMahon et al., 1998, Saleh et al., 1998).

HDACs carry out their effects by removing acetyl groups from lysines in the N-terminal tails of histones and are correlated with transcriptionally repressed state. Various proteins have been shown to possess intrinsic HDAC activity (Grozinger and Schreiber, 2002). Mammalian HDACs are ordered into three classes (class I, II and III) based upon sequence similarity to the yeast HDACs (Rpd3, Had1 and Sir2). Class I consist of HDACs 1, 2, 3 and 8, class II of HDACs 4, 5, 6, 7, 9 and 10. HDAC1 and HDAC2, members of class I HDACs, are the best characterized HDAC proteins and are usually found in stable, multicomponent complexes of proteins, which are then recruited by DNA binding proteins (such as coreppressors). Sin3, NCoR/SMRT, Mi2/NuRD and CoREST complexes are among these complexes. The Myc antagonist, Mad proteins has been shown to interact with Sin3/NCoR complexes to exert their function and to repress expression of myc target genes (Ayer et al., 1995, Hassig et al., 1997, Laherty et al., 1997, Schreiber-Agus et al., 1995, Sommer et al., 1997).
There is accumulating evidence that transcription might also be regulated by acetylation of non-histone proteins, such as p53, tubulin and various transcription factors (Gu and Roeder, 1997).

Another way of modifying chromatin structure is by ATP-dependent chromatin remodelling complexes as SWI/SNF, ISWI, RSC and Mi-2/NuRD, which alter chromatin structure by changing the location or confirmation of the nucleosome without covalent modifications. These complexes all have ATPase activity and they increase accessibility to DNA as well as to histones. The yeast SWI-SWF complex is the founding member of this family of ATP-dependent chromatin-remodelling complexes. Its hallmark subunit which possesses intrinsic ATPase activity is SWI2/SNF2. Brahma (Brm) is the Drosophila homolog of SWI2/SNF2 whereas hBRM/hSNF2a and BRG1/hSNF2b are the human homologues. Some activators have been shown physically or functionally to interact with SWI/SNF activity. For instance C/EBPβ activator and c-Myc proto-oncoprotein interacts directly with subunits of mammalian SWI/SNF complexes (for review see (Naar et al., 2001)). SWI/SNF complexes can also function as repressor of transcription. This has been demonstrated by physical presence of SWI/SNF components at repressed promoters (for review see (Martens and Winston, 2003)).

Myc/Max/Mad Network

The *myc* proto-oncogene family is shown to be involved in a number of essential cellular processes and deregulation of this gene is frequently observed in a variety of human cancers. This has led to an extensive study of *Myc* to understand its function and its involvement in carcinogenesis (for review see (Oster et al., 2002)).

The *myc* oncogene was originally identified in avian myelocytomatosis retrovirus for over two decades ago (*v-myc*) (Sheiness and Bishop, 1979, Sheiness et al., 1978). Shortly after, a cellular homolog of *v-myc* was isolated in chicken (Vennstrom et al., 1982) which was capable to induce myeloid leukaemia, sarcomas, liver, kidney and other types of tumours in chicken (Gonda et al., 1982). It was also discovered that the human c-*myc* homolog is constitutively active in Burkitt's lymphoma due to translocation involving c-*myc* and *Ig*-locus (Dalla-

Favera et al., 1982, Taub et al., 1982). c-*myc* is evolutionary conserved and has been identified in all vertebrates, including zebrafish (Schreiber-Agus et al., 1993) and in some non-vertebrates such as the sea star *Asteria vulgaris* (Walker et al., 1992) and *Drosophila* (Gallant et al., 1996). *myc* has not yet been identified in yeast or *Caenorhabditis elegans*. Other *myc* family members, the N- and L-*myc*, were subsequently discovered as amplified or highly expressed genes in neuroblastoma (Kohl et al., 1983, Schwab et al., 1983) and in small lung cancer (Nau et al., 1985), respectively. B- and S-*myc*, are two additional members of the *myc* family, that have only been identified in rodents and are not well-characterized (Ingvarsson et al., 1988, Sugiyama et al., 1989). c-, N-, and L-*myc* share similar genomic organisation and as well as evolutionary conserved domains that are functionally important protein regions. They all consist of three exons: an untranslated exon (exon 1) and two exons containing protein coding sequence (exon 2 and 3). Expression of these members of the *myc* family is deregulated in many different tumours. L-*myc* seems to have a weaker oncogenic potential compared to c- and N-*myc* (Barrett et al., 1992). c-, N-, and L-*myc* seem to have similar functions and to various degrees they seem to be able to substitute for each other's activities in different biological processes. For instance, it has been shown that insertion of N-*myc* into c-*myc* locus rescued the c-*myc* null phenotype (Malynn et al., 2000) indicating similar function of c- and N-*myc*. c-*myc* and N-*myc* encodes essential genes and embryos carrying c-*myc* deletion die before birth at day 10.5 (Davis et al., 1993). It should also be mentioned that the expression pattern of these three genes differ during development and in the adult pointing towards different roles (Charron et al., 1992, Davis et al., 1993, Jakobovits et al., 1985, Stanton et al., 1992, Zimmerman et al., 1986).

Myc/Max/Mad Network and the structure of its members

The Myc family members belong to basic/helix-loop-helix/leuzine zipper (bHLHZip) family of transcription factors and they encode short-lived phoshoproteins (64 and 67 kDa). The structure of Myc is shown in figure 3. The bHLHZip domain located at the C-terminus of the protein is responsible for DNA-binding and protein-protein interaction (Luscher and Larsson, 1999).

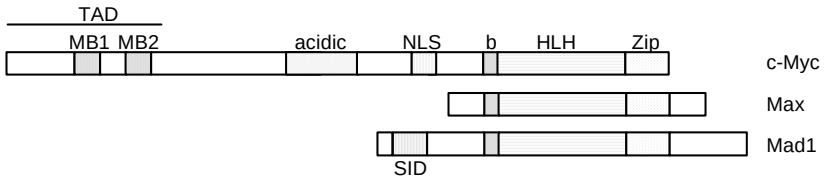

Figure 3: Structure of c-Myc, Max and Mad. Domains of functional importance are indicated. TAD: transcriptional activation domain, MB: Myc box, NLS: nuclear localisation sequence, b: basic region, HLH; helix-loop-helix, Zip: leucine zipper, SID: Sin3 interaction domain

The N-terminus of the Myc protein harbours a transactivation domain (TAD) involved in Myc-regulated transcription. The TAD contains two evolutionary

conserved regions, namely Myc box I and II (MBI, II). MBI contains several phosphorylation sites that are important for the regulation of Myc protein stability by the ubiquitin/proteasome pathway (Flinn1999, Sears2000, Salghetti 2000, Bahram 2000,Gregory&Hann2000.Welcker 2004 (PNAS) Nakayama 2004 (EMBO)). It has been shown that phosphorylation of Thr58 serves as a recognition signal for the E3 ligase Fbw7 (Welcker et al., 2004, Yada et al., 2004). Further, MBI is also required for cell transformation. MBII mediates interaction with many partners (see below) and is necessary for all biological activities of Myc, including transcriptional activation and repression, proliferation, block of differentiation, apoptosis and transformation (Grandori et al., 2000). Additionally, MBII serves as an interaction surface for the E3 ligase Skp2, leading to ubiquitylation and subsequent degradation by the proteasome (Kim et al., 2003, von der Lehr et al., 2003). Additional sites/domains in Myc protein are: a nuclear localisation site (NLS) important for import into the nucleus and a central acidic domain that contain several phosphorylation sites that might be important for function.

Myc needs to dimerize with another bHLHZip protein, Max; to be able to activate transcription. This discovery was one of the major breakthroughs in Myc research (Blackwood et al., 1992, Prendergast and Ziff, 1992). The Myc:Max dimers bind specific DNA sequences called E-boxes (CACGTG) and this interaction is of importance both for transcriptional activation and for the biological functions of Myc (Amati et al., 1993).

In addition to Myc, Max can also dimerize with other bHLHZip proteins including Mad family proteins (Mad1, Mxi1, Mad3 and Mad4), Mnt and Mga (Ayer et al., 1993, Hurlin et al., 1995a, Hurlin et al., 1997, Hurlin et al., 1999, Zervos et al., 1994). These proteins contain conserved regions: the bHLHZip domain in the C-terminus and a Sin3-interaction domain (SID) in the N-terminus which mediates transcriptional repression. Mad/Mnt proteins have been shown to act as antagonists to Myc in certain functions. They bind to similar if not identical E-boxes and they compete with Myc to bind Max and exert their function. Mad/Mnt family proteins together with Myc and Max form the Myc/Max/Mad network there Max plays a central role (see figure 4). Different cellular responses can be achieved depending on whether Max form complex with Myc or Mad.

