Regulation and Function of the Mad/Max/Myc Network during Neuronal and Hematopoietic Differentiation

BY

ANNE HULTQUIST
Dissertation for the Degree of Doctor of Philosophy, Faculty of Medicine, presented at Uppsala University in 2001

ABSTRACT


The Mad/Max/Myc transcription factor network takes part in the control of vital cellular functions such as growth, proliferation, differentiation and apoptosis. Dimerization with the protein Max is necessary for the Myc-family of oncoproteins and their antagonists, the Mad-family proteins, to regulate target genes and carry out their intended functions. Myc functions as a positive regulator of proliferation, antagonized by the growth inhibitory Mad-proteins that potentially functions as tumor suppressors. Deregulated Myc expression is found in a variety of tumors and signals negatively regulating Myc expression and/or activity could therefore be of potential use in treating tumors with deregulated Myc.

Our aim was to therefore to investigate possible negative effects on Myc expression and activity by growth inhibitory cytokines and by the Myc antagonists, the Mad-family proteins. Two different cellular model systems of neuronal and hematopoietic origin have been utilized for these studies.

Our results show that Mad1 is upregulated during induced neuronal differentiation of SH-SY5Y cells. Further, the growth inhibitory cytokine interferon-γ (IFN-γ) was shown to cooperate with retinoic acid (RA) and the phorbol ester TPA in inducing growth arrest and differentiation in N-myc amplified neuroblastoma cell lines. In contrast to treatment with either agent alone, the combined treatment of TPA+IFN-γ and RA+IFN-γ led to upregulation of Mad1 and to downregulation of N-Myc, respectively, thus correlating with the enhanced growth inhibition and differentiation observed after combination treatment. Ectopic expression of an inducible Mad1 in monoblastic U-937 cells led to growth inhibition but did not lead to differentiation or enhancement of differentiation induced by RA, vitamin D₃ or TPA. In v-Myc transformed U-937 cells Mad1 expression reestablished the TPA-induced G₁ cell cycle arrest, but did not restore differentiation, blocked by v-Myc. The growth inhibitory cytokine TGF-β was found to induce Mad1 expression and Mad1:Max complex formation in v-Myc transformed U-937 cells correlating with reduced Myc activity and G₁ arrest.

In conclusion, our results show that the Myc-antagonist Mad1 is upregulated by growth inhibitory cytokines and/or differentiation signals in neuronal and hematopoietic cells and that enforced Mad1 expression in hematopoietic cells results in growth inhibition and increased sensitivity to anti-proliferative cytokines. Mad1 and cytokine-induced signals therefore seem to cooperate in countering Myc activity.

Key words: Myc, Mad1, neuroblastoma, hematopoiesis, phorbol ester, retinoic acid, interferon-γ, TGF-β, differentiation.

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"THERE IS A THEORY WHICH STATES THAT IF EVER ANYONE DISCOVERS EXACTLY WHAT THE UNIVERSE IS FOR AND WHY IT IS HERE, IT WILL INSTANTLY DISAPPEAR AND BE REPLACED BY SOMETHING EVEN MORE BIZARRE AND UNEXPLICABLE.

THERE IS ANOTHER WHICH STATES THAT THIS HAS ALREADY HAPPENED."

FROM THE BOOK "THE RESTAURANT AT THE END OF THE UNIVERSE" BY DOUGLAS ADAMS

"THE TRUTH IS OUT THERE"

X-FILES

THIS THESIS IS DEDICATED WITH ALL MY LOVE TO GUSTAV, ARVID AND LOVISA
This thesis is based upon the following papers, which are referred to in the text by their Roman numerals:

* I. Guzhova and A. Hultquist contributed equally to this work.


IV Wu, S, Hultquist, A, Öberg, F and Larsson, L-G. TGF-β induced cell cycle arrest in v-Myc transformed monocytic cells is linked to increased expression of the Myc-antagonist Mad1. Manuscript.

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<tr>
<td>bHLHZip</td>
<td>basic/helix-loop-helix/leucine zipper</td>
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<tr>
<td>cad</td>
<td>carbamoyltransferase/dihydrorotase</td>
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<tr>
<td>CKI</td>
<td>cyclin dependent kinase inhibitor</td>
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<td>Cdk</td>
<td>cyclin dependent kinase</td>
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<td>CLP</td>
<td>common lymphoid progenitor</td>
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<td>CMP</td>
<td>common myeloid progenitor</td>
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<td>CFU</td>
<td>colony forming unit</td>
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<td>CFU-GM</td>
<td>CFU-granulocyte/macrophage</td>
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<td>DAG</td>
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<td>G-proteins</td>
<td>guanine triphosphate (GTP)- binding proteins</td>
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<td>histone acetyltransferase</td>
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<td>IFN-γ</td>
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<td>interferon-γ receptor</td>
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<td>Inr</td>
<td>initiator</td>
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<td>Janus kinase</td>
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<td>Myc homology box</td>
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<td>NB</td>
<td>neuroblastoma</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>ornithine decarboxylase</td>
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<td>protein tyrosine kinase</td>
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<td>Rb</td>
<td>retinoblastoma protein</td>
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<td>RA</td>
<td>Retinoic acid</td>
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<td>retinoic receptor X</td>
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<td>Sin3 interaction domain</td>
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<tr>
<td>Stat</td>
<td>signal transducers and activators of transcription</td>
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<td>TAD</td>
<td>transactivational domain</td>
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<td>transforming growth factor β</td>
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<td>TPA</td>
<td>12-0-tetradecanoylphorbol-13-acetate</td>
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<td>TR</td>
<td>thyroid receptor</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VitD₃</td>
<td>vitamin D₃</td>
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INTRODUCTION

Cancer development is associated with mutations in genes regulating functions such as proliferation, apoptosis, differentiation, DNA repair, cell adhesion and angiogenesis. The oncoprotein Myc is a transcription factor controlling genes involved in several of these functions and is deregulated in many types of cancer such as neuroblastoma, Burkitt’s lymphoma and small cell lung cancer. Myc was identified over 20 years ago and despite impressive amounts of work dedicated to this gene much about Myc function still remains unclear. The identification of Max, the dimeric partner of Myc and the antagonists of Myc, the Mad-family, were milestones contributing to an increased understanding of Myc and its role in normal and malignant cells. Research on this network of proteins is in an expansive phase; a plethora of putative Myc target genes have been identified and new functions of the Myc and Mad-families, such as their involvement in chromatin modification, have recently been unveiled. However, less is known regarding signals negatively regulating Myc function and increased knowledge about pathways diminishing Myc expression and/or activity is of importance, not only for the basic understanding of tumor biology, but may potentially also be of interest in the treatment of tumors with deregulated Myc.

This thesis is focused on two potential pathways that could be effective in counteracting Myc activity. Firstly, the Myc antagonist Mad1. Little is known today about the regulation of Mad1 and its biological activities. I have investigated the expression of Mad1 in response to differentiating and growth inhibitory signals in neuronal and hematopoietic cell systems. Further, the effect of enforced Mad1 expression with respect to growth, differentiation and apoptosis has been measured in hematopoietic cells. Secondly, the thesis has addressed the question whether certain cytokine signals have the capacity to abrogate Myc function. This is based on previous findings in v-Myc transformed monocytic cells and is now extended to studies of N-myc amplified neuroblastomas, one of the classical Myc-type of tumor.
BACKGROUND

Development of cancer

Cancer is a genetic disease caused by progressive accumulation of lesions in multiple genes governing vital cellular functions thereby overriding normal mechanisms. Generally, there are two major types of genes that are targets for these lesions. Firstly, so-called proto-oncogenes, i.e. genes that normally function to promote growth or survival. Carcinogenic mutations affecting such genes usually result in deregulated expression of the gene or enhanced activity of the gene product, thereby creating an activated, so-called oncogene. The other major type of genes are so-called tumor suppressors, i.e. genes protecting the organism from cancer by inhibiting cell proliferation, survival or by ensuring the fidelity of the DNA code through DNA repair (for review [1, 2]. Carcinogenic events targeting tumor suppressors usually lead to an inactivation of the gene. It has been suggested that four to seven such carcinogenic events hitting such genes are necessary to create a cancer cell; thereby explaining the increased incidence of cancer with age. Recently, Hahn et al. reported that tumorigenic conversion of human primary fibroblasts and epithelial cells can be achieved by the combinatorial action of three oncogenes, an oncogenic allele of H-ras, the simian virus large-T oncoprotein (SV40LT) and the catalytic subunit of telomerase (hTERT), thereby implicating that fewer mutations than earlier thought are necessary to develop cancer [3]. One should, however, bear in mind that one of these genes, SV40 LT, is a viral oncogene with multiple functions. It has also been speculated that the origin of cancer lies in the disturbance of genes maintaining the genetic stability [4] and once those genes are inactivated further mutations would easily follow. There are ongoing discussions whether the normal somatic mutation rate leading to a selective growth advantage allowing for clonal expansion and additional mutations of these cells is sufficient for tumor development in humans, or whether an underlying genetic instability leading to an increased mutation rate is needed [4, 5].

The transformation of a normal cell into a cancerous one requires deregulation of several distinct control mechanisms. A normal cell proliferates only in response to mitogenic signals in form of, for instance, growth factors, ligands on neighbouring cells or extracellular matrix components transmitting their signal through interaction with cell surface or intracellular receptors. In a cancer cell this need of mitogenic signals has been circumvented, either by autocrine stimulation where the cell produces its own mitogenic signals or by mutations activating the transmembrane receptors, leading to signalling in the absence of ligand or by mutations in genes encoding components of the intracellular pathways transmitting the signals. Tumors can also influence fibroblasts and immune cells adjacent to the tumor to produce proliferative signals.

Cell proliferation is usually limited by growth inhibitory factors or intracellular inhibitory proteins. In many tumors the receptors or intracellular modules necessary for transmitting anti-growth signals are inactivated by different mutations. For example, the receptor of the growth inhibitory polypeptide TGF-β and the downstream effectors of the this pathway, the Smads, are mutated in several forms of cancer [6]. p53, a tumor suppressor gene that can arrest cell proliferation in response to DNA damage or induce apoptosis in the presence of overexpression of oncogenes such as c-myc or E1A [7], is inactivated in about 50% of human cancers. Further, many tumors have acquired mutations that deregulate or disable the growth
suppressive retinoblastoma protein (Rb) or other proteins taking part in the regulation of Rb activity [8]. Evidence of the importance of inactivating growth inhibitory pathways for tumors to developed is the fact that the majority of cancers, if not all, have inactivated genes of the Rb-pathway or p53.