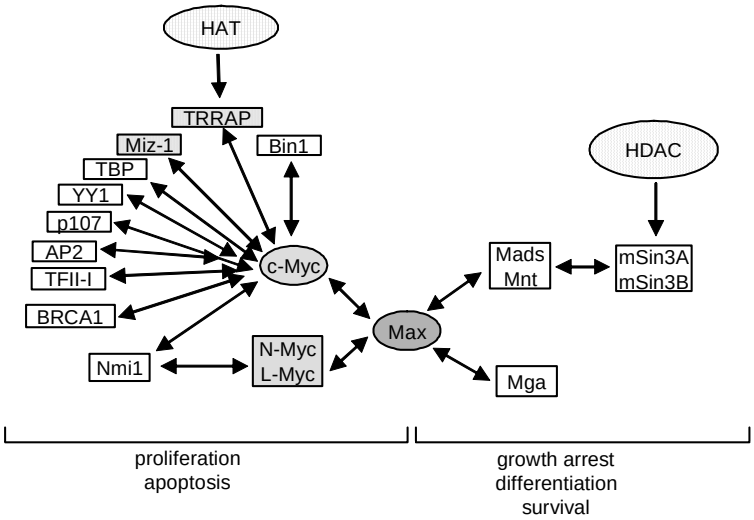

Figure 4: The Myc-Max-Mad network and some associated proteins

Further a Max-like protein, Mlx; was identified and characterized as a Mad1 binding protein (Billin et al., 1999, Meroni et al., 2000). Mlx can form dimers with Mad1, Mad4 and Mnt. The function of Mlx in Myc/Max/Mad network is not well understood yet. It is known that Mad/Mlx heterodimers bind specifically to E-boxes and have the ability to repress transcription in an msin3 dependent manner. An additional partner to Mlx is MondoA and Mlx/MondoA complexes are believed to activate transcription by binding E-boxes in target gene promoters. Mlx consequently, can both activate and repress transcription depending on the protein it is interacting with. Another Max interacting protein is Mga which only share the bHLHZip domain in common with other Myc/Max/Mad network proteins (Hurlin et al., 1999). Mga binds E-boxes but in contrast to Mad/Mnt proteins it does not contain SID and therefore believed that it exert its function not dependent on mSin3.

c-Myc can interact with many other proteins both in the N- and C-terminus. For instance TBP, TRRAP, Bin1 and p107 interact with N-terminus (Beijersbergen et al., 1994, Gu et al., 1994, Hateboer et al., 1993, McMahon et al., 1998, Sakamuro et al., 1996) while TFII-I, YY1, Miz-1, Nmi, BRCA1 and AP-2 bind the C-terminus of c-Myc (Bao and Zervos, 1996, Gaubatz et al., 1995, Peukert et al., 1997, Roy et al., 1993, Shrivastava et al., 1993, Wang et al., 1998b).

Transcriptional Regulation

Myc/Max/Mad network proteins can regulate transcription both by involving histone-modifying enzymes and interacting with the basal transcription machinery. Antagonistic biological functions of Myc and Mad can depend on the recruitment of counteracting enzymes, HATs and HDACs.

Myc activates transcription by interacting with the coactivator TRRAP (Transformation-transactivation domain- Associated Protein) through its TAD

domain or more specifically via MBII (McMahon et al., 1998, Patel et al., 2004a). TRRAP is a component of various complexes with HAT activity, for instanceGCN5/PCAF or Tip60 (Fernandez et al., 2003, Frank et al., 2003, McMahon et al., 2000). Interaction of Myc with HAT containing complexes seem to be essential for Myc induced transformation, since dominant negative mutants of TRRAP has been shown to inhibit transformation by Myc (McMahon et al., 2000). It has also recently been shown that Myc interacts with CBP (cAMP response element binding protein) which is another coactivator with HAT activity (Vervoorts et al., 2003). Further, Myc has been observed to interact with other chromatin-remodeling enzymes, the Tip48 and Tip 49 which contain ATPase helicase activity. These proteins interact with Myc via MBII and have been shown to be essential for Myc-induced oncogenic transformation (Wood et al., 2000). Tip48 and Tip 49 can further bind Baf53 which is a component of STAGA (containing GCN5) and Tip60 complexes. GCN5, Tip48, Tip49, and Baf53 proteins are part of various complexes which are involved in chromatin regulation either by ATP-dependent remodelling or histone acetylation (Frank et al., 2003, McMahon et al., 1998, Nikiforov et al., 2002b, Park et al., 2001, Patel et al., 2004a). It has further been shown that interaction of Myc with the E3 ligase Skp2 does not only lead to ubiquitylation and proteasomal degradation (as mentioned above) but is also needed for the transcriptional activity of Myc (Kim et al., 2003, von der Lehr et al., 2003). Additionally, Myc has also been reported to interact with other protein complexes involved in transcriptional regulation. For instance, Myc binds INI1 which is a homologue of yeast SNF5 protein and a subunit of SWI/SNF complex involved in chromatin remodelling in an ATP dependent manner. Dominant negative mutants of INI1 inhibit c-Myc induced transcription (Cheng et al., 1999). Additionally, Myc can directly interact with TBP which is a component of the basal transcription machinery. Importance of this interaction for Myc function has unfortunately not been explored yet. But more recently it has been shown that Myc interact with cyclin T1 and Cdk9, components of a polII CTD kinase. This interaction has been shown to be sufficient for activation of a synthetic promoter construct suggesting that Myc stimulates elongation of transcription (Eberhardy and Farnham, 2002).

In addition to its role as transcriptional activator Myc has also the ability to repress transcription. Transcriptional repression is, at least partially, mediated by interaction with Miz1 (Myc-interacting zinc finger protein 1) which binds the HLH-domain of Myc. c-Myc has been shown to suppress expression of several cell cycle/growth arrest genes, including gas1, $p15^{Ink4b}$, $p21^{Cip1}$, $p27^{Kip1}$ and gadds (Gartel and Shchors, 2003). Miz-1 binds to initiator (Inr) like elements of these genes promoters and upregulate their expression. By forming complexes with Miz1, c-Myc antagonizes this process and inhibits Miz-1 mediated transcriptional activation of these genes (Herold et al., 2002, Peukert et al., 1997, Seoane et al., 2001, Staller et al., 2001, Wanzel et al., 2003) (Paper IV). Transcriptional repression of c-Myc through Miz1 will be further discussed in the Results and Discussion section.

Mad proteins repress transcription by recruiting HDACs to the target gene promoters. The recruitment of HDACs is mediated through SID domain of Mad which directly bind to mSin3. mSin3, in turn, is part of a multisubunit complex

including, HDAC1 and 2, the corepressors N-CoR/SMRT and many other (Alland et al., 1997, Hassig et al., 1997, Heinzel et al., 1997, Laherty et al., 1997, Sommer et al., 1997). Removal of the acetyl groups by HDACs leads to a more compact state of chromatin resulting in transcriptional repression. In addition to HDACs mSin3 proteins has been shown to interact with other chromatin modifiers, such as hBrm ATPase of the SWI/SNF complex or Suv39H1 methyltransferase (Czermin et al., 2001, Kuzmichev et al., 2002, Sif et al., 2001, Vaute et al., 2002). However, so far, HDAC is the only chromatin modifying enzyme that is recruited by Mad.

Target Genes of Myc and Mad

Myc and Mad exert their functions by regulating expression of specific genes. At present there are several hundred of Myc target genes that have been identified (Boon et al., 2001, Coller et al., 2000, Orian et al., 2003). Recent studies indicate that Myc binds and regulates up to 10-15 % of all genes (Fernandez et al., 2003, Patel et al., 2004b). However, since different approaches have been used to identify these genes it is not well known how many of them are regulated directly by Myc. Many of potential target genes were identified by micraoarray-based screens. Several other approaches ranged from differential expression screens, promoter analysis and educated guesses to more recent methods such as microarray screens, SAGE and ChIP have been used to identify putative target genes. Some examples of Myc target genes are: *cyclin D2, p21, p15, cdc25a, cdk4, cul1*, involved in cell cycle progression; *gadd45, p53* involved in apoptosis; *ornithine decarboxylase (ODC), cad, α-prothymosin, eIF4E, eIF-2α* involved in growth and metabolism, *hTERT* involved in immortalization and *tip48, pcaf* involved in transcription, *VEGF, tsp-1, activin A* involved in angiogenesis (for review see (Dang, 1999, Levens, 2002).

So far the identified Mad family target genes are not as many as Myc target genes because it has been studied less. Since Myc and Mad have antagonistic effects it was reasonable to assume that they regulate same set of genes. To address this question the basic region of Myc was replaced with the basic region of Mxi. Subsequent microarray analysis revealed that Myc and Mxi1 has both common and distinct target genes (O'Hagan et al., 2000). A screen for Mad1 target genes also showed that Myc and Mad1 have both common and distinct target genes (Iritani et al., 2002). Some of the common genes are *ODC, hTERT, gas1* and *cul1*. Many of the nonoverlapping genes do not posses E-box sequences and possibly involve alternative partners for Myc and Mad/Mnt. For instance, Myc-dependent repression of transcription involves the zinc finger protein Miz-1 which binds initiator-like sequences. Mad proteins probably can not recognize these sites and thereby is not able to control this particular set of genes (James and Eisenman, 2002, Nikiforov et al., 2002a, Nikiforov et al., 2003, Orian et al., 2003).

Biological function of Myc and Mad

Myc is a multifunctional protein which regulates various distinct biological activities, including proliferation, differentiation, cell cycle progression, apoptosis and tumorigenesis. Myc is believed to regulate these functions by regulating the

transcription of specific target genes discussed above. Mad family proteins function as Myc antagonists in many of these biological functions.

Several lines of evidence suggest that Myc functions as a positive regulator of proliferation in response to various mitogenic signals. For instance, it has been shown that Myc is expressed in proliferating cells and Myc expression is induced when cells are treated with different growth factors, cytokines and mitogens (Armelin et al., 1984, Campisi et al., 1984, Kelly et al., 1983, Rabbitts et al., 1985). For the proliferation to take place it is necessary for cells to overcome the G1/S checkpoint. c-Myc has been reported to be sufficient to overcome this checkpoint and induce S phase in absence of mitogens (Eilers, 1999) The essential role of Myc in cell cycle has been supported by a large body of evidence. For instance, antisence Myc inhibits S phase transition (Heikkila et al., 1987). Eliminating Myc expression by other techniques such as SiRNA also interferes with G1/S transition for review see (Oster et al., 2002)). Further, it has been shown that null *myc* Rat1 fibroblasts grow slower and have a prolonged G2 phase G2?? (Mateyak et al., 1997). MEF *c-myc* knock-out cells support mentioned data above (de Alboran et al., 2001). Several genes involved in cell cycle regulation are among Myc target genes, including *cyclin D1, D2,* and *cdk4*. Myc has been shown to activate Cyclin E/Cdk2, by directly activating cyclin D1and D2, which sequester p27Kip1. This leads to activation of Cyclin E/Cdk2 complexes which are needed to overcome the G1/S checkpoint (Coller et al., 2000, Hermeking et al., 2000, Luscher, 2001, Oster et al., 2002). Expression of other CKIs, such as p15INKB and p21Cip1 are also repressed directly by Myc, again leading to activation of CyclinD/Cdk4 and CyclinE/cdk2, respectively. p21Cip1 will be discussed further in results and discussion section. Inactivation of pRb is another important aspect of cell proliferation. c-Myc upregulates Id2 which in turn binds and inactivates pRb (Lasorella et al., 2000).

Mad proteins are involved in pathways that negatively affect cell proliferation and are generally expressed in differentiated cells (see below). There is also evidence that some *mad* family genes, *mxi1*, *mad3* and *mnt,* are expressed during proliferation and in non-differentiated cells (Hurlin et al., 1996, Pulverer et al., 2000, Queva et al., 1998, Zervos et al., 1993). One possible explanation to this is that these Mad/Mnt proteins have a function to antagonize or modulate the effects of Myc during certain periods of cell proliferation and differentiation. Ectopically overexpressed Mad/Mnt family proteins have been studied in tissue cultures and transgenic mice. Mad1, Mxi1, Mad3 andMad4 interfered with the proliferation of the cells and blocked cooperative transformation by Myc and Ras (Cerni et al., 1995, Chen et al., 1995, Chin et al., 1995, Lahoz et al., 1994, Roussel et al., 1996). Further, cells from mad1-/-, mxi1-/-, or mnt-/- mice showed increased proliferative capacity (Foley et al., 1998, Hurlin et al., 2003, Schreiber-Agus et al., 1998). The capacity of Mad to inhibit proliferation is tightly linked to its transcriptional function since Mad mutants that do not bind DNA, associate with Max, or contain a functional repression domain are inactive in biological assays. The effects of Mad on proliferation and differentiation can also be due to the ability of Mad to alter cell cycle. There are studies that indicated Mad1 overexpression resulted in delayed G0 to S phase transition, G1 arrest or accumulation of cells in the G1 (or

G2) phase of the cell cycle, reduced CyclinE/Cdk2 activity and growth arrest at reduced cell density {Queva, 1999 #249; Bejarano, 2000 #250; Chen, 1995 #31; {Roussel, 1996 #313}(see Paper III).

It has been shown that c-Myc stimulates cell growth (defined as increase in cell mass and size) independent of cell cycle status in certain cell types such as B cells, hepatocytes, *Drosophila* wings and T cells (Iritani and Eisenman, 1999, Johnston et al., 1999, Schuhmacher et al., 1999). In *Drosophila melanogaster* loss of *c-myc* homolog *dmyc* results in delayed growth and reduced cell size (Johnston et al., 1999). Overexpression of *dmyc,* on the other hand, gives rise to larger cells. Cell division rate of these cells is not affected neither by loss nor overexpression of *dmyc* indicating that distinct Myc activities control cell cycle and cell growth. For instance, primary B cells with overexpressed Myc are larger at every stage of their development (Iritani and Eisenman, 1999) supporting previous data. In addition, N-myc null progenitor cells from conditional knock-out mice show impaired proliferation, an increase of neuronal differentiation and changed morphology and size (Knoepfler et al., 2002). Several of Myc target genes are involved in metabolism. Some of these genes are believed to be involved in the control of molecular mechanism of cell growth. For instance, rate-limiting translation factors eEIF4 and eIF2α are among these genes. Mad genes are generally considered to have growth inhibitory effects. It has been reported that Mad1 reduces cell size in thymocytes (Iritani and Eisenman, 1999). It is however not clear if cell growth and cell cycle are coupled in cells with overexpressed Mad. Mad proteins and cell growth will be discussed in detail in Results and Discussion section.

In addition to its positive regulatory effect of proliferation Myc often has a negative effect on differentiation. In several cell types, including neuroblastoma, myeloid cells, erythroleukemia cells and teratocarcinoma cells, Myc is downregulated during differentiation (Gonda and Metcalf, 1984, Lachman and Skoultchi, 1984, Thiele et al., 1985). Further, it has been shown in many of these cells that overexpression of Myc can inhibit differentiation (Coppola and Cole, 1986, Freytag, 1988, Larsson et al., 1988). Myc can exert its inhibitory effect in differentiation for instance by repressing certain target genes important for differentiation process. It has been observed that Myc represses C/EBPα which is important in inducing differentiation of 3T3-L1 adipocytes (Mink et al., 1996). However, it has also been shown that differentiation of certain cell types is not inhibited, and in a number of cases even promoted by Myc (Gandarillas and Watt, 1997, Lerga et al., 1999).

Mad is usually expressed in differentiating and/or growth arrested cells. Several studies has shown a clear correlation between terminal differentiation and increased *mad*-family mRNA levels. In hematopoietic cells both *mad1* and *mxi1* transcripts and proteins are detected rapidly after induced differentiation (Ayer and Eisenman, 1993, Larsson et al., 1994, Zervos et al., 1993). The increase in Mad1 protein resulted in a switch from Myc:Max to Mad:Max complexes after induced differentiation (Ayer and Eisenman, 1993, Hurlin et al., 1995a, Larsson et al., 1997). This model is supported by the evidence that the occupancy of the target genes promoters, cyclin D2 and hTERT, *in vivo* shifts from Myc.Max toward Mad1:Max predominance during hematopoietic differentiation (Bouchard

et al., 2001, Xu et al., 2001). In situ hybridization analyses of embroyonic tissues during mouse development indicate distinct but overlapping expression pattern of *mad* family RNAs in different tissues. (Hurlin et al., 1995a, Hurlin et al., 1995b, Vastrik et al., 1995). These studies confirmed that c-*myc* and N-*myc* is primarily expressed in proliferating cells. Both *mxi1* and *mad4* transcripts were detected at low levels in increasing cells but however their expression levels were increased in differentiating cells. *mad1* gene was observed to be expressed in cells late in the differentiation whereas *mad3* was expressed in the S-phase of the proliferating cells (Hurlin et al., 1995b, Pulverer et al., 2000, Queva et al., 1998). There are however also some contradictory results concerning mad1 and its function in differentiation. For instance, Mad1 has been shown to stimulate differentiation in mouse erythroleukemia (MEL) cells while it blocks differentiation of 3T3-L1 adipoblasts (Cultraro et al., 1997b, Pulverer et al., 2000). Further it has been observed that granulocytic precursor cells of mad1$^{-/-}$ mice have an abnormal cell cycle resulting in increased proliferation and delayed terminal differentiation (Foley et al., 1998). Recently it has also been suggested that during granulocytic differentiation Mad1 and p27 can cooperate and downregulate Myc. These results indicate that during differentiation of certain cell types Mad proteins might disrupt cell cycle progress (McArthur et al., 2002). For further discussion see Results and discussion section.

A number of oncoproteins that stimulates proliferation such as Myc and E1A can also induce apoptosis. It has been shown that deregulated c-Myc expression sensitizes cells to a range of pro-apoptotic stimuli, such as DNA damage and hypoxia, and depleted survival factors, such as serum, IL-3. Myc can also potentiate apoptosis by sensitising cells to signalling through CD95, TNF and TRAIL (Hueber et al., 1997, Klefstrom et al., 1994, Lutz et al., 1998) Myc induced apoptosis has also been reported to involve activation of the Arf/Mdm2/p53 pathway (Zindy et al., 1998).Arf is upregulated by Myc resulting in accumulation of p53 which in turn can induce apoptotic effectors such as Bax. Myc has also been shown to repress anti-apoptotic factors such as Bcl2 and Bcl-x. These factors suppress apoptosis by blocking the membrane permabilization (MOMP) that is necessary for cytochrome C release (Martinou and Green, 2001). Not surprisingly Mad proteins has been shown to restrain apoptosis induced by various stimuli. For instance, targeted disruption of Mad1 has been observed to enhance the apoptosis of granulocytes upon cytokine withdrawal (Foley et al., 1998). Apoptotic response to DNA damage and death receptor signalling has been shown to be impaired when Mad1 is overexpressed (Bejarano et al., 2000, Gehring et al., 2000).

Neuroblastoma

Neuroblastoma (NB) is a childhood tumour that originates from sympathetic neuroblasts of the peripheral nervous system. It is one of the most common solid tumors of childhood primarily affecting children under five years (the median age 18 months). NB accounts for 7-10% of the childhood cancers (although compromising 15% of childhood cancer death) and approximately 10-15 children under the age of 15 are diagnosed in Sweden every year. The tumours are usually

located in the adrenal gland and sympathetic ganglia of the abdomen, neck, throax, and/or pelvis. Neuroblastomas most commonly spread to regional lymph nodes, bone and bone marrow. NB has broad clinical characteristics and patient outcome. The symptom of the disease differs depending on location of the tumour, metastatic spread and the age of the patient (for review see (Brodeur, 2003)).