Differentiation can be defined as stepwise progression from multipotent cells lacking overt specialization into a cell that has acquired this specialized phenotype and therefore differ from the cell from which it originates [9]. In most cell types terminal differentiation is coupled to an irreversibel block of cell proliferation. Neoplasia seems always accompanied by disturbances in the differentiation process, although these alterations can be more or less accentuated. Differentiation can be discussed in terms of tissue differentiation, meaning the overall morphology and the relation among the cells and the surrounding tissues, and cellular differentiation, meaning the changes in the phenotype and the capacity of the single cell to proceed along a lineage specific pathway of differentiation. Cells in a tumor can range from highly differentiated, still containing the functions characteristic of that particular cell type, to cells that hardly have any resemblance to its original phenotype. Cancerous genetic lesions responsible for disturbing or blocking the process of differentiation include mutations enhancing positive regulators of proliferation or inhibiting growth arrest molecules since terminal differentiation in many cases requires growth arrest. Mutations or inactivations of factors necessary for the differentiation process per se, such as the transcription factor C/EBPα during adipocyte differentiation or as in acute promyelomonocytic leukemia (APL) where fusion of the RA receptor RARα to the transcription factor PML leads to inhibition of genes regulated by retinoic acid (RA), are other ways for a tumor to evade terminal differentiation.

Apoptosis, or programmed cell death, is another safe-guarding system, where cells subjected to DNA damage, overexpression of oncogenes, hypoxia or absence of survival factors can be induced to die through this active, highly organized and energy-dependent process [10, 11]. Tumor cells at an early stage usually exhibit a high degree of apoptosis due to overexpression of oncogenes or hypoxic conditions caused by inadequate presence of blood supply, frequently occur and for tumor development to proceed the cancer cells therefore have to develop resistance towards apoptosis. Apoptosis is regulated and carried out by a large group of molecules including surface receptors such as the death receptor Fas, mitochondria associated molecules of the Bcl-2 family, both exhibiting pro- and anti-apoptotic characteristics, and different caspases, proteases that are the actual effector enzymes of apoptosis. Another player in apoptosis is the tumor suppressor p53 which induces apoptosis in response to for example DNA damage. Mechanisms involved in evading apoptosis are inactivation of pro-apoptotic factors or enhancement of survival molecules.

Mammalian cells have a limited life-span due to an inherent inability of infinite replication determined by a biological clock [12]. This limited number of divisions is due to shortening of telomers, repetitive DNA-sequences and associated proteins protecting the ends of chromosomes. Telomerase, the enzyme maintaining the length of telomers, is expressed in germline, but not in somatic human cells. In contrast, most malignant cells maintain their telomers by high expression of telomerase or by another mechanism called alternative lengthening of telomers (ALT) thereby allowing for immortalisation of these cells [13].

Blood supply, carrying oxygen and nutrients, is necessary for the survival of normal and tumor cells. For the tumor to be able to expand it has need of new blood vessel formation, a process called angiogenesis, since oxygen and nutrients can only travel a short distance into a
tissue. Vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factor-2 (FGF-1/2) are two factors that stimulate angiogenesis [14] and tumor cells have either found ways to increase transcription of these pro-angiogenic factors or to inhibit factors negatively regulating angiogenesis.

Finally, to expand into neighbouring tissues or invade distant organs the cancer cell has acquired changes in genes encoding proteins that regulate the cell-cell and cell-matrix interactions. These proteins include integrins and E-cadherins, which connect adjacent cells and provide anti-growth signals via the β-catenin/Lef/Tcf proteins [15]. E-cadherin function is lost in a majority of epithelial cancers. Extra-cellular matrix-degrading proteases are upregulated in tumor cells and are also envisioned to be involved in the metastatic process [16].

**Signalling pathways for growth and differentiation**

Cells need information from the environment to decide upon what actions to take. These signals are molecules are in the form of hormones, growth factors, cytokines, neurotransmitters, immunomodulatory molecules, ions, adhesion molecules on neighbouring cells. To receive the signals there are several groups of receptors including receptor protein tyrosine kinases, G-protein coupled receptors, cell adhesion receptors, receptor protein-serine/threonine kinases, nuclear receptors and ion channels which specifically recognize the ligand and transmit the signal to the interior of the cell. The signals are transmitted via intracellular pathways into the nucleus to regulate specific genes. The actual interpretation and the final outcome of the signal is dependent upon the cellular context. The accumulated knowledge that has been gained in this field is overwhelming and I will try only to cover a limited set of signal transducing pathways of direct relevance for this thesis.

**Tyrosine kinase receptor- and G-protein mediated signalling**

Growth factors, cytokines, neurotrophins and many differentiation factors bind to transmembrane receptor protein tyrosine kinases (PTKs) or to receptors associated with PTKs. A unique feature of receptor PTKs is a tyrosine kinase domain in the intracellular part of the receptor. Based on their structural similarities the RTKs can be divided into families including for example the PDGF receptor family and the EGF receptor family. The initiating step in signalling by growth factors is its binding to the receptor. Growth factors often bind as dimers to their receptors, thereby facilitating receptor dimerization leading to autophosphorylation in trans, i.e. each member of the receptor pair phosphorylates the other on specific tyrosine residues. The phosphorylated residues serve as docking sites for proteins containing Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Some of these proteins have enzymatic function, while others appear to function as adaptor proteins without any catalytic activity. Proteins containing SH3, pleckstrin-homology (PH) and several other domains are also involved in these signalling pathways. Subsequently, the formation of signalling complexes lead either to recruitment of cytoplasmic proteins to the plasma membrane where they can encounter their membrane-associated substrates or to phosphorylation of the receptor-bound molecules by the intrinsic tyrosine activity of the receptor (for review Kavanaugh and Williams 1996, [17-19]. Several signalling pathways are activated by receptor tyrosine kinases. In addition to receptor PTKs there is a large family of
receptor associated protein tyrosine kinases which includes the Janus kinases (JAKs), Src and Abl, among others.

Another group of receptors transduce their signal through guanine triphosphate (GTP)-binding proteins (G-proteins). The best known subgroup of these receptors is the seven transmembrane receptors which are activated by for example hormones, neurotransmitters and growth factors. As the name implies these receptors contain seven hydrophobic segments that form transmembrane domains. The G-proteins are heterotrimeric proteins composed of $\alpha$, $\beta$ and $\gamma$ subunits and are associated with the cytoplasmic phase of the plasma membrane. The receptor can interact with one or several specific heterotrimeric proteins since several different $\alpha$, $\beta$ and $\gamma$ subunits have been identified thus giving rise to many potential $\alpha\beta\gamma$ combinations. Upon activation of the receptor by appropriate signals, the receptor interacts with the G-protein causing bound GDP to be exchanged for GTP in the $\alpha$ subunit. This induces dissociation of the $\alpha$ and $\beta\gamma$ subunits from each other and from the receptor and by regulating other effector enzymes, receptors or ion channels the subunits initiate signalling pathways responsible for diverse cellular activities (for review [20, 21]).

The receptor tyrosine kinase-, receptor associated protein tyrosine kinase- and G-protein-mediated signalling affect many downstream target signalling molecules such as components of the Ras/Raf/MAPK pathway, adenylyl cyclase, phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC). The Ras/Raf/MAPK-pathway is one of the major pathways in transmitting signals in response to different receptors and intracellular molecules. Ras is a monomeric G-protein that is activated, as the heterotrimeric G-proteins, by exchange of GDP for GTP. When activated Ras-GTP interacts with a series of effector proteins including the Raf protein-serine kinase and the PI3K. Raf can further activate the mitogen activated protein kinase kinase (MAPKK) MEK1 or 2 which in turn activates the MAPK ERK [22]. PLC mediates cleavage of phosphatidylinositol thereby producing diacylglycerol (DAG) and 1,4,5-inositoltriphosphate (IP3). DAG will subsequently activate PKC that is a family of serine/threonine kinases. Several different isotypes of PKC have been identified, including $\alpha, \beta, \gamma, \delta$ among others. Another distinction is also made between classical, novel and atypical PKCs where the classic and novel PKCs are dependent upon DAG for kinase activation while the atypical are insensitive to this activation [23] [24]. Phorbol esters can mimic the effect of DAG and also activates PKC. PKC can for example activate ERK, thereby shortcutting the Ras/Raf/MAPK-pathway.

Phorbol esters such as 12-0-tetradecanoylphorbol-13-acetate (TPA) were originally described as tumor promoters, but has also been shown to induce growth arrest/inhibition and differentiation of several cell types such as neuronal and myeloid cells [25, 26], and can also promote proliferation of certain cells. TPA activates classical and novel types of PKC, thereby shortcutting the normal PKC activating pathway. Inhibitors of PKC has been reported to inhibit differentiation in neuroblastoma cells [27, 28]. The anti-proliferative activity of TPA has been attributed to downregulation of the c-myc proto-oncogene and its upregulation of the cyclin-dependant kinase inhibitor p21cip1/WAF1 in for example hematopoietic, neuroblastoma and breast cancer cell lines [29-33].
Interferon-γ/Jak/Stat-pathway

Interferon-γ (IFN-γ) plays an important role in regulation of the immune system, in particular in combating viral infections and tumor cells. IFN-γ induces a number of genes whose products participate in defence against viruses, such as the 2-5A-synthetase involved in a pathway that ultimately cleaves single stranded RNA. IFN-γ is also an important immunomodulatory molecule partly responsible for regulating the expression of proteins of the major histocompatibility complex class I (MHC) and uniquely capable of inducing expression of MHC class II proteins, thereby promoting development of CD8+ and CD4+ T-cell responses, respectively (for review [34]. IFN-γ also activates macrophages triggering processes such as production of reactive oxygen and reactive nitrogen intermediates, to kill microbial targets. IFN-γ also induces apoptosis and growth inhibition, activities important for fighting infections and cancer.

Interferon-γ initiates signalling by binding to its specific receptor consisting of the two subunits IFNγR1 and IFNγR2. The intracellular Jak/Stat pathway further transmits the signal to the cell nucleus. This pathway is utilized by a majority of cytokine receptors and also by tyrosine kinase receptors, and involves different Janus kinases (Jaks) and signal transducers and activators of transcription (Stats) to propagate the signal from the receptor since cytokine receptors lack intrinsic kinase activity (for review [34-36]. Different receptors, both cytokine and growth factor receptors, utilize distinct sets of Jaks and Stats.

The Jaks constitute a family of four members, Tyk2, Jak1, Jak2 and Jak3. Jaks uniquely contain two kinase-homolgy domains (JH1 and JH2). The interferon-γ pathway utilizes Jak1 and Jak2 and Jaks associate with membrane proximal regions of the typeI receptor. The Stat family contains seven members, Stat1, 2, 3, 4, 5a, 5b and 6, and as suggested by their names function as transcription factors. They contain a domain called Src homology region 2 (SH2), which is present in many adaptor proteins and important for binding to phosphorylated tyrosine residues.

Interferon-γ signalling

In unstimulated cells, the receptor subunits IFNγR1 and IFNγR2 are preassociated with Jak1 and Jak2, respectively. Interferon-γ bind as a homodimer to two IFNgR1 subunits thereby generating binding sites for two IFNgR2 subunits. The recruitment of the IFNgR2 subunits into the complex upon interferon-γ stimulation brings the intracellular domains of the receptor subunits in close proximity of each other and the preassociated Jak1 and Jak2 are activated by autophosphorylation in trans. The activated Jaks phosphorylate a tyrosine-containing sequence near the C terminus of the IFNγR1 subunit thereby creating paired docking sites for Stat1 [37]. The tyrosine reside Y440 has been shown to be the critical phosphorylation site for Stat1 binding. Two Stat1 molecules bind to these docking sites via their SH2-domain and becomes phosphorylated by the receptor-bound kinases at tyrosine 701 in their carboxy-terminal [38-40]. The phosphorylated Stat1 molecules dissociate from the receptor complex and form a homodimer which translocates into the nucleus. The Stat1 homodimer bind specific gamma activated sequences (GAS) elements in the DNA and transactivates a number of IFN-γ responsive genes. Phosphorylation of serine 727 is necessary for full transcriptional activation by Stat1, although the identity of the kinase mediating this phosphorylation in response to IFN-γ is still
ubknown [41, 42]. The Stat molecules can bind other factors to enhance the transcriptional effect including Nmi, a protein discovered as a N-Myc [43, 44] and p300/CBP [45].

IFN-γ has been observed to affect myc, a positive regulator of growth, and early reports state that interferon-induced growth inhibition is linked to downregulation of c-myc mRNA in Burkitt’s lymphoma and in murine macrophage cells [30, 46]. Conflicting results of the effect of IFN-γ treatment on c-myc expression in fibroblasts or pro-B cells and whether this is dependent upon Stat1 were recently published. c-myc downregulation was observed in fibroblasts, while upregulation was shown in the pro-B cell line [47, 48].

The TGFβ pathway

Transforming growth factor-β (TGF-β) is part of a family of related polypeptide growth factors including, besides TGF-β, activins and bone morphogenetic proteins (BMPs) for review [49-51]. These molecules play an important role both during development and in the adult organism, regulating a wide variety of cellular responses such as proliferation, growth inhibition, lineage determination, apoptosis and migration. For example, molecules of the TGF-β superfamily function as morphogens during development, acting in a graded fashion to specify cell [52]. The effects of these molecules depend to a large degree upon the cellular setting in that TGF-β for example induces growth arrest of epithelial and hematopoietic cells while it promotes cell proliferation of mesenchymal cells [53, 54]. Disturbances of the TGF-β pathway have also been implicated in tumor development and severe developmental defects [6].

Smads, the intracellular effector molecules

The TGF-β family of factors signals through serine/threonine kinase receptors at the cell surface which transmit the signal through intracellular effector proteins, Smads, that translocates to the cell nucleus and act as transcriptional regulators of TGF-β responsive genes.

Based on structural and functional similarities, the Smads can be divided into three groups; receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and the inhibitory Smads (I-Smads). The R-Smads include Smad1, 2, 3, 5 and 8, while Smad4 is the only Co-Smad identified so far in humans and mice and Smad6 and 7 constitute the I-Smads. The different TGF-β superfamily members utilizes distinct sets of R-Smads and Smad2 and 3 are mediators of the TGF-β and activin signals while the BMPs utilize Smad 1, 5 and 8. R-Smads contain two conserved Mad-homology regions, MH1 and MH2, that form globular structures separated by a linker region. The N-terminal MH1 domain contain DNA-binding activity while the MH2 domain mediates nuclear translocation and transcriptional activity (Liu 1996). It appears as if the MH1 and MH2 domains interact with each other to keep the protein in its inactive state until a proper signal arrives [51].

To function as transcriptional regulators the R-Smads must interact with Co-Smads and these are shared by all R-Smads [55]. There are only one Co-Smad identified so far in human and mouse, Smad4. The Co-Smad contain MH1 and MH2 domains, but is not phosphorylated by the receptor as are the R-Smads.

Smad6 and 7, the inhibitory Smads, negatively control TGF-β superfamily signalling by acting as a Smad4 decoy and blocking activated receptors, respectively [56, 57].
TGF-β signalling

TGF-β signalling is initiated by binding of a TGF-β molecule to its serine/threonine kinase type II receptor thereby recruiting the type I receptor into a complex. The role of the type II receptor is to phosphorylate and thereby activate the type I receptor. The recruitment of the intracellular effectors, the Smads, to the TGF-β receptor complex is mediated by a membrane-associated FYVE-containing protein, termed Smad anchor for activation (SARA) [58]. SARA is also partly responsible for tethering the inactive Smads in the cytoplasm by occluding a nuclear import signal in the MH2 domain [59]. The Smad proteins are phosphorylated by the type I receptor leading to activation of the R-Smads and dissociation from SARA and the receptor complex. The activated R-Smads form heterodimers with Co-Smad4 after which the complex translocates to the nucleus where it regulates target genes by binding the sequence AGAC-3, termed Smad-binding elements (SBEs), within their regulatory regions [60].

Several cofactors have been identified that cooperate with Smads at specific [60]. In addition, subgroups of R-Smads only bind certain sets of cofactors thereby adding to the specificity of the different pathways of the TGF-β family. FAST, a winged-helix/forkhead family member, c-Jun and TFE3 are examples of such DNA binding cofactors. The Smad complex can also recruit transcriptional corepressors or coactivators when bound to DNA. Ski and SnoN are inhibitory factors of TGF-β signalling. They interact with Smad2, 3 and 4 and inhibit the transcriptional activation mediated by the Smad complex by recruiting a complex containing the corepressors N-Cor/SMRT and Sin3 together with the histone deacetylases (HDACs) [61-65](See section Transcriptional regulation). TGIF is another corepressor inhibiting Smad mediated transcription by recruiting HDACs [66]. The Smads also interact with the coactivators p300/CPB [67, 68], containing intrinsic histone acetyltransferase activity (HAT). HDACs and HATs are involved in chromatin structure modifications and thereby regulating transcriptional activity [69].

Other pathways have also been reported to feed into the TGF-β pathway, modulating its activity in different ways. The interferon-γ/STAT-pathway has, for example, been observed to inhibit TGF-β responses by inducing the expression of the inhibitory Smad7 and oncogenically activated Ras has been reported to negatively regulate Smad2 and [70, 71]. The vitamin D3 pathway, on the other hand, seems to converge with the TGF-β pathway and cooperatively activates Smad3 [72]. There are also some evidence that the TGF-β pathway could activate several MAP kinases including JNK, p38 and Erk [6].

As mentioned earlier, TGF-β can inhibit proliferation of many cell types and escape from this growth inhibitory effect is a trait of many tumors. Inhibition of growth by TGF-β is accompanied by down-regulation of the c-myc proto-oncogene [73, 74] and upregulation of the cyclin-dependant kinase inhibitors p21cip1/WAF1 and p15INK4b [75-79], for review [54] (See section Myc in growth and proliferation). TGF-β has also been reported to induce growth arrest in cells lacking p15Ink4b [80] through repression of cdc25A phosphatase, which is required for activation of cyclin-dependant kinases. (See section Eucaryotic cell cycle)

Nuclear receptors

The nuclear receptor superfamily is involved in regulating development, homeostasis and metabolism. The nuclear receptor family consists of three groups of proteins, namely the steroid receptors including the glucocorticoid receptor and the sex hormone receptors, the non-
steroidal receptors such as the vitamin D3 (VDR), thyroid receptor (TR) and retinoic acid receptors (RAR), and the orphan receptors where the ligands have yet to be identified. These nuclear receptors, unlike membrane-bound receptors, function as transcription factors and directly regulate a plethora of different genes, mainly in a ligand-dependent manner. Many of these receptors directly bind signalling molecules which easily can enter the cell because of their lipophilic character (for review see [81-84]).

Structure of nuclear receptors

The nuclear receptors have similar structures with a C-terminal ligand-binding domain (LBD) connected by a hinge region to a central DNA-binding domain (DBD) and the N-terminal domain which contains the activation function 1 (AF-1) domain, involved in transactivation and is the least conserved region across the superfamily. A short sequence within the LBD, referred to as the activation function 2 (AF-2) is necessary for ligand-dependent transactivation. The DBD is composed of two zinc-finger motifs that allow for recognition and binding to specific repeats in the DNA. The functional entity of the nuclear receptors is the homo- or heterodimer. These dimers bind DNA recognition sequences composed of two hexanucleotide repeats separated by a specific number of nucleotides characteristic for each type of dimer. The retinoid x receptor RXR unit functions as a common dimerization partner for the nuclear receptors of the non-steroidal group such as RAR, VDR and TR. Some of the nuclear receptors, such as retinoic acid and thyroid hormone receptors binds DNA in the absence of ligand and actively repress transcription of their target genes. Nuclear receptors interact with several different coregulator complexes including chromatin modifying complexes, such as the N-CoR/HDAC corepressor proteins or the NCoA-family of coactivators, and also with components of the Mediator (for review see [85]. (see section Transcriptional regulation).

Retinoic acid (RA) and vitamin D3

Vitamin A and its derivatives (the retinoids) are essential for normal embryonal development and maintenance of differentiation in the adult organism. Lack or excess of vitamin A is not compatible with normal embryonal development since the embryo dies in the absence of vitamin A and an excess of vitamin A is highly teratogenic. In the adult vitamin A is necessary for tissue homeostasis, vision and reproduction [86]. Vitamin A (retinol) is the inactive precursor of retinoic acid and other retinoids and it is oxidized to the active acid molecule by two enzyme steps. Beta-carotene from plants or retinol derived from retinyl esters in meat are absorbed by the intestinal enterocytes. The retinol is stored as retinyl esters in the liver and released into the circulation bound to a transport protein RBP (retinol binding protein) to maintain a steady plasma level of retinol. Retinol enters the target cells and is metabolized to different retinoids such as all-trans RA (atRA) and 9-cis RA and the different forms of active RA have been found to bind different receptor subunits. atRA binds mainly the RAR receptor subunit, while 9-cis-RA interacts with both the RAR and RXR subunit. The heterodimer RAR/RXR binds classical retinoid response elements (RARE) and when atRA or 9-cis-RA is bound to the RAR moiety. The homodimer RXR/RXR binds RXREs in response to 9-cis-RA stimulation [81]. The retinoid receptors also bind DNA in the absence of ligand and repress genes by interacting with HDACs [85].