The clinical hallmark of neuroblastoma is its variability. It can vary from very benign, untreatable tumours to tumours that can differentiate spontaneously despite their metastatic spread. Spontaneous regression is an enigmatic clinical behaviour of NB that is particularly seen in infants. NB is classified into four stages according to the International Neuroblastoma Staging System (INSS) (Brodeur et al., 1993, Evans et al., 1971). This system is based on local growth of the tumour, growth of differentiation, local and distant infiltration and the age of the child at diagnosis (stage 4S tumours). A higher stage indicates a more advanced disease. Tumours of stage 1 show a localised growth pattern, while stage 4 tumours display metastatic growth. Stage 4S can undergo spontaneous regression despite its colonalisation of several organs (infants under the age of 1 year). In general, stages 1, 2 and 4S have a good prognosis with or without treatment, whereas stages 3 and 4 have a poor prognosis despite treatment. In addition, patients diagnosed under the age of two years usually have a better prognosis than older patients. The INSS have been a valuable tool in prediction of prognosis and choice of appropriate treatment method. Unnecessary treatments can be avoided using INSS.

Recent years, various genetic and biological features in NB have been investigated to better understand the behaviour of NB and to find genetic markers which can improve the diagnosis/prognosis of the disease. Several tumour markers have been revealed including N-*myc* amplification, ploidy, deletion or loss of heterozygosity of chromosome 1p and gain of chromosome 17q. Many genetic features of NB are correlated to the clinical outcome. For instance, N-*myc* amplification or allelic loss (such as 1p) is correlated with more aggressive tumours and poor prognosis, while near-triploidy is associated with better outcome (Bown et al., 1999, Brodeur, 2003, Brodeur et al., 1984, Caron et al., 1996, Seeger et al., 1988)**.** Amplification of N-*myc* was originally described in NB cell lines (Schwab et al., 1983) and shortly after in some neuroblastoma tumours (Brodeur et al., 1984) correlating with advanced tumours and poor prognosis. Occurrence of amplification of N-*myc* is 25% in NB and 40% in the advanced stages of NB (Brodeur, 2003, Kohl et al., 1984, Schwab et al., 1984, Seeger et al., 1985). The reason why N-*myc* amplification is correlated to a more aggressive phenotype is still not known. There is corresponding overexpression of N-*myc* mRNA and protein (Seeger et al., 1988), which probably ensures through its target genes that cells stay in the cell cycle and not enter G0, and maintains cells at an immature stage (Nakagawara et al., 1992). N-*myc* amplification has strong correlation with loss of 1p heterozygosity (Fong et al., 1989). A large body of evidence shows the involvement of N-*myc* in NB development. For instance, the ectopic expression of N-Myc into neuroblastoma cells in culture leads to enhanced proliferative potential. Further, targeting N-*myc* to neuroectormal cells leads to development of NB in transgenic mice (Knoepfler et al., 2002, Thiele and Israel, 1988, Weiss et al., 1997). It has been shown that differentiation of N-*myc* amplified NB cell lines

in vitro with RA, IFN-γ and TPA correlate with N-Myc downregulation (Hammerling et al., 1987, Thiele et al., 1985, Watanabe et al., 1989). Further it has also been observed that inhibition of N-Myc expression by antisence technology promote the inhibition of growth and a more differentiated phenotype (Schmidt et al., 1994).

Cancer cells usually have increased telomerase activity. In neuroblastoma, telomerase activity has been shown to correlate inversely with patient outcome. There is also a direct correlation between high expression of telomerase and N-*myc* amplification (Hiyama et al., 1995). *Telomerase* (*hTERT*) has been shown to be a direct target gene of c-Myc and N-Myc (Lutz et al., 1996, Mac et al., 2000, Wang et al., 1998a) Neurotrophins (NT) are growth factors that regulate growth, development, survival and repair of the nervous system. They can both bind the high-affinity tyrosine kinase receptors belonging to trk family and low-affinity $p75^{NTR}$ receptor. The main ligands for the TrkA, TrkB and TrkC receptors are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) respectively. TrkB can also interact with NT-4 (Patapoutian and Reichardt, 2001). $p75^{NTR}$ receptor bind all NT but the function of this receptor is still unclear. Activation of the TrkA receptor by NGF leads to survival and differentiation of neuroblasts into ganglion cells. TrkA is expressed in NB tumours with favourable prognosis and is inversely correlated to N-Myc expression, whereas TrkB is expressed mostly in tumours with poor prognosis and N-*myc* amplification (Brodeur, 2003).

Neuroblastoma is conventionally treated by surgery, autologous bone marrow transplantation, radiation and chemotherapy. However, as the knowledge about important genes, proteins and pathways involved in pathology of neuroblastoma increases, it will probably lead to development of more biologically based therapies, such as differentiation therapy, induction of apoptosis and immunotherapy. Differentiation threapy is an approach that is promising in neuroblastoma since retinoic acid derivates have been shown to induce differentiation and decrease growth rate of neuroblastoma cells (Sidell et al., 1983, Thiele et al., 1985). In clinical trials, high-risk neuroblastoma patients have been treated with 13-cis retinoic acid after chemotherapy and bone marrow transplantation. A significantly increased survival rate with minimal toxicity was observed in this study (Matthay et al., 1999).

In vitro models of neuronal differentiation

Cell lines derived from tumours of the nervous system has been extensively used as model systems to study aspects of neuronal differentiation or biology of neuroblastoma. In murine systems, the rat pheochromocytoma cell line PC12 is the most frequently used for neuronal differentiation studies. There are several cell lines established from high-stage (mostly stage 3 and 4) human neuroblastoma tumours with N-*myc* amplification, such as frequently used IMR-32, LAN-2 and LAN-5 cell lines. Attempts to establish cell lines from low-stage tumours have not been so successful. One exception is the SH-SY5Y cell line with no N-*myc* amplification which is a well-established model for neuronal differentiation. SH-SY5Y is a subclone of the SK-N-SH cell line that was established from the bone

marrow biopsy of a 4-year-old girl with metastatic neuroblastoma (Biedler et al., 1973). The phorbol ester TPA, retinoic acid (RA) and certain growth factors are used to differentiate these cells. SH-SY5Y cells has been shown to differentiate and get growth arrested when treated with TPA and RA (Pahlman et al., 1981, Pahlman et al., 1984) (paper I). There are a number of characteristics of the differentiation process, such as extension of neurites and expression of certain neuronal differentiation markers. Growth-associated-protein-43 (GAP43), neuronal specific enolase (NSE), neuropeptide tyrosine (NPY) and choline acetyltransferase activity (ChAT) are among these markers (Pahlman et al., 1995). It has also been shown that neuroblastoma cell lines with amplified N-*myc* can differentiate to some extent in response to TPA, RA and IFN-γ (Haussler et al., 1983, Montaldo et al., 1992, Parodi et al., 1989, Sidell, 1982, Spinelli et al., 1982, Watanabe et al., 1989) (Paper I). For instance, RA and IFN-γ has been observed to differentiate LAN-5 cells and lead to neurite outgrowth, inhibition of proliferation and increased levels of neurotransmitters (Sidell, 1982, Wuarin et al., 1991).

In vitro models for myeloid differentiation

Establishment of leukaemia and lymphoma cell lines have made important contributions to the studies of these types of cancer, but also to studies of hematopoiesis, haematology in general and immunology. Some of these cell lines, which are relevant to this thesis, are U-937, HL-60 and K-562 cells. The human monoblastic U-937 cells are established from a patient with histiocytic lymphoma and are arrested at the monocytic stage of the differentiation (Sundstrom and Nilsson, 1976). It is well-documented that these cells can be induced to undergo terminal differentiation along the monocytic lineage with differentiation agents such as TPA, RA and VitD3. This makes U-937 cells an excellent model system to study myeloid/monocytic differentiation (Nilsson et al., 1981). Distinct agents give rise to different differentiation phenotypes (Larsson et al., 1988, Oberg et al., 1993, Olsson et al., 1983, Olsson and Breitman, 1982). The common characteristics for terminal differentiation of U-937 cells by these agents are growth arrest in G0/G1, increased expression of monocytic differentiation marker CD11c associated with β1 integrins and mature monocyte and macrophage morphology. The human HL-60 cell line is established from a patient with acute myeloid leukaemia (Collins et al., 1977), is bipotential and carry amplified c-myc gene. HL-60 cells, arrested at an early stage of myeloid differentiation, can be induced to differentiatiate by RA, TPA or DMSO treatment (Collins and Dorshkind, 1987). K-562 cells are established from a patient with chronic myeloid leukemia (CML) in blast crisis. K562 is frequently used as model system for studies of erythroid and megakaryocytic differentiation.

Aims of the present investigation

The general objective of this thesis was to gain more knowledge about the role of Myc/Max/Mad-network proteins in the control of growth and differentiation of human neuronal and hematopoietic tumour cells. Since deregulation of *myc*-family genes is frequently observed in these types of human cancers, an import aim is to identify signals that negatively regulate Myc function, and which potentially may contribute to novel therapeutic strategies for treatment of such cancers.
The thesis can be divided into three parts. The first part (Paper I and II) is based on previous results from hematopoietic cells demonstrating that IFN-γ can restore induced terminal differentiation and growth arrest in v-Myc-transformed human monoblasts despite continuous v-Myc expression. The aim of this part was to clarify whether IFN-γ when combined with differentiation signals could restore terminal differentiation and growth arrest also in N-*myc*-amplified human neuroblastoma cells, and if so whether the effects of the combinational therapy were due to regulation of Myc/Max/Mad-network proteins. In Part II (Paper III) the biological role of Mad1 during differentiation and growth inhibition in hematopoietic cells was investigated. The aim of the last part was to shed light on the ability of c-Myc to block hematopoietic differentiation and how this relates to its function to repress transcription. Since the mechanism by which Myc represses transcription is poorly understood, another objective in this part of the work was to gain more knowledge about this process.