It has long been known that RA is important for development and differentiation and a link between lack of vitamin A and neoplasia was reported in 1925 when rats developed squamous
metaplasia at several epithelial sites when deprived of vitamin A. These lesions were reversed with readdition of vitamin A [87]. It has also been shown that RA can promote differentiation of several different cells in vitro including neuroblastoma and hematopoietic cells [88, 89]. The hypothesis that vitamin A could be useful in treatment of cancer prompted many clinical trials using different retinoids on patients suffering from various neoplastic and preneoplastic conditions. RA treatment of some premalignant lesions such as oral leukoplakia as well as in acute promyelocytic leukemia, juvenile chronic leukemia, mycosis fungoides and some secondary cancers has been successful [90]. Recently, evidence of the effect of 13-cisRA in treatment of minimal residual disease in neuroblastoma patients was reported [91].

Vitamin D₃ is important for calcium homeostasis and for bone formation. The active metabolite of vitamin D₃ (VitD₃), 1,25a-dihydroxycholecalciferol, is formed in several steps from cholecalciferol which is synthesized in the skin in a reaction catalyzed by ultraviolet light. The last step to form active 125a-dihydroxycholecalciferol is strictly regulated by parathyroid hormone, serum calcium and by feedback mechanisms. VitD₃ binds to the VDR which form heterodimers with RXR and bind vitamin D responsive elements (VDREs), found in promoters of genes regulating VitD₃ metabolism and bone formation/remodelling. Vitamin D₃ has also been found to exert functions in other organs such as the parathyroid gland, skin and immune system. VitD₃ induces growth arrest and differentiation of different cell types in vitro such as U-937 myelomonocytic cells and keratinocytes [92, 93] and to be able to upregulate the CKI p21cip1/WAF1 in the process [94]. The c-myc proto-oncogene is also downregulated in hematopoietic cells following VitD₃ treatment [95].

Transcriptional regulation

The main regulation of gene expression occurs at the level of initiation of transcription. For initiation of transcription the basal transcriptional machinery needs to be assembled at the promoter of the gene. Generally, the basal transcriptional machinery contains three major components that are necessary for transcription; (i) the RNA polymerase II together with several general transcription factors including TFIIB, TFIIE, TFIIF and TFIIH, (ii) the TATA binding complex TFIID, containing the actual TATA-box binding protein, TBP, and several TBP associated factors, so called TAFs and (iii) the Mediator complex, constituted by so called Med and Srb proteins, that is thought to mediate the response of RNA polymerase II to activators [96]. The process of initiating transcription can be divided into two parts; (1) A derepression process where transcription factors bind to specific DNA sequences in promoter or enhancer regions and recruit chromatin modifying complexes with histone acetyltransferase activity or recruit ATPase-dependent remodelling complexes. The activity of these complexes leads to a more relaxed chromatin structure thereby exposing TFIID and RNA polymerase II binding sites at TATA-boxes and Inr sequences, (2) the TFIID complex and the RNA polymerase II enzyme together with the general transcription factors can thereby bind DNA and initiate transcription.

During recent years, the modification of chromatin by acetylation/deacetylation of histones or ATP-ase dependent conformational changes of chromatin has been recognized as an important level of regulating transcription. Two major classes of chromatin modifying complexes are reported to exist, depending upon usage of covalent modification or not. The first class consists of histone acetyltransferases (HATs) or histone deacetylases (HDACs) that will add or remove acetyl groups from lysines in the N-terminus of histones, repectively (for [69, 97].
Hyperacetylated regions of chromatin have been connected with active transcription while hypoacetylated chromatin is transcriptionally silent. Transcription factors, such as p53 or GATA-1, have also been described to be acetylated by cofactors such as p300/CBP and this will modify their function. The second class consists of ATP-dependent chromatin remodelling complexes, such as SWI/SNF, that alter chromatin structure by changing the location or conformation of the nucleosome without covalent modification.

Many of the histone modifying complexes and also components of mediator-like complexes have been found to interact with DNA binding transcription factors as corepressors or coactivators to inhibit or enhance transcription mediated by these factors [85, 98] (Fig. 1). Well described examples of this are the nuclear receptors, where, generally, factors negatively affecting transcription by for example deacetylation seem to be bound in complex with nuclear receptors in the absence of ligand, while ligand-binding will induce a switch towards binding of coactivator complexes mediating acetyltransferase activity and other chromatin remodelling effects.

Coactivator complexes are made up of different groups of proteins including (i) the NCoA-or p160-family made up of the SRC-1/NCoA-1, TIF-2/GRIP-1/NCoA-2 or pCIP/ACTR/AIB1 proteins, (ii) the global coactivator CBP/p300 (iii) the p/CAF complex which contains members of the Ada family as well as TAFs and therefore bears resemblance to the SAGA complex in yeast, (iv) the Brg (SWI/SNF) ATPase-dependent chromatin remodelling complex and (v) the TRIP/DRIP/ARC-complexes [85]). The three first coactivator complexes, the NCoA-family, CBP/p300 and the p/CAF complex, contain histone acetyltransferase activity, while the Brg complex functions in a non-covalent manner by remodelling chromatin in an ATPase-dependent manner. The TRAP/DRIP/ARC complexes have been shown to be necessary for transcription by a number of transcription factors, including the nuclear receptors. This complex does not contain any intrinsic acetyltransferase activity [99], but instead are sharing several components with the Mediator like complexes CRSP, NAT and SMCC. In short, these complexes have all been observed to associate with nuclear receptors in a ligand-dependent manner and to enhance or be necessary of transcription induced by the nuclear receptors.

A number of nuclear receptors and other DNA-binding transcription factors also interact with corepressor complexes containing histone deacetylase activity. These receptors, including the RA and TR receptor, have been found to bind DNA in the absence of ligand and interact with the corepressors N-CoR/SMRT, via the CoR-box in the ligand-binding domain of the receptor [100, 101]. It has also been shown that N-CoR/SMRT is part of a complex containing the repressor protein mSin3 and the histone deacetylase, HDAC1, homologous to the yeast Rpd3p protein [102-104]. Mad1 and Mxi1, members of the Mad-family of proteins and antagonists of Myc, are also dependent upon interaction with the Sin3/N-CoR/HDAC complex through binding to mSin3 to exert their function [105-109]. It has been shown that other transcriptional repressors, such as the retinoblastoma protein, also recruit deacetylases and chromatin remodelling complexes for their repressional activity [110, 111]. Purification of a murine Sin3 complex resulted in copurification of HDAC1 and 2, the histone binding proteins RbAp46 and 48 and the small proteins SAP 18 and 30 [112, 113]. It is therefore plausible that recruitment of factors regulating modification of histones and remodelling of chromatin are major functions of transcriptional repressors and activators. In the case of nuclear receptors, they function both as repressors and activators depending upon the presence or absence of ligand. Basally they repress target genes by binding to deacetylase complexes, while ligand-
binding induces a switch towards recruitment of acetyltransferases and other complexes positively regulating transcription. In the Myc/Max/Mad-network, on the other hand, this switch takes place by replacing repressive Mad:Max complexes linked to deacteylases with Myc:Max complexes which bind acetyltransferases and members of the SWI/SNF-complex [114-116].

**Figure 1** The chromatin connection. Many transcriptional regulators interact with chromatin modifying complexes that negatively or positively regulate transcription. Some of these transcription factors, for example nuclear receptors such as TR and RAR, switch between interaction with histone deactetylase (HDACs) containing complexes, negatively affecting transcription by inducing tighter chromatin structure, to interaction with histone acetyltransferases HATs, which facilitates transcription. In the case of the Mad/Max/Myc-family of transcriptional regulators the Mad-proteins interact with HDAC complexes, functioning as transcriptional repressors, while Myc has been found in complexes with HAT’s.
The eucaryotic cell cycle

For embryonal development to proceed and for an adult organism to maintain regenerating tissues, cells must multiply. The cells do this by replicating their DNA followed by cell division to produce two identical daughter cells. This process has been called the cell cycle and is a highly structured process that involves a multitude of regulatory proteins. Morphologically, the cell cycle can be subdivided into interphase, encompassing the G_1, S and G_2 phases of the cell cycle, and the mitotic phase which include prophase, metaphase, anaphase and telophase. G_1 and G_2 denotes the "gaps" of the cycle where the cell prepares for DNA synthesis and mitosis, respectively (for review [117, 118]).

The main players driving the cell cycle are the cyclin-dependant kinases (Cdks) together with the periodically expressed cyclins. There are at least nine Cdks in higher eukaryotes where cdk1, cdk2, cdk4 and cdk6 are directly regulating the cell cycle, while other Cdks have other regulatory roles. The different Cdks need to interact with a cyclin regulatory subunit to become active. The main cyclins involved in cell cycle regulation are cyclin A, cyclin B1 and 2, cyclin D1, 2 and 3, cyclin E. Cyclin D and cyclin E-Cdk complexes are active during the G_1 phase of the cell cycle, ensuring that the cells enters S phase, whereas cyclin A and cyclin B-Cdk complexes are involved in the S and G2/M transitions. The different Cdk-cyclin complexes are active in specific periods of the cell cycle depending upon the sequential upregulation and degradation of distinct cyclins. Phosphorylation of a conserved Cdk threonine residue and dephosphorylations on some tyrosine residues are also needed for full Cdk activation. One of the main tasks for the G_1 Cdk-cyclin complexes in ensuring G_1 to S transition is to inactivate the retinoblastoma (Rb)-protein. In its active, unphosphorylated state Rb arrests cells in the G_1 phase of the cell cycle and this function of Rb is, at least in part, dependent upon interactions with the E2F-family of DNA-binding transcription factors (Harbour and Dean 2000). The E2F-family of proteins form dimeric complexes consisting of one E2F subunit binding to a DP partner. There are six different E2Fs, E2F1-E2F6, and two DPs, DP1 and DP2 identified and Rb mainly interacts with E2F1-E2F4 dimerizing with DP1. The ability of Rb to arrest cells in the G_1 phase of the cell cycle and this function of Rb is, at least in part, dependent upon interactions with the E2F-family of DNA-binding transcription factors (Harbour and Dean 2000). The E2F-family of proteins form dimeric complexes consisting of one E2F subunit binding to a DP partner. There are six different E2Fs, E2F1-E2F6, and two DPs, DP1 and DP2 identified and Rb mainly interacts with E2F1-E2F4 dimerizing with DP1. The ability of Rb to arrest cells in the G_1 phase of the cell cycle and this function of Rb is, at least in part, dependent upon interactions with the E2F-family of DNA-binding transcription factors (Harbour and Dean 2000). The E2F-family of proteins form dimeric complexes consisting of one E2F subunit binding to a DP partner. There are six different E2Fs, E2F1-E2F6, and two DPs, DP1 and DP2 identified and Rb mainly interacts with E2F1-E2F4 dimerizing with DP1. The ability of Rb to arrest cells in the G_1 phase of the cell cycle and this function of Rb is, at least in part, dependent upon interactions with the E2F-family of DNA-binding transcription factors (Harbour and Dean 2000). The E2F-family of proteins form dimeric complexes consisting of one E2F subunit binding to a DP partner. There are six different E2Fs, E2F1-E2F6, and two DPs, DP1 and DP2 identified and Rb mainly interacts with E2F1-E2F4 dimerizing with DP1. The ability of Rb to arrest cells in the G_1 phase of the cell cycle and this function of Rb is, at least in part, dependent upon interactions with the E2F-family of DNA-binding transcription factors (Harbour and Dean 2000). The E2F-family of proteins form dimeric complexes consisting of one E2F subunit binding to a DP partner. There are six different E2Fs, E2F1-E2F6, and two DPs, DP1 and DP2 identified and Rb mainly interacts with E2F1-E2F4 dimerizing with DP1. The ability of Rb to arrest cells in the G_1 phase of the cell cycle and this function of Rb is, at least in part, dependent upon interactions with the E2F-family of DNA-binding transcription factors (Harbour and Dean 2000).
synthesis. One of the targets of E2F is cyclin E and A that both are required for G1/S transition. Since cyclin E-Cdk2 induce phosphorylation of Rb and release of E2F a positive feed-back loop is created.

There are several different levels of regulation of the cell cycle. These include sequential and transient expression of the different cyclins, post-translational changes of the Cdks, including phosphorylations and dephosphorylations, cellular localization of the cell cycle regulators and also the activity of cyclin-dependent kinase inhibitors (CKIs) (See below). Phosphorylations can invoke both negative and positive effects on Cdk activity. The CAK (Cdk activating kinase), consisting of cyclin H and Cdk7, activate the Cdks by phosphorylating a conserved threonine residue, while the Wee1 kinase adds negatively regulating phosphorylations on two tyrosines. The family of Cdc25 phosphatases removes these negatively acting phosphorylations. The phosphorylation of the threonine residue and the dephosphorylation on tyrosines are necessary for full Cdk activation (for review [117,122]).

Inhibitors of cyclin-dependent kinases include two families of proteins; the Cip/Kip family consisting of p21Cip1/Waf1, p27Kip1 and p57Kip2, and the Ink4 family including p16Ink4a, p15Ink4b, p18INK4c and p19INK4d (for review see [121, 123]). The Ink4 family proteins bind to and specifically inhibit the catalytic subunit of Cdk4 and Cdk 6 and prevent association with the D-type cyclins. The Cip/Kip family, on the other hand, acts in a broader fashion and affect cyclin D-, E- and A-dependent kinase activity. The Cip/Kip-family also associates with both subunits of the cyclin-Cdk complex. Recently, it has been observed that Cip/Kip proteins associate with active cyclin D-Cdk4/6 complexes without inhibiting their activity. Furthermore, p21 and p27 were actually suggested to promote interaction between D-type cyclins and their Cdk partner by stabilizing the complex. Supporting this conclusion, it was also reported that the assembly of cyclinD1/D2-Cdk4 complexes was impaired in mouse embryo fibroblasts lacking p21 and p27 and that Cip/Kip proteins increased the stability of the D cyclins and directed the cyclinD-Cdk-p21/p27 complex to the nucleus. A non-catalytic function of D cyclins is to sequester p21 and p27 after the cell has received mitogenic signals, thereby relieving cyclin E-cdk2 complexes of inhibition and facilitating for cyclin E-cdk2 activation later in G1.

Many growth inhibitory molecules upregulate CKIs as a way to arrest the cell cycle progression and many of these factors, for example TGF-β, upregulate both Ink4 and Cip/Kip family members. The upregulation of the Ink4 family of CKIs result in Ink4 binding to Cdk4 and Cdk6, leading to disruption of Cdk-cyclinD-Cip/Kip complexes and release of Cip/Kip inhibitors which in turn can interact with and inhibit the cyclinE-Cdk2 complex thereby arresting the cell cycle. Upregulation of Cip/Kip proteins leads to a direct binding and inhibition of cyclin E-dependent kinase activity.

**Apoptosis**

Apoptosis is a physiological form of cell death necessary in development of an organism, for tissue homeostatis and for the immune defense. It can be triggered by a variety of death signals including withdrawal of survival factors and genotoxic damage caused by irradiation, chemical compounds or endogenous molecules such as oxygen radicals. Apoptosis is characterised by a certain set of morphological and biochemical features such as chromatin condensation, membrane blebbing and proteolytic cleavage of vital cellular constituents. Deregulated cell death is associated with several kinds of diseases such as cancer,
autoimmunity and neurodegenerative diseases. It is therefore vital that the initiation and effectuation of apoptosis are strictly regulated (for review see [10, 124]. The regulation and effectuation of apoptosis include a large variety of factors. Two major initiating pathways can be discerned; the death receptor (Fas/TNFα) and the bcl-2 family pathway. These two pathways interconnect at later stages of the apoptotic process and activate caspases, proteolytic proteins that are the actual effectors of the apoptotic pathway. Upon binding of its ligand the Fas receptor recruits adaptor proteins and procaspase-8, leading to generation of active caspase-8 which in turn activates downstream effector caspases. The Bcl-2 family members are both pro- and anti-apoptotic and are regulated by different factors including the PI-3K/Akt pathway inducing survival and the p53 tumor suppressor protein inducing apoptosis in response to DNA damage or overexpression of oncogenes. The Bcl-2 family of factors seems mainly to regulate apoptosis by regulating cytochrome c release from mitochondria [10]. The pro-apoptotic members induce release of cytochrome c which, together with Apaf-1, procaspase-9 and ATP form the apoptosome where procaspase-9 is activated and in turn activate the downstream effector caspases. The death receptor pathway can also induce release of cytochrome c via Bid, a Bcl-2 member. The anti-apoptotic members of the Bcl-2 family stabilizes the mitochondria by preventing cytochrome c release thereby protecting the cell from executing the apoptotic program.

The Mad/Max/Myc-network

The myc gene family consists of three well characterised members; c-myc, N-myc and L-myc that share similar genomic organisation as well as several evolutionarily conserved domains of functionally important protein regions. B- and S-myc are two additional members only identified in rodents. The Myc family play a role in many important functions of the cells such as proliferation, apoptosis, differentiation and cell growth (for review (144-145]. c-, N- and L-myc members are implicated in tumorigenesis as they are deregulated in many different tumors.

The myc gene was initially discovered about two decades ago as the retroviral transforming sequence (viral myc or v-myc) of the MC29 avian leukemia virus (ALV) [125] and c-myc was identified in chicken as the cellular homologue of v-myc [126]. It was also observed that c-myc was activated through promoter insertion in chicken lymphomas and c-myc was also found to be translocated in human Burkitt’s lymphoma, murine plasmacytoma and rat immunocytoma [127, 128]. The additional myc family members, the N- and L-myc genes, were discovered as amplified or highly expressed genes in the childhood tumor neuroblastoma [129, 130] and in small cell lung cancer [131], respectively. These genes are considered to be proto-oncogenes because mutations in their structure or deregulated expression have been linked to a wide variety of human and animal forms of cancer (for review [122]. The myc gene is evolutionary conserved and has been cloned from vertebrates and several other species including Drosophila, and sea star Asteria sralgaris [132, 133], but has not been identified in yeast or Caenorhabditis elegans. The c-, N- and L-myc genes consist of three exons, where exon 2 and 3 contain the coding sequences. The three members seem to exert similar functions and can to various degrees substitute for each other’s activity in certain biological assays. The expression of the three myc genes during development and in the adult, on the other hand, show distinct
patterns which differ between the genes pointing towards distinct roles [134-138]. In addition, L-myc seems to exhibit a weaker oncogenic potential than c- or N-myc [139]. However, insertion of N-myc into the c-myc locus showed that N-myc rescued the c-myc null phenotype suggesting that at least c- and N-myc carry out similar functions, but in distinct cells [140].

Structural domains and function of Myc

The Myc members are short-lived phosphoproteins and belong to the basic/helix-loop-helix/leucine zipper (bHLHZip) family of transcription factors, where the basic region mediates DNA-binding and the helix-loop-helix and the leucine zipper mediate protein-protein interactions [141-143] for review [144, 145]. Myc has been found to dimerize with the bHLHZip protein Max via the C-terminal HLHZip region and this interaction has proven necessary for Myc to bind DNA and exert its functions [146-154]. The Myc:Max complex bind E-boxes, regulatory recognition elements found in many promoters, with the preferred sequence CACGTG or CATGTG, but can also bind to non-canonical [155-157]. In contrast to Myc, Max is stable protein and can also bind DNA as a homodimer [150]. The N-terminus of Myc harbors a transactivational domain (TAD) containing two conserved regions Myc box I (MBI) and Myc box II (MBII) roughly spanning amino acids 45-63 and amino acids 129-141, respectively (Fig. 1) [152]. Myc thereby functions as a transcriptional activator and can induce transcription from synthetic reporter genes containing promoter-proximal E-boxes and also from natural E-box containing regulatory elements derived from suggested Myc target genes [146, 151, 158-161]. Like the bHLHZip region, MBII seems to be necessary for all biological functions of Myc including proliferation, block of differentiation, transformation, its ability to induce apoptosis [154, 162-164]. The role of MBII in transactivation is somewhat unclear since MBII mutants have been reported to have unaltered transactivation properties when artificial reporter promoter fusion constructs have been used. Other reports, however, state that deletion of MBII leads to decreased transactivation activity and activation of natural Myc target gene promoters has been suggested to be dependent upon [148, 152, 164-166]. MBII has recently been shown to interact with a histone acetyltransferase SAGA-like complex via the protein TRRAP thereby implicating Myc in chromatin modification [115, 116]. The binding of Myc to the histone acetyltransferase containing complex seems necessary for Myc-induced transformation. Myc has also recently been described to interact with the SNF5 homolog INI1, involved in cromatin remodelling, and dominant negative forms of INI1 negatively affect Myc-induced transcription [114]. Further, Myc has been observed to interact with two ATPase/helicase proteins, TIP48 and 49, which seem necessary for Myc-dependent transformation [167]. The N-terminal of Myc has also been observed to interact with the TATA-box binding protein (TBP) [168]. MBI has in some studies been shown to be dispensable for transformation and apoptosis while partial deletion has been observed to attenuate transactivation activity [148, 158, 162, 163, 166, 168].