Specific aims:

1. To determine whether IFN-γ in combination with RA or TPA would have synergistic effects in inducing differentiation and/or growth arrest in N-*myc* amplified neuroblastoma cells. (Paper I)

2. To clarify whether induced neuronal differentiation of human neuroblastoma cells, with or without amplified *N-myc,* involves regulation of Myc/Max/Mad network proteins (Paper II)

3. To shed light on the role of Mad1 during differentiation and growth arrest in human hematopoietic cells (Paper III)

4. To determine whether the ability of Myc to block hematopoietic differentiation involves repression of transcription of cyclin-dependent kinase inhibitors such as p21Cip1 and to reveal the mechanism behind such transcriptional repression by Myc (Paper IV)

Results and Discussion

Interferon-γ cooperates with retinoic acid and phorbol ester to induce differentiation and growth inhibition of human neuroblastoma cells (Paper I)

Advanced stages of neuroblastoma exhibit amplifications of N-*myc* at 30-40% of the cases and is correlated to poor prognosis despite therapy. These circumstances necessitate new approaches for therapy. It has previously been shown that neuroblastoma cells, including cells with N-*myc* amplification, treated with differentiation agents such as retinoic acid (RA), phorbol ester (TPA) and IFNs in vitro undergo cellular differentiation and stop to divide (Haussler et al., 1983, Pahlman et al., 1981, Pahlman et al., 1984, Parodi et al., 1989, Sidell, 1982, Spinelli et al., 1982, Watanabe et al., 1989). In clinics 'differentiation therapy' with RA has been used after autologous bone barrow transplantation with encouraging results. We investigated here the possibility to improve differentiation therapy by combining agents known to induce differentiation of neuroblastoma cells *in vitro*. Our group has earlier shown that combination of RA or TPA with IFN-γ will overcome the Myc induced growth arrest and differentiation in monoblastic U-937 despite continuous expression of v-Myc protein (Bahram et al., 1999, Oberg et al., 1991). Further, in previous studies RA and IFN-γ has been shown to have synergistic effects on differentiation and cell growth in LAN-5 cells (Cornaglia-Ferraris et al., 1992, Wada et al., 1997, Wuarin et al., 1991). Therefore we chose to combine IFN-γ with RA and TPA to study their effects on cell growth and differentiation in neuroblastoma cells. The aim of this study was to investigate if the synergistic effects of IFN-γ and RA could be observed in other neuroblastoma cell lines than LAN-5 and also if IFN-γ synergize with TPA.

In our study we utilized 5 neuroblastoma cell lines with (LAN-1, LAN-2, LAN-5 and IMR-32) or without (SH-SY5Y) N-*myc* amplification. These cell lines were subjected to substances RA, TPA, IFN-γ alone or in combination. The effects of these treatments on proliferation and viability were determined by cell counting. Differentiation was evaluated by morphology changes, by measurement of neurite length and number, and by studying the expression of neurofilaments as a marker of neuronal differentiation.

Our results showed that RA induced differentiation to various extents in all cell lines studied. The most prominent effect was seen in SH-SY5Y cells which lack N-*myc* amplification. Further, RA had also growth inhibitory effects in all cell lines treated. In cell lines LAN-1 and LAN-2 lower cell viability might have contributed to growth inhibitory effects. RA in combination with IFN-γ enhanced differentiation and/or growth inhibition in all five cell lines, but particularly in cell lines with elevated N-Myc expression. Enhanced differentiation was observed as increased percentage of differentiated cells and increased length of neurites. Complete growth inhibition was seen in LAN-5, IMR-32 and LAN-1 containing N-*myc* amplification after 2-4 days of RA+IFN-γ treatment. LAN-2 cells, which express much less N-*myc* mRNA despite amplification and SH-SY5Y cells with

normal N-*myc* alleles, responded to RA treatment effectively by decrease in cell number. Addition of IFN-γ enhanced the effect to some extent.

TPA affected differentiation and growth to different degrees in cell lines included in the study. In SH-SY5Y cells, TPA had strong effects on proliferation and differentiation in agreement with previous reports (Pahlman et al., 1981, Pahlman et al., 1984, Spinelli et al., 1982). LAN-1 and LAN-5 cells were growth inhibited to a lesser extent and LAN-2, IMR-32 cells were only affected poorly. TPA had poor or none effect on differentiation of these four cell lines. Combination of TPA with IFN-γ led to enhanced growth inhibition in various degrees in all cell lines. LAN-5 and IMR-32 cells, with high N-Myc levels, responded strongest to TPA+IFN-γ treatment. The viability of IMR-32 cells was somewhat lower and this might have contributed to the inhibitory growth effects. TPA+IFN-γ had pronounced effects on differentiation in all cell lines but LAN-2. Percentage of differentiated cells and neurite length were increased in LAN-1, LAN-5, IMR-32 and SH-SY5Y cells. Further, expression of neurofilament in LAN-5 and IMR-32 after TPA, IFN-γ or TPA+IFN-γ supported data on proliferation and differentiation suggesting that TPA+IFN-γ stronger effects than the either agent alone.

IFN-γ alone induced differentiation to some extent in all five cell lines but the strongest effects were seen in LAN-1 and SH-SY5Y cells. Growth inhibition was observed after IFN-γ induction in cell lines LAN-5 and SH-SY5Y and to a lesser extent in IMR-32 cells.

In summary, the combined treatments RA+IFN-γ or TPA+IFN-γ had synergistic or enhancing effects on morphological differentiation, neurite outgrowth and proliferation in all cell lines, in particular in cell lines with amplified N-*myc*. Single agent treatment were mostly effective in SH-SY5Y cells lacking N-*myc* amplification. These results may be of potential interest for the development of combinational differentiation therapy in the treatment of high risk neuroblastoma.

Mad 1 is upregulated of during human neuronal differentiation (Paper II)

The expression of the *mad*-family genes is regulated during development and differentiation of a variety of cell types as discussed above, including neuronal cells (Ayer and Eisenman, 1993, Hurlin et al., 1995a, Hurlin et al., 1995b, Queva et al., 1998, Vastrik et al., 1995). For instance, high expression of *mad*1 and *mad*4 is observed in differentiated cells during murine neural tube development, whereas *mxi1*, *mad*3 and *mnt* are coexpressed with N-*myc* in proliferating cells undergoing differentiation (Hurlin et al., 1997, Hurlin et al., 1995b). Expression of *mxi1* and *mnt* can also be found in more differentiated cells. Little is however known about Mad family expression during human neuronal differentiation.

To study the expression of *mad/mnt* family genes and proteins during human neuronal differentiation, we made use of the SH-SY5Ycell line, which is a widely used model system for various studies of symphatetic neuronal cells (Pahlman et al., 1981, Pahlman et al., 1983). We first investigated the expression patterns of *mad1, mxi1, mad3, mad4, mnt, max, c-myc and N-myc* mRNA during TPA-

induced SH-SY5Y differentiation by northern blot analysis. Increased expression of *mad*1 mRNA was detected already after one hour of TPA treatment while the low expression of c-*myc* declined, but expression of the other genes were either unchanged (*mxi*1, *mnt, max*), or too weak to be detected (*mad*3, *mad*4, N-*myc*)

To study the corresponding proteins during the differentiation process immunoprecipitations of lysates from ^{35}S-methionine in vivo labelled cells were performed. The synthesis of Mad1 began to increase after 1 hour and reached a peak after 4 hours. A decrease in Mad1 to pre-induction levels was observed after 24 hours of TPA induction with a subsequent upregulation again at 48 and 96 hours demonstrating a biphasic expression pattern. The expression of *mad1* mRNA exhibited a similar kinetic pattern. This biphasic expression pattern was also observed in another neuroblastoma cell line, SK-N-BE, in a recent study by Smith et al (Smith et al., 2004) and as well as in murine erytroleukemia (MEL) cells (Cultraro et al., 1997b). The myeloid and keratinocyte in vitro differentiation system also exhibit an early upregulation of Mad1 but unlike the cell lines mentioned above they do not exhibit a biphasic pattern of Mad1 expression. The differences in the Mad1 kinetics indicate that the regulation of Mad1 expression is complex and that various differentiation signals might act on *mad1* promoter. In addition, differences in cell type and level of differentiation may also contribute to the dissimilarities.

c-Myc protein synthesis did also display a biphasic expression pattern. Max protein levels remained constant during the first 24 hrs of TPA induction and increased a little bit at later time points. The expression level of Mad1 was lower than U-937 myelomonocytic cells used as reference. Since c-Myc levels were also low the upregulation of Mad1 might still be enough to cause a switch from c-Myc:Max to Mad1:Max heterodimers on c-Myc/Mad1 target genes. Unfortunately, we did not posses antibodies of high enough quality to analyse other Mad/Mnt-family proteins. N-Myc was not detectable in these cells.

In summary, the expression of Mad1 is upregulated and c-Myc downregulated in SH-SY5Y cells during TPA induced differentiation. This result thus resembles the expression of *mad*1 in differentiated murine neuornal cells as described above and indicates a switch towards Mad1 predominance in Myc/Max/Mad network, preceding differentiation and growth arrest of human neuronal cells.

Regulation of the Myc/Max/Mad network in human neuroblastoma cells with N-*myc* amplification using IFN-γ together with RA or TPA (Paper I and II)

In paper I we demonstrate that IFN-γ can synergize with RA or TPA to enhance differentiation and growth arrest in a number of neuroblastoma cell lines, in particular in cell lines with amplified N-*myc*. It was therefore of interest to investigate mechanisms involved in the effects of IFN-γ and whether a shift in the N-Myc/Mad1 expression pattern would correlate with IFN-γ enhanced differentiation and growth arrest in these cells. We utilized the N-*myc* amplified cell lines IMR-32 and LAN-5 for these studies. The cells were treated with RA, TPA, IFN-γ alone or in combination, for three days. The protein expression of

Mad1, N-Myc and Max was studied by immunoprecipitations of ^{35}S-methionine labelled cell lysates.

In contrast to non-N-*myc* amplified SH-SY5Y cells, Mad1 was only upregulated weakly in IMR-32 and even less in LAN-5 cells after TPA treatment. Combination of TPA with IFN-γ led to a strong upregulation of Mad1 in LAN-5 cells and to a lesser degree in IMR-32 cells. This was correlated with synergistic effects of TPA+IFN-γ on differentiation and growth inhibition. N-Myc protein levels were not at all or weakly reduced after TPA or TPA+IFNγ treatment.

RA+IFN-γ treatment did also increase Mad1 expression levels, although not to the same extent as TPA+IFNγ but on the other hand, led to a downregulation of N-Myc levels in both cell lines. Reduced N-Myc expression was in good agreement with downregulated N-*myc* mRNA expression in LA-N-5 cells (Wada et al., 1997). Treatment with RA alone had less effect on the expression of N-Myc, approximately 2-fold reduction or less in repeated experiments in both cell lines.

In conclusion, the cooperative effect of TPA and IFN-γ on differentiation and growth arrest correlated with increased Mad1 protein levels. The similar synergistic effects of RA and IFN-γ primarily correlated with decreased N-Myc levels. This suggests that different signals affect the Myc/Max/Mad network in distinct ways but with similar outcomes.

IFN-γ costimulation results in reduced N-Myc/Max complex formation and Mad1/Max predominance at ODC promoter (Paper II)

It has earlier been reported that cellular concentration of Max containing complexes shifts from c-Myc/Max to Mad1/Max predominance during induced differentiation of hematopoietic cells and keratinocytes (Ayer and Eisenman, 1993, Hurlin et al., 1995a, Larsson et al., 1997) resulting in preferential Mad1 occupancy of the cyclin D2 and hTERT target gene promoters (Bouchard et al., 2001, Xu et al., 2001).

We performed coimmunoprecipitations of N-Myc/Max complexes from IMR-32 cell extracts followed by western blot analysis to study whether the cellular amounts of N-Myc:Max heterodimers were changed in response to TPA, RA and IFN-γ or their combinations in N-*myc*-amplified neuroblastoma cells. Slight reductions in N-Myc/Max complexes were observed after RA, TPA and IFN-γ treatments. The combined treatments of RA+IFN-γ and TPA+IFN-γ, on the other hand, resulted in considerable reductions in the concentrations of N-Myc/Max heterodimers. This reduction in the case of RA+IFN-γ seem in part to be due to decreased N-Myc protein expression and partially due to posttranslational effects on N-Myc:Max complex formation and/or complex stability. In the case of TPA+IFN-γ posttranslational effects seem to predominate. Therefore, the phenotypic changes in response to combinational treatments are likely not only due to changed Mad1 and N-Myc expression. Similar results were reported in v-Myc transformed U-937 monoblasts, where IFN–γ in combination with TPA

inhibited v-Myc activity rather than affecting the v-myc expression (Bahram et al., 1999).

Further, we asked whether occupancy of N-Myc, Max and Mad1 on the N-Myc and Mad target gene *ODC* promoter will also shift towards Mad1 predominance. We performed ChIP assays using undifferentiated and RA+IFN- γ differentiated IMR-32 cells. A dramatic decrease in N-Myc occupancy was observed, while Mad1 binding increased in differentiated cells. The presence of Max was essentially unchanged after RA+IFN-γ treatment. In addition, RA+IFN-γ induced differentiation led to decreased acetylation of histone H4 indicating alteration of the chromatin structure into a more condensed state.

In summary, these results suggest that IFN-γ in combination with RA or TPA regulates the expression of N-Myc and/or Mad1 resulting in a shift in balance towards Mad1/Max heterodimers. Mad1/Max occupy target gene promoters which results in altered chromation structure and transcriptional repression, correlating with terminal differentaion and growth arrest. It is possible that also other factors can also be regulated and cooperate with Mad1 in inducing differentiation and inhibiting growth, such as $p21^{Cip1}$ and $p27^{Kip1}$. $p21^{Cip1}$ has been shown to be regulated by IFN–γ via the JAK-STAT pathway (Chin et al., 1996). RA treatment of neuroblastoma and hematopoeitic cells has lead to upregulation of both $p21^{Cip1}$ and $p27^{Kip1}$ (Borriello et al., 2000, Jiang et al., 1994, Liu et al., 1996). TPA has also been shown to induce $p21^{Cip1/Waf1}$ (Biggs et al., 1996, Blagosklonny et al., 1997, Jiang et al., 1994, Zeng and el-Deiry, 1996).

Our obtained results with the neuroblastoma cells with N-*myc*-amplification suggest that combined RA+IFN-γ "differentiation therapy" potentially may be of clinical interest in treatment of advanced stages of childhood neuroblastoma.

Mad1 inhibits cell growth and proliferation but does not promote differentiation or overall survival in human U-937 monoblasts (Paper III)

Mad is usually expressed in differentiating and/or growth arrested cells as mentioned earlier. Utilizing U-937 monocytic differentiation model system we and others have previously shown that Mad1 is rapidly induced during RA, TPA and vitamin D3 (VitD3) induced differentiation. The biological role of Mad1 during differentiation and growth inhibition is, however, not well understood. The aim of this study was to shed light on this role by investigating the effects of ectopic expression of Mad1 during these processes.

For these studies, we established U-937-1 clones with stable integration of inducible Mad1 constructs by using the tet-activator and lac-repressor systems. This tet-on system (Gossen et al., 1995) was regulated by the rtTA-activating ligand doxycycline (Dox). The clones were studied with regards to proliferative capacity, differentiation and cell cycle distribution. The question asked here was whether Mad1 expression would be enough to induce growth inhibition and differentiation in the absence of differentiation signals, alternatively whether it would enhance differentiation induced by these signals.

First, a number of clones were selected and *mad1* mRNA and protein levels were studied by northern blot analysis and by immunoprecipitation of 35S-methionine labelled cell lysates with Mad1 antibodies, respectively. The clones exhibited various basal and inducible Mad1 levels. Kinetic analysis revealed that Mad1 expression was upregulated after 6 hours of Dox treatment and the protein levels remained stable at least 48 hours after induction. Coimmunoprecipitations showed that increased levels of Mad1 correlated with increased levels of Mad1/Max heterodimers. In a control clone containing the *luciferase* gene instead of *mad1* the endogenous amount of Mad1 in complex with Max was very low. Further, we studied whether the elevated basal level and/or the Dox-induced expression of Mad1 affected the expression of Myc/Mad target gene *ODC* by realtime RT-PCR. Our results showed that induction of Mad1 led to reduced expression of *ODC*. Taken together, these results indicate that our inducible system was working and that exogenous Mad1 proteins were active and had a functional role.

We next investigated the proliferative potential of Mad1-expressing U-937 cells and found that it was substantially lower than U-937 control cells. Mad1 clones also formed fewer colonies on semi-solid medium. Addition of Dox only partially enhanced this effect indicating that elevated basal expression of Mad1 may have exceeded a threshold level effecting proliferation. A mutant clone which lack NH2 terminus of Mad1 containing SID domain did not exhibit reduced proliferative capacity suggesting that NH2 terminus of Mad1 is required for the antiproliferative action in agreement with previous reports (Grandori et al., 2000). Cell cycle analysis showed that the Mad1-clones exhibit an increased number of cells in the G1 phase of the cell cycle. Addition of nocodazole to block cells in G2/M phase showed that the Mad1 expressing clones contained a larger subpopulation of cells arrested in G1 than the control clone. The majority of the Mad1 cells continued to cycle until the nocodazole block G2. These results agree with the results suggesting that Mad1 increases the probability of cells to arrest in (or exit cell cycle at) G1 rather than affecting duration of the cell cycle (Holzel et al., 2001). In summary, our results indicate that Mad1 has growth inhibitory effects supporting previous data.

Both Myc and Mad have been shown to affect cell size. In certain but not all cellular settings, Myc seems to stimulate cell growth independent of the cell cycle whereas Mad1 has been reported to reduce cell size in thymocytes (Iritani et al., 2002, Iritani and Eisenman, 1999, Johnston et al., 1999, Schuhmacher et al., 1999). Our results show that Mad1-expressing cells are smaller than control cells in all phases of the cell cycle and this difference is particularly pronounced in RA differentiated cells. The reason why Myc and Mad affect cell size independent of cell cycle phase in some but not all cellular settings is not clear but may reflect variations in the link between cell growth and cell cycle control in different cells.