In contrast to Myc, Max does not contain a transcriptional domain and ectopic expression of Max has been shown to repress transcription in reporter gene assays and also to inhibit Myc-induced transformation and transactivation, probably by forming inert complexes at the Myc binding sites [146-148, 151, 161, 169].
In addition to its role as a transcriptional activator, c-Myc also represses transcription of a number of genes such as gadd45, gas1, p21 Cip1/Waf1, p27Kip1 and C/EBP-α [169, 190-191,208,310,311] for review see [170]. Many of these genes are involved in growth arrest and differentiation, and one can easily imagine that their repression would enhance Myc’s positive effects on proliferation and block of differentiation. The mechanism whereby Myc represses genes are still unclear. As mentioned above, MBII has in several studies been observed to be important for Myc mediated repression implicating MBII in both transrepressional and transactivational functions. It has been shown that Myc is capable of directly or indirectly repressing transcription via the Inr elements. Further, c-Myc has been shown to interact with Inr-binding proteins such as Miz-1, TFII-I and YY-1 thereby affecting their activity [171-174]. Inr elements are initiator elements that often are present at TATA-less promoters and from which transcription can be initiated. Myc was recently shown to inhibit Miz-1 induced transactivation of p15Ink4b [175]. The induction of p15INK4b by the cytokine TGF-β was also reported to depend on relief of Myc inhibition of Miz-1 transactivational effect [176].

Figure 2. Schematic illustration of Myc, Mad and Max protein structure. 
TAD: transactivational domain, MBI and II: Myc homology region I and II, NLS: nuclear localisation signal, bHLHZip: basic/helix-loop-helix/leucine zipper region, SID: Sin3 interacting domain.

The function of Myc in proliferation and growth

A large amount of evidence suggests that Myc functions as a positive regulator of proliferation. First, Myc expression is correlated with proliferating cells and Myc expression is induced in response to many growth factors, mitogens and cytokines [177-181]. Secondly, forced expression of Myc in resting cells has been shown to induce S phase entry in the absence of mitogens [182]. Further, substances such as TGF-β, TPA and RA which normally down regulate c-myc expression are unable to induce growth arrest in cells engineered to constitutively express Myc [205, 312-313]. Thirdly, cells harboring mutant growth factors that are unable to induce Myc cannot enter the cell cycle [183, 184], antisense Myc inhibits S phase entry [185] and rat fibroblasts with targeted disruption of both c-myc gene alleles proliferate very slowly [186]. Effects of targeted deletion of the c- or N-myc genes in mice further establish Myc as an important player in cell proliferation since absence of c- or N-myc causes embryonal lethality, retarded development, multiple organ defects, smaller size of the organs and of the embryos as a whole [134, 135, 137].

Several groups have investigated the connection of Myc to the cell cycle machinery (for review see [187]. Myc has been reported to stimulate cyclin E/Cdk2 activity and to antagonize
p27Kip1 activity by upregulating gene expression of cyclin D1 and D2 that sequester the 
p27Kip1 protein thereby relieving its inhibition of cyclinE/Cdk2 [188, 189]. And, as 
mentioned above, c-Myc represses the p27Kip1 and p15Ink4b transactivation via direct or 
indirect binding to Inr sequences present in the promoters of these genes [174, 175]. p21Cip1 
has also been reported to be negatively regulated by c-Myc [32, 190, 191] Wu et al. in prep.). cdc25A, the phosphatase activating the Cdks, is suggested to be a target gene activated by Myc 
[192].

Several lines of evidence also implicate Myc in regulation of cell growth (i.e the 
accumulation of cell mass). Cell growth is a prerequisite for the doubling of cell mass needed 
for maintenance of the cell size throughout cell division. The two processes, cell growth and 
cell cycle progression, are coordinated during cell proliferation in ways that have not been 
clearly elucidated [193]. In Drosophila melanogaster loss of the c-myc homolog dMyc leads 
to retarded growth and reduced cell size [194] and overexpression of the c-myc transgene 
under the Ig heavy-chain enhancer in B lymphocytes lead to increased protein synthesis and 
increased cell size [195]. In rat fibroblasts with a targeted deletion of both c-myc alleles, 
protein synthesis and turn-over are decreased [186]. Some target genes of Myc, such as the 
translation initiator factors eIF-4E and eIF-2a and MrDb, a RNA helicase involved in rRNA 
processing, could also explain Myc’s effect on cell [Grandori, 1996 #161; Rosenwald, 1993 
#219; for review see[196]]. In the era of micro arrays many new presumptive Myc target 
genes have been identified and several of those are involved in translational control or 
metabolism [191, 197]. Recently, identification of target genes for N-Myc has also shown 
these to include many genes for ribosomal proteins, factors regulating translation and RNA 
processing proteins, thereby further establish Myc’s importance in cell growth [198]. The 
effect of Myc on proliferation therefore seems to depend upon both regulation of the cell cycle 
machinery and regulation of factors involved in cell growth.

**Differentiation and Myc**

Not surprisingly, since Myc is a positive regulator of proliferation, the opposite is often true for 
the effect of Myc on differentiation. Myc is downregulated during differentiation of several types 
of cells including neuroblastoma, myeloid cells, erythroleukemia cells and teratocarcinoma 
cells [30, 178, 199-201]. Conversely, overexpression of Myc can inhibit differentiation in 
several of these cell systems, while antisense Myc enhances differentiation [202-207]. Myc 
also represses genes such as C/EBPα that are important for inducing differentiation of 3T3-L1 
adipocytes [164, 208]. However, Myc has also been observed to have a positive role during 
certain differentiation processes. For example, murine erythroleukemia cells induced to 
differentiate exhibit a biphasic pattern of c-myc expression with an early, transient 
downregulation. Cells with ectopic c-myc expression exhibit faster return of c-myc levels, 
correlating with differentiation with faster kinetics [209]. A positive correlation between c-Myc 
expression, proliferative burst and differentiation was also observed during in vitro differentiation 
of B-type CLL cells [210]. Further, introduction of an inducible myc construct into 
keratinocytes was reported to cause progressive growth arrest and to induce terminal 
differentiation [211].
Myc and Apoptosis

The process of apoptosis is one safeguard mechanism to protect cells from deregulated oncogenes such as c-myc. Deregulated expression of c-Myc increases apoptosis in cells in specific situations such as serum deprivation or isoleucine starvation [163] and to sensititize cells for TNF-α and IFN-γ induced apoptosis [163, 212]. N-Myc has also been observed to sensitize neuroblastoma cells to IFN-γ induced apoptosis [213]. The mechanism through which Myc induces apoptosis was observed to depend on the integrity of the CD95/Fas death receptor pathway [214]. It was also shown that IGF-1 and Bcl-2 could protect cells from CD95 induced apoptosis. Recently it has been observed that c-Myc can induce release of cytochrome c from mitochondria, although signals from the Fas receptor pathway are still needed for apoptosis [215]. Myc induced apoptosis has also been reported to rely on activation of the ARF-Mdm2-p53 pathway [216, 217].

The Mad/Mnt-family

Apart from Myc, Max also interacts with several other bHLHZip proteins; Mad1 [218], Mxi1 [219], Mad3 [220], Mad4 [220], Mnt [221, 222] and Mga [223]. Mad1-4, constituting the Mad-protein family, and Mnt/Rox have been shown to act as antagonists of certain aspects of Myc function. Conserved regions within these proteins are the bHLHZip region in the C-terminus, a short α-helical domain in the N-terminus, constituting the mSin3-interacting-domain (SID) and regions C-terminal of the bHLHZip [218, 219, 221, 222, 224]. Like Myc, the Mad proteins and Mnt/Rox must dimerize with Max through their HLHZip domain to be able to bind DNA via their basic region and exert their functions. The SID is also crucial for Mad and Mnt function and this region has been shown to interact with the repressor protein mSin3a/b [105, 108], mSin3 in turn is part of a multiprotein complex including the histone deacetylases HDAC 1 and 2, the co-repressors N-CoR/SMRT and several other [102, 103, 106, 107, 109]. Recruitment of this complex is necessary for Mad function suggesting that Mad proteins exert their effect by modification of chromatin. c-Ski and its related gene product Sno is also part of the N-CoR/mSin3/HDAC complex and are necessary for the repressional activity of Mad and also for the thyroid receptor [62]. Recently, importance of the C-terminus of Mad1 has been described where specific deletions can abolish the effect of Mad1 in growth inhibition and reversal of Myc-induced block of differentiation [225].

The Mad proteins function as antagonists of Myc in repressing Myc-induced transcription and transformation and in inhibiting cell proliferation [105, 218, 219, 225-236]. The role of Mad in differentiation and apoptosis is more unclear since it has been reported that Mad proteins can both either enhance or inhibit differentiation and the apoptotic process [226, 230, 231, 234, 237] Paper III).

Mga is a transcription factor harboring both the bHLHZip motifs and a second DNA binding domain; the T-box that is characteristic of the Tbx family of transcription factors [223]. Mga can activate transcription both from Myc:Max and T-box binding sites in a Max-dependent manner.

The Mad-family, Max and Myc-family are therefore proposed to constitute a network with Max in the center, that has been suggested to function as a switch between growth/growth arrest or growth/differentiation. Since the levels of Max stay stable, the amount of Myc or
Mad in the cell would decide if the balance will tip in favour of growth or growth inhibition, respectively (Fig. 3).

Figure 3 The Mad/Max/Myc network.

Recently, a Max-like bHLHZip protein, Mlx, has been identified that only dimerizes with Mad1 and Mad4 of the Mad-family [238]. Mlx, in turn, can interact with a recently described group of bHLHZip proteins, the so called Mondo family which can, when localized to the nucleus, activate transcription from CACGTG E-boxes [239].

To cite the title of a recent review, "The Max network gone Mad", a high degree of complexity now emerges with the identification of so many new Myc antagonists and also perhaps parallel networks with Mlx as the central protein [240].