We next investigated whether induced Mad1 expression could affect the stage of differentiation or the capacity to differentiate. The state of differentiation was determined by measuring CD11c and CD14, surface antigens specific for monocyte differentiation, by flow cytometry at different time points after addition of Dox. No significant change was detected in the expression of surface antigens

on Mad1-containing clones compared to control clones both in presence and absence of Dox treatment. This suggests that Mad1 expression can not result in spontaneous differentiation of U-937 cells. Further, no enhancing effects of Mad1 on differentiation induced by RA, TPA or VitD3 could be observed. In contrast, the RA induced differentiation was rather inhibited (indicated by a lower percentage of cells expressing CD11c) in Mad1-containing clones at day 1 and 3 after induction. However, by 5 days, the percentage of CD11c expressing cells was approximately the same as in the control cells suggesting that RA-induced differentiation was delayed in Mad1-expressing cells. The mutant Mad1 clone, lacking SID containing NH2 terminal part of Mad1, did not exhibit the delay suggesting that interaction of Mad1 with the Sin3 transcriptional repressor complex is required for this process to take place.
Our results contrast to the data from Cultraro et al. (Cultraro et al., 1997a) which suggest that enforced Mad1 expression induces differentiation of MEL cells. On the other hand, our results agree with results from Pulverer et al. (Pulverer et al., 2000) suggesting inhibitory effects of Mad1 on adipocyte differentiation. This discrepancy may have several explanations. The two reports mentioned above and our results all agree on Mad1 as a growth inhibitor. The antiproliferative effect of Mad1 might effect differentiation in different ways depending on cell type and in some cases acting indirectly. Usually, differentiation and proliferation are well-matched at earlier stages but in conflict at later stages of differentiation. For example, U-937 cells keep proliferating during the first days of RA-induced differentiation before arresting in G1 phase of the cell cycle. Mad1-expressing U-937 cells on the other hand stop proliferating prematurely and exhibit reduced size in response to RA, correlating to delayed differentiation. Mad1 seem to interfere with the proliferative burst at the early stage of differentiation which seems to be necessary for the continuous differentiation. In addition, some growth inhibitory signals for U-937 cells, such as transforming growth factor β (TGF-β) that induce Mad1 expression, can inhibit differentiation (Oberg et al., 2001).

We also investigated whether Mad1 expression favours or disfavours apoptosis, since there are conflicting results regarding this in the literature. The cells were treated with TNF-α or CD95-Fas, well-known inducers of apoptosis, and the percentage of apoptotic cells was measured by Annexin V staining. TNF-α sensitized the Mad1-expressing cells to apoptosis whereas α-Fas did not but rather desensitized the cells to some extent. The increase in TNF-α sensitivity was not observed in a Mad1 mutant clone lacking the N-terminus of Mad1 containing the SID domain, suggesting that it involved Mad1-induced repression of target genes. Mad1 induced desensitisation by Fas-induced apoptosis also required the SID domain. The mechanism by which Mad1 sensitizes U-937 cells to TNF-α induced apoptosis is still unknown. Both Fas and TNF-α utilize a common signalling pathway to induce apoptosis involving FADD/caspase 8 and 10. It has been reported that Mad1-induced inhibition of Fas-induced apoptosis in osteosarcoma cells correlated with reduced levels of caspase activity (Gehring et al., 2000). TNF-α receptor signalling is much more complex than Fas-signaling since it both involves pro- (FADD/caspase 8 and 10) and anti-apoptotic (TRAF2/RIP1/NFκB) pathways (Nilsson and Cleveland, 2003, Varfolomeev and Ashkenazi, 2004). In addition, TNF-α also activates c-*jun* NH2-terminal kinase (JNK) pathway which

exhibit both pro- and anti-apoptotic abilities. Since in our system, Mad1 sensitized TNF-α-induced apoptosis but not Fas-induced apoptosis, this might indicate that Mad1 inhibits the antiapoptotic NFκB pathway or enhances the proapoptotic JNK pathway. This hypothesis needs to be investigated further.

In conclusion, our results suggest that enforced expression of Mad1 in U-937 cells inhibits cell growth and proliferation but does not promote differentiation or survival in general. The effects of Mad1 in differentiation and apoptosis seem to be more complex and necessitate further investigation.

Myc represses differentiation-induced p21Cip1 expression via Miz-1-dependent interaction with the p21 core promoter (Paper IV)

One of the well-known biological activities for c-Myc family proteins is its block of cellular differentiation. The mechanism behind this is however not well understood. The expression of c-Myc is generally downregulated in response to differentiating signals (Oster et al., 2002). Our group has earlier shown that constitutive expression of v-Myc blocks induced differentiation and growth arrest of human U-937 cells (Bahram et al., 1999, Larsson et al., 1988). The question we asked here was how c-Myc is involved in the block of differentiation. $p21^{Cip1}$ is rapidly induced in hematopoietic cell lines, including U-937 cells (Liu et al., 1996), after treatment with differentiating agents and is believed to be an important component of the cellular growth/differentiation switch. We therefore hypothesized that v-Myc might interfere with the expression of p21 to block differentiation. As mentioned earlier $p21^{Cip1}$ belongs to Cip/Kip family of Cdk inhibitors and plays an important role in cell cycle arrest, differentiation, DNA repair, cell senescence and apoptosis (Sherr and Roberts, 1999, Steinman, 2002).

The ability of TPA to induce differentiation and growth arrest in U-937-1 and v-Myc transformed U-937-myc6 cells were determined by measuring expression of the differentiation marker CD11c and by the incorporation of ^{3}H-labeled thymidine, respectively. U-937-1 cells differentiated as aspected, exhibiting increased expression of CD11c and reduced incorporation of ^{3}H-labeled thymidine. The v-Myc-expressing U-937-myc6 cells on the other hand showed no induction of CD11c and almost no reduction of ^{3}H-labeled thymidine indicating that differentiation and growth inhibition by TPA were blocked by v-Myc, in agreement with previous report (Larsson et al., 1988). We next asked if the expression of $p21^{Cip1}$ in response to TPA was altered in the v-Myc expressing cells. In U-937-1 cells $p21^{Cip1}$ mRNA and protein are detected after 4 hours post-induction whereas no induction at all could be seen in the U-937-myc6 cells. c-Myc levels declined as $p21^{Cip1}$ levels increased in both cell lines, whereas v-Myc levels was unaltered in U-937-myc6 cells. These results suggest that v-Myc directly or indirectly repress TPA-induced expression of $p21^{Cip1}$.

Next, we utilized transient transfection assays to investigate whether Myc can repress the $p21^{Cip1}$ promoter. U-937 cells were transfected with a full length p21 promoter/reporter construct (0-luc) together with increasing amounts of c-Myc.

Both in absence or presence of TPA, coexpression of c-Myc led to a repression of reporter activity already at low levels of c-Myc suggesting that c-Myc affects $p21^{Cip1}$ expression at least partially at the level of transcription. A number of $p21^{Cip1}$ deletion mutants were utilized to map the Myc-responsive region of the $p21^{Cip1}$ promoter. Our analysis showed that deletions of sequences -2326 bp to -94 bp relative to the $p21^{Cip1}$ start site did not abolish the ability of c-Myc to repress basal- and TPA-dependent transcription of $p21^{Cip1}$ expression. To further map c-Myc responsive region two exchange mutants were generated by replacing either nucleotides -94/-50 of the p21 promoter (generating CMV/p21Luc) or nucleotides -49/+16 with the corresponding region of CMV promoter (generating p21/CMVLuc). c-Myc was unable to repress the activity of p21/CMVLuc indicating that the c-Myc responsive region is situated between -49 and +16 of the *p21* core promoter. This also suggest that the four Sp1/3 binding sites located in the upper region of the promoter, which has previously been shown to be required for p21 induction by multiple signals (Gartel and Tyner, 1999), is not essential for the repressing effect of c-Myc. Further, to map domains of c-Myc required for repression a panel of c-Myc deletion mutants were used. We found that deletion of conserved HLH, Zip and MBII domains abolished the ability of c-Myc to mediate repression whereas deletion of the basic domain did not affect the transcriptional repressing indicating that direct binding of c-Myc to DNA is not required.

The HLH domain of c-Myc interacts with the initiator (Inr) binding transcription factor Miz-1 (Peukert et al., 1997) which has been shown to bind p15Ink4b at the core promoter (Peukert et al., 1997, Seoane et al., 2001, Staller et al., 2001). Since we had observed that HLH domain of c-Myc was involved in repression of *p21* promoter, we investigated the possibility that c-Myc exerts its repressive function through Miz-1. Using transient transfection and luciferase assays we studied ability of Miz-1 to activate *p21* promoter. We observed that Miz-1 activated p21 via the same core promoter region as c-Myc utilizes in repression. Three potential Miz-1 binding sites in this region of the promoter were identified and mutations of these sites eliminated most of the activating effect of Miz-1 suggesting that Miz-1 act through these sites. When c-Myc was cotransfected it inhibited Miz-1-dependent transcription in a dose dependent manner in both TPA treated and untreated cells. Further to investigate whether Miz-1 could be involved in differentiation processes, the expression of Miz-1 was studied. Miz-1 mRNA and protein were upregulated during differentiation in several myeloid cell lines, whereas c-Myc was downregulated as expected.