Expression patterns and knock-outs of the mad-family

Studies of the expression of the mad/max/myc network during murine embryogenesis, in the adult mouse and in cell lines show that the myc-genes generally are expressed in proliferating cells and are downregulated when the cells start to differentiate, while the mad-genes show the opposite pattern. The mad-gene expression does exhibit certain differences where mad3 and mxi1 expressions generally are found in proliferating cells, but also in more differentiated cells, while mad1 and mad4 are expressed during later stages of differentiation in the developing spinal cord, limbs, chondrocytic system and [220, 241, 242]. mnt has been found to be coexpressed with myc in proliferating tissues [221]. In the adult mouse, mad1 was expressed in more differentiated cells of the skin and gut, while c-myc was found in the proliferating cells [236, 241]. The expression of Mad in hematopoietic cell lines or keratinocytes in culture show low levels of Mad in proliferating cells and upregulation when cells are induced to differentiate and this is also followed by a switch from Myc:Max heterodimers to Mad:Max [220, 241, 243-245]. Mice with targeted deletion of mad1 had
delayed cell cycle exit of precursors of granulocytes, but the number of mature granulocytes was normal due to enhanced apoptosis [246]. Ectopic expression of mxi1 and mad3 was found in the spleen, suggesting that the different mads can substitute for each other. More marked changes were found in mice with deletion mxi1 where the mice displayed a hyperplastic phenotype [247]. Increased splenic cellularity and extramedullary hematopoiesis were found, together with degenerative changes in the kidney cortex. mxi1 knockouts did also display increased susceptibility to tumorigenesis induced by chemical carcinogens or on a homozygous null Ink4a/p16 cyclin dependent kinase inhibitor knockout background. Some spontaneous B-cell lymphomas were also observed. mad3 knockout mice displayed no defects in cell cycle exit or differentiation, but had increased cell death of thymocytes and neuronal progenitors after gamma irradiation [242].

Ectopic expression of Mad1 in transgenic mice led to early postnatal lethality and dwarfism [248]. Fibroblasts derived from these mice cycled more slowly, arrested at reduced density at confluence and their Go to S phase transition was delayed, thereby further establishing the role of Mad1 as a growth inhibitory factor. Cyclin D1-associated activity was drastically reduced in the Mad1-expressing fibroblasts and HPV-E7, capable of binding the proteins of the Rb family and inactivating them, could rescue the Mad1-induced phenotype implicating the Rb-pathway to be targets of Mad1.

**Target genes of Myc and Mad**

To exert their functions Myc and Mad regulate specific genes (for review see [144, 249, 250]). Before the era of microarrays several putative Myc target genes had been identified for example by subtractive hybridisation, immunoprecipitation of chromatin or educated guesses. Examples of those are ornithine decarboxylase (ODC), a-prothymosin, cdc25A, eIF-4E, eIF-2a and carbamoyltransferase/dihydroorotase (cad) [158, 182, 192, 251, 252]. Strangely, investigation of several putative Myc target genes in myc null fibroblasts found that only the expression of cad and gadd45, that is repressed by Myc, was affected [253]. Recently, many genes supposedly regulated by c- or N-myc have been identified using microarray techniques [191, 197, 198]. These genes can be categorized into different functional groups such as those linked to the cell cycle, cell growth, signaling, adhesion and apoptosis etc. To solve the question of which of these genes that will prove to be important for Myc function is a formidable task and it will require adequate biological assays.

Not many target genes of the Mad-family have yet been identified, but recently O’Hagan et al. replaced the basic region of Myc with that of Mxi1 (Myc(Mxi1-BR)) and micro array analysis with both Myc and Myc(Mxi-BR) showed that these proteins influence transcription of both overlapping and distinct genes [197]. For example, the hTert gene, encoding the rate-limiting enzyme of the telomerase complex, has been reported to be a transcriptional target of Myc and has recently also been observed to be repressed by Mad1 and Mxi1 [197, 254-256]. ODC is another target gene of Myc that has been observed to be repressed by Mxi1 [197, 257].

**Differentiation**

During embryogenesis pluripotent stem-cells generate a plethora of different cell types making up the tissues of the organism. A large effort has been put into this area of research to
understand how the specific cell fate decisions are made and how the offspring of pluripotent cells know where to go and what to become. Using models such as the fruit fly *Drosophila melanogaster* the knowledge in this field has increased dramatically and we now know that, for example, lateral inhibition involving the delta/notch pathway and concentration gradients of morphogenic molecules seem to play an important role. The constant regeneration in the adult organism of tissues such as the blood and skin, requires stem cells that are both self-renewing and give rise to progenitor cells that will differentiate into the actual effector cells of the organ. The differentiation process, both in the embryo and in the adult, requires a fine-tuned balance between proliferative and growth arrest/differentiation signals. During tumor development both of these processes have been deregulated to different degrees. Well differentiated tumors exhibit similar tissue morphology and cell characteristics as the normal, non-malignant cells, while the low differentiated or anaplastic tumor has lost these hallmarks.

I will here give a short overview of the development of the sympathetic nervous system and the hematopoietic system followed by a description of the neuroblastoma and lymphoma differentiation model systems used in this thesis.

**Development of the sympathetic nervous system**

The nervous system is comprised of two parts; the central nervous system (CNS) that is made up of the brain and the spinal cord and the peripheral nervous system (PNS) consisting of the cranial and spinal nerves together with their ganglia. Another distinction of the nervous system is the division into the motoric nervous system and the autonomous nervous system, regulating functions mainly without conscious control. The autonomic nervous system can be divided into the sympathetic (SNS), the parasympathetic and the enteric nervous systems and it lies mainly within the PNS. The sympathetic and parasympathetic systems innervate the same internal organs such as the gut, heart, blood vessels, but exert opposite functions. The sympathetic nervous system acts mainly by preparing the body for physical encounters, the so called “fight-or-flight” reactions by, for example, increasing the blood pressure and heart rate using the neurotransmitter norepinephrine at the post-ganglionic nerve endings. The parasympathetic nervous system is mainly concerned with the body’s vegetative functions.

The SNS originates from cells of the neural crest which is a structure that is induced at the dorsolateral border of the neural plate. Neural crest cells are multipotent and give rise to the sensory and autonomous neurons and glia cells of the peripheral nervous system, as well as melanocytes, smooth muscle and cartilage. The neural crest cells delaminate from the neural plate when it is closing to become the neural tube around the fourth week of human development (for review see [258, 259]). Neural crest cells move ventrolaterally on either side of the neural tube and form a mass between the neural tube and the overlying ectoderm from where it can migrate out into the body (Fig.4). It has been suggested that the neural crest, at the beginning of migration, consists of heterogenous cell types that are both unspecified stem cells and cells that have adopted a more restricted phenotype [260]. Already before the neural crest cells start to migrate out into the organism different cell populations can be identified and some of the earliest markers are receptor tyrosine kinases. Subsets of premigratory cells destined for sensory neurogenesis express the trkC receptor which binds the neurotrophin NT-3, while melanocytic precursors express c-kit, the Steel receptor. The fate of the neural crest cells is then further directed by different signals along the migration routes. Bone
morphogenetic proteins 2/4 (BMP 2/4), Wnt molecules and TGFβ1-3 have been shown to act on migrating neural crest cells to induce development of different cell types. The Wnt/β-catenin pathway is suggested to be important for expansion of neural crest cells and for induction of pigment cell formation. Continuous signalling by BMP2 and BMP4 induce development of autonomic neurons. BMP signalling has also been suggested to be important for neural crest cell migration as is also the Rho-family of GTPases [258].

Figure 4. Neural crest development. The notochord induces cells in the ectoderm to form the neural plate (A). The neural plate invaginates, fuses and forms the neural tube (B), and around closure the neural crest cells bud off and migrate out into the body (C).

The bHLH gene Mash-1 is a downstream target of BMP2 and 4 and is expressed in autonomic precursor cells before differentiation is initiated. Mice lacking Mash-1 have no sympathetic neurons suggesting that it is required for neuronal differentiation [261]. The homebox genes Phox2a and Phox2b are implicated in development of autonomous ganglia and Mash-1 has been observed to induce Phox2a. GGF, a neuregulin has been implicated in promoting the development of Schwann cells which are glial cells supporting the neurons. TGFβ1 can also support autonomic and sensory neurogenesis in the presence of basic fibroblast growth factor and nerve growth factor, but is also important for cardiac smooth muscle formation. dHAND and eHAND, two bHLH proteins are expressed in cardiac smooth muscle and catecholaminergic neurons, indicating that they may function downstream of TGF-β. N-myc is also expressed in neural crest cells and seems to promote ventral migration and neuronal differentiation of these cells [262]. Further, N-myc deficient mice have reduced numbers of neurons of the dorsal root ganglia and also sympathetic ganglia [135, 137, 263]. The sympa-tho-adrenal lineage of the SNS give rise to neurons, mostly found in the sympathetic ganglia, and chromaffin cells of the adrenal gland. Extra-adrenal chromaffin cells
making up the paraganglia and SIF cells also originate from the sympatho-adrenal cells, but these structures are practically absent in adult tissues (Fig. 5).

**Figure 5** Schematic illustration of the cell types derived from the sympatho-adrenal progenitor cells. Sympathetic neurons comprise the sympathetic ganglia, SIF cells are located among the neurons of the sympathetic ganglia and chromaffin cells are located in the adrenal medulla.

**Neuroblastoma**

Neuroblastoma (NB) is one of the most common solid tumors of childhood, actually the most common solid tumor of the neonatal period, and was first described by the German pathologist R.L.K Virchow in 1864. The tumors are derived from neural crest precursors of the sympatho-adrenal lineage and can originate wherever there is sympathetic nervous tissue. The most common locations include the adrenal gland and sympathetic ganglia of the abdomen, thorax, neck and pelvis. The median age at diagnosis is 22 months and few patients are diagnosed over 5 years of age. Spread of the tumor occurs most commonly to lymph nodes, bone marrow, bone, liver and skin. The symptoms from the tumor differ depending upon location and metastatic spread [264].

**Classification**

Neuroblastoma is a heterogenous tumor varying from very aggressive tumors that are untreatable to tumors that differentiate spontaneously despite metastatic spread (4S tumors). One commonly used classification of neuroblastoma tumors is the International Neuroblastoma Staging System (INSS) [265, 266] where 4 stages are recognized depending upon local growth of the tumor, local and distant infiltration and the age of the child at diagnosis (4S tumors). Tumors of the lower stages show localized growth, while tumors of stage 4 show dissemination to distant organs. Generally, stages 1, 2 and 4S have a good prognosis and can be treated or even differentiate spontaneously, while stage 3 and 4 tumors
progress despite aggressive treatment. In addition to the INSS classification system there are other systems classifying the tumor according to histology.

**Prognostic factors**

In addition to the clinical stage of the tumor, including local growth and metastatic spread, there are also other prognostic factors of importance such as location of the tumor, the age at diagnosis, DNA content, structural chromosomal aberrations, telomerase activity and expression of growth factor receptors. Adrenal tumors and diagnosis over the age of 12 months correlates with worse outcome than extra-adrenal tumors and younger age at diagnosis [264]. Tumors with a diploid DNA content have been shown to have worse prognosis than hyperploid tumors [267]. Deletion of the short arm of chromosome 1 (1p) and gains of the long arm of chromosome 17 (17q) are also correlated with poorer prognosis [268, 269]. The genes residing at these chromosomal locations have yet to be identified. Amplification of N-Myc is one of the more prominent chromosomal abnormalities in NB and is found in 25% of all neuroblastomas and in 40% of the aggressive stages and correlate with advanced stages of disease, worse prognosis and insensitivity to chemotherapy [129, 270-274]. N-myc amplification is often observed together with deletion of chromosome 1p. Telomerase activity has been shown to correlate with the stage of disease and with features corresponding to an adverse outcome for the patient [275]. Interestingly, telomerase has been reported to be a target gene of c-Myc [255].

Neurotrophins (NT) are growth factors for different classes of neurons and bind via high-affinity tyrosine kinase receptors belonging to the trk family [276]. TrkA is the high affinity receptor for nerve growth factor (NGF), while trkB is the receptor for brain-derived growth factor (BDNF) and also binds NT-3 and NT-4/5. TrkC is the main receptor for NT-3. A low-affinity receptor p75NTR bind all of these neurotrophins, but the function of this receptor is still unclear. TrkA is expressed on cells of the sympatho-adrenal lineage during embryonal development and binding of NGF ligand induces differentiation into mature sympathetic ganglion cells and rescues cells from undergoing apoptosis. In neuroblastoma, trkA expression is expressed in tumors with favourable prognosis and is inversly correlated with N-Myc expression. TrkB expression, on the other hand, is mainly correlated with an adverse outcome and with N-myc amplified tumors, perhaps reflecting a more immature phenotype since trkB expression is normally found earlier in the development of sympathetic neurons than trkA.

Prognostic factors are important for deciding upon the mode of action against the tumor. Since certain stages of neuroblastoma tumors are fairly "benign" in the sense that they are easily treated or even can differentiate spontaneously it is important not to overtreat these patients. In contrast, patients with tumors exhibiting prognostic factors predicting a progressing tumor need very aggressive treatment.

**N-myc in neuroblastoma**

Amplification of N-myc has been observed to correlate with more aggressive tumors and with poorer prognosis [270, 273, 274]. Further evidence for implicating N-myc in neuroblastoma development are observations that ectopic expression of N-Myc led to enhanced proliferative potential, inhibition of trkA/NGF- and RA-induced differentiation in neuroblastoma cell lines with normal endogenous N-myc alleles and the development of neuroblastoma in transgenic mice with N-myc targeted to neuroectodermal cells [207, 213, 277-
N-myc is downregulated during in vitro differentiation of N-myc amplified cell lines with agents such as RA, interferon-γ and TPA and inhibition of N-myc expression using anti-sense technology has been reported to promote growth inhibition and a more differentiated phenotype [31, 201, 281, 282].

Treatment

Treatment of neuroblastoma tumors include surgery, chemotherapy, irradiation and autologous bone marrow transplantation (ABMT). Despite these different therapeutic strategies, in aggressive tumors with factors predicting an adverse outcome, such as N-myc amplification, deletion of 1p and gain of chromosome 17q, the survival rate is very low due to relapse of the majority of tumors even after initially successful treatment. Differentiation therapy is one strategy that theoretically could have potential to function in neuroblastoma tumors since a subgroup of these tumors have been shown to differentiate spontaneously in vivo. Retinoic acid is a molecule naturally occurring in the body and several different tumor types, including neuroblastoma, can be induced in vitro to differentiate in response to this agent [89, 283]. Clinical trials in neuroblastoma patients have shown some success in treating minimal residual disease after ABMT with 13-cis RA [91].

In vitro models of neuronal differentiation

To study aspects of neuronal differentiation or neuroblastoma biology it would be optimal to study primary cells or tumors. Most available sources of primary neuronal cells come from the animal kingdom such as cells from rat, chicken and [284-286]. Primary human neuroblastoma cells are difficult to come by since there are quite few patients diagnosed in Sweden each year and it can still be difficult to obtain primary cells from the patients and establish cultures to study. Therefore cell lines derived from tumors of the nervous system is a way of studying human neuronal differentiation and aspects of neuroblastoma biology. As mentioned earlier, most of the established neuroblastoma cell lines exhibit amplified N-myc and are derived from stage 3 or 4 tumors, since it has been difficult to establish clones from low-stage and non-myc amplified tumors. One exception is the non-N-myc amplified cell line SH-SY5Y which is a well-established model for neuronal differentiation and is a subclone of the cell line SK-N-SH which was established from metastatic tissue taken from a patient with a progressive neuroblastoma tumor [287]. SH-SY5Y has been shown to differentiate and exhibit growth arrest in response to TPA and RA [26, 288]. The differentiation process is characterised by extension of neurites and upregulation of certain markers specific for neuronal differentiation such as neuronal specific enolase (NSE), catecholamine production, the axonal growth cone protein GAP43, neuropeptide tyrosine (NPY), tyrosine hydroxylase (TH) and choline acetyltransferase activity (ChAT) (for review [289]). LA-N-5 is a neuroblastoma cell line with N-myc amplification that has also been used quite extensively in differentiation studies [89, 290]. LA-N-5 can be induced to differentiate with substances such as RA and interferon-γ and this is characterized by neurite outgrowth, inhibition of proliferation and increase of neurotransmitters specific for a cholinergic or adrenergic phenotype. LA-N-5 therefore seems to be of a mixed phenotype, being able to differentiate in two directions depending upon which differentiation inducing agent that is used.
Hematopoietic differentiation

Hematopoiesis is the process by which the body produces all the different blood cells necessary for survival of the organism. The different blood cells are responsible for carrying out different functions of the body such as the erythrocytes, which transport oxygen from the lungs out into the tissues, the leucocytes, including monocytes, granulocytes and lymphocytes, which protect the organism against foreign invaders such as viruses, parasites and bacteria, and platelets which arrest bleeding by clotting of the blood. The mature blood cells are rather short-lived and there is constant generation of new blood cell progenitors originating from a small number of stem cells residing in the bone marrow. Stem cells are self-renewing and totipotent to be able to keep generating blood cells of all lineages [291] (Fig. 6). Infections or trauma can lead to an increased demand for blood cells thereby promoting a larger number of stem cells to enter the cell cycle and produce more effector cells [292].

Development of all different blood cells is a step-wise progression of pluripotent stem cells through intermediate stages of multi- and bipotent progenitor cells that progressively and irreversibly get more restricted in their developmental capacity. The stem cell gives rise to the common lymphocytic progenitor (CLP), which gives rise to the lymphocytic lineage, while the rest of the sub-lineages originate from the common myeloid progenitor (CMP). The term CFU, colony forming unit, denotes the ability of specific progenitors to create morphologically distinct colonies in cell culture assays under the influence of growth factors, so called cytokines. The different CFUs of the myeloid lineage develop from the common myeloid progenitor via the intermediate CFU-mix cell. While the self-renewal and differentiation of stem-cells and their progenitors are suggested to be a stochastic process, survival and proliferation of the cells are regulated by cytokines [293]. These factors are characterised, to different degrees, by pleiotropy (multiple biological actions) and redundancy (shared biological actions), where some cytokines support development of multiple lineages, whereas others are more specified. Cytokines can be divided into (1) factors triggering the proliferation of cell cycle dormant primitive progenitors and ensure their survival, including the stem cell factor, Tpo, IL-6 and IL-3, (2) factors supporting proliferation of multipotent progenitors after their exit from G0, including IL-3, GM-CSF and IL-4 and (3) lineage-specific factors supporting proliferation and maturation of committed progenitors, including M-CSF, Tpo, G-CSF and Epo. Many of the cytokines are synergistic factors, meaning that they have no effect on their own, but need to cooperate with other factors. To induce cell cycle entry of dormant progenitor cells in vitro stimulation of combined cytokines is needed. Stem cell factor acts in synergy with most of the factors in inducing proliferation of quiescent stem cells. Hematopoietic commitment and differentiation is also dependent upon induction of transcription factors and one early acting factor is Tal-1/SCL, a bHLH protein, that is critical for blood formation since it induces the expression of the stem cell factor receptor, c-kit. Several other transcription factors important for hematopoietic development include Ikaros, GATA-1,2,3 and PU.1, where Ikaros is important for development of lymphoid cells and GATA-1,2,3 for myeloid cells and PU.1 for both myeloid cells and B lymphocytes. I will not dwell any further on this subject, but to say that there are several other factors involved and that they interact to ensure the differentiation of the hematopoietic cells (for review see [294]).
Figure 6. The hematopoietic tree showing the differentiation of multipotent stem cells via intermediate multipotent and bipotent progenitors into all different layers of mature blood cells. HSC: hematopoietic stem cell; CMP: common myeloid progenitor; CFU-Meg: macrophage; CFU-M: monocyte; BFU-E: erythrocyte; CFU-GEMM: mast cell; CFU-B: basophil; CFU-E: eosinophil; CFU-Meg: megakaryocyte; CFU-M: monocyte; CFU-GEMM: mast cell; CMP: common myeloid progenitor.
Monocytic/macrophagic differentiation

The hematopoietic differentiation system used in this thesis is the monoblastic cell line U-937. I will therefore in some more detail describe the monocytic/macrophagic lineage differentiation.

The mononuclear phagocyte system, consisting of blood monocytes and various tissue macrophages, play a major role in regulation of the immune response and in protecting against foreign invaders by phagocytosis, antigen processing, antigen presentation, T cell activation and cytokine secretion. Monocytes are produced in the bone marrow and rapidly enter the circulation from which the cells are recruited into various tissues to become tissue macrophages. These cells exist in many organs where encounters with foreign particles or need for phagocytosis of endogenous waste is expected.

Differentiation into monocytes/macrophages proceeds via the intermediate stages of the CFU-mix progenitor and the CFU-GM cells, which give rise to both granulocytes and monocytes, under the influence of cytokines and other components of the hematopoietic microenvironment. GM-CSF functions as a proliferative and differentiation inducing factor of early myeloid progenitors, but also induces growth arrest and differentiation of later stages of the monocytic lineage. M-CSF is the most specific factor in monocytic development (for review see [294, 295]. The activation of monocytes into macrophages also involves different cytokines, adhesion, chemoattractants and bacterial lipopolysaccharides (LPS).

There are several differentiation markers such as cell-surface antigens, functional assays and morphology that has been used to identify monocytes. Markers expressed preferentially by monocytes are seldom absolute in their lineage-restriction, but can also be expressed by other myeloid cells. Since there are also many different monocytes and tissue macrophages, the expression patterns of these different groups is not homogenous, but there are some genes that can be considered as typical monocytic markers. β2-integrins are markers for leukocytes and the function as a unit consisting of an α-subunit (CD11a,b,c) in complex with a β-subunit (CD18). CD11a is expressed on all leukocytes, while CD11b and c are more restricted to monocytes, macrophages, neutrophils and natural killer cells. CD11b and c expression is regulated during monocyte differentiation and CD11b is first expressed at the monoblastic stage