To investigate whether Miz-1 and c-Myc are able to bind the *p21* core promoter *in vitro,* DNA oligo pulldown assays were utilized. Miz-1- and/or c-Myc-transfected Cos7 cells were precipitated with a double stranded oligonucleotide representing nucleotides -49 to +16 of the *p21* promoter. Our results showed that Miz-1 alone bound to the promoter whereas c-Myc could not bind on its own. Once c-Myc was cotransfected with Miz-1 it could interact with the promoter indicating that c-Myc bind *p21* promoter through Miz-1 interaction. Interestingly, we also observed that Miz-1 DNA binding was enhanced when cotransfected with c-Myc suggesting that c-Myc might stabilize the DNA binding of Miz-1. The dependence of c-Myc on Miz-1 for DNA binding was further demonstrated in this assay by using a c-Myc HLH point mutant, MycV394D, with impaired binding to

Miz-1 but normal binding to Max (Herold et al., 2002). The mutant MycV394D showed a reduction both in Miz-1 and DNA binding to approximately 25-30% of that of wt c-Myc. Another mutant, Myc(BR)-Mad, where the HLHZip domain is replaced by the corresponding region in Mad1, is unable to bind Miz-1 but binds to Max and activates transcription from E-boxes (Staller et al., 2001). Unlike wildtype c-Myc, this mutant and MycV394D was unable to repress transcription of the *p21* core promoter. Further, to demonstrate that repression of the endogenous *p21* gene by Myc was Miz-1 dependent we utilized K562 leukaemia cells containing a Zn^{2+} inducible wt c-Myc or MycV394D genes. TPA induced endogenous p21 expression was repressed by wt c-Myc but not by the mutant confirming the results from the *p21* promoter/reporter assay. Next, we used chromatin immunoprecipitation (ChIP) assay to study whether c-Myc and Miz-1 interact with the *p21* promoter also *in vivo*. This analysis indeed showed presence of c-Myc, Miz-1 and Max on the p21 promoter. In addition, the analysis also demonstrated that the association of c-Myc with the *p21* promoter was reduced after TPA-induced differentiation of HL-60 cells while the binding of Max and Miz-1 was only slightly affected. On the other hand, binding of v-Myc, Miz-1 and Max to *p21* promoter increased after TPA treatment in v-Myc transformed cells, correlating with the expression of p21.

Taken together our results suggest that c-Myc represses both differentiation induced and basal *p21* expression at least partially at the level of transcription. This is in agreement with previous reports suggesting that c-Myc inhibits both basal and TGF- -, TPA-, and p53-induced *p21* expression in other cell lines (Ceballos et al., 2000, Claassen and Hann, 2000, Coller et al., 2000, Gartel et al., 2001, Mitchell and El-Deiry, 1999). Our results further suggest that the Myc-responsive region on *p21* promoter is located around the transcriptional start site and that c-Myc binds this region via interaction with Miz-1, which associates directly with the *p21* promoter. It has earlier been shown that c-Myc also utilizes Miz-1 for repression at the $p15^{INK4B}$ promoter (Seoane et al., 2001, Staller et al., 2001). We could also show that repression of the endogenous p21 gene depends on sites specifically interacting with Miz-1 by using mutants of c-Myc that are unable to bind Miz-1.

c-Myc/Miz-1 functions as a growth/differentiation switch (Paper IV)

The mechanism through which Myc represses transcription is not well understood. However, different models have been proposed on how c-Myc might mediate this process (for review see (Wanzel et al., 2003)). One of these models is that c-Myc activates the synthesis of transcriptional repressor proteins and in this way acts indirectly to repress genes. Another suggested model is that the Myc/Max heterodimer represses transcription by direct DNA-binding to E-boxes. However, so far Myc/Max dimers have not been found to interact directly to core promoters of repressed genes *in vivo*. It has also been suggested that c-Myc might sequester a necessary component of the basal transcription machinery by binding to the TFIID subunit TBP and thereby interfere with the preinitiation complex via an active

repression mechanism (Facchini and Penn, 1998). The interaction of c-Myc with activators, such as Smads, Sp1 or NFY, might prevent their activation and lead to repression thereby (Gartel and Shchors, 2003). Another possibility is that Myc is recruited to the core promoters by protein-protein interactions, for example, Inr-binding proteins, such as TFI-II, YY1, Miz-1 and Sp1 (Gartel et al., 2001, Peukert et al., 1997, Roy et al., 1993, Shrivastava et al., 1993).

Our results are in agreement with the model suggesting recruitment of c-Myc to the promoter by protein-protein interactions. Based on our results we suggest a model there Miz-1 plays a dual role (figure 5). Miz-1 can act as an activator of the

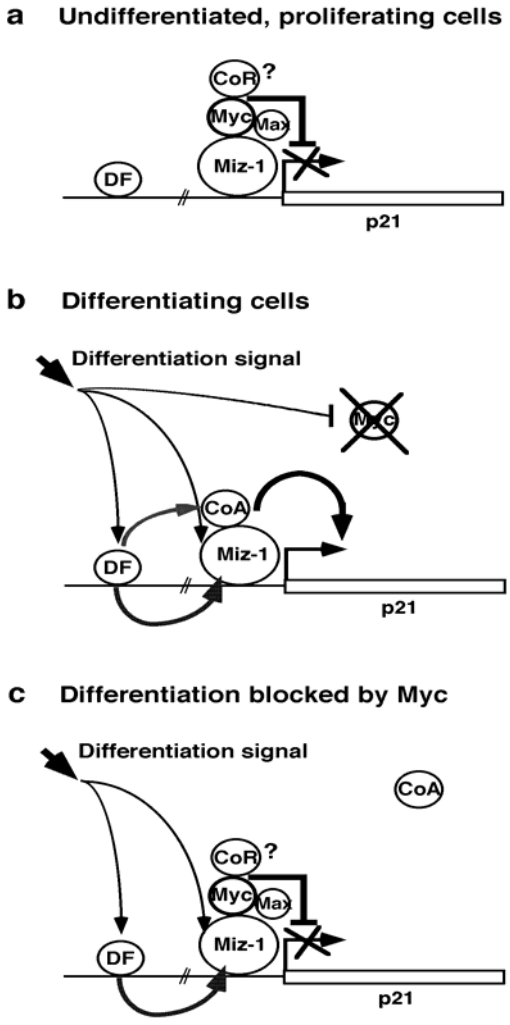

Figure 5: A Myc/Miz-1 switch model for regulation of *p21* gene expression during differentiation. DF: differentiation factor, CoR: corepressor, CoA: coactivator

p21 promoter and since its expression is increased during hematopoietic differentiation it may have a positive regulatory role in this process. However, Miz-1 can act as a repressor of the same promoter when it is cooperating with c-Myc. This is supported by our data indicating that c-Myc utilizes Miz-1 for repression of *p21* and it enhances DNA binding activity of Miz-1 *in vitro* and possibly also *in vivo*. Myc has also been reported to facilitate the transport of Miz-1 to the nucleus (Peukert et al., 1997). Here we suggest that c-Myc/Miz-1 functions as a growth/differentiation which resembles the E2F/Rb switch for G1/S transition. In our suggested model, Miz-1 in complex with Myc represses *p21* and thereby promoting cell growth and differentiation. When c-Myc is downregulated by differentiation signals, Miz-1 operate together with cofactors and other transcription factors to activate transcription of *p21,* which in turn stimulates terminal differentiation. Our model is in agreement with other findings from studies concerning Myc/Miz-1 interaction. For instance, p15Ink4b promoter has been shown to be regulated by Miz-1 and c-Myc (Seoane et al., 2001). In another paper it has been suggested that TGF-β downregulates expression of *c-myc* and release Miz-1 from an inhibitory complex with c-Myc. This allowed an interaction between Miz-1 and p300 and thereby activated transcription from the p15Ink4b promoter (Staller et al., 2001).

Miz-1 seems to be a keyplayer for both negative and positive signals which regulate the *p15* and *p21* core promoter. A question that can be asked is how Myc transform Miz-1 from an activator to a repressor. In p15 repression it has been suggested that c-Myc prevents binding of the coactivator p300 to Miz-1 by binding to through its HLH domain to the same surface on Miz-1 as p300 (Staller et al., 2001). However, we have found apart from the HLH region, that both Zip and MBII are required for c-Myc to be able to repress the *p21* promoter. This contradicts the results from Staller et al. A possible scenario is that a corepressor is recruited to the *p21* promoter possibly via MBII as shown in our model. Interestingly, in a recent paper, it has been shown that Myc recruits a DNA methyltransferase corepressor, Dnmt3a, to the $p21^{Cip1}$ promoter and thus leading to mehtylation and gene silencing (Brenner et al., 2005). c-Myc, Miz-1 and Dnmt3a form a ternary complex to repress the promoter. These results are therefore in good agreement with our proposed model.

Conclusions

1 The expression of Mad1 is upregulated while c-Myc is downregulated during TPA-induced neuronal differentiation of human SH-SY5Y neuroblastoma cells.

2 Combined treatment with IFN-γ and RA or TPA has synergistic or enhancing effects on morphological differentiation, neurite outgrowth and growth arrest in human neuroblastoma cell lines, in particular in those cell lines with amplified N-*myc*.

3 IFN-γ -induced terminal differentiation of N-myc-amplified neuroblastoma cells involves downregulation of N-Myc and/or upregulation of Mad1 expression resulting in reduced N-Myc/Max heterodimerisation, shift in the occupancy of the ODC target promoter in vivo from N-Myc/Max towards Mad1/Max and repression of N-Myc/Mad1 target genes.

4 Enforced expression of Mad1 in human U-937 monoblasts inhibits cell growth and proliferation but does not promote differentiation or survival in general.

5 Differentiation-induced p21Cip1 expression is repressed by Myc in a Miz-1 dependent manner, correlating with inhibited differentiation. c-Myc binds to the p21Cip1 core promoter in vivo through interaction with Miz-1, and seems to switch Miz-1 function at the promoter from an activator to a repressor.

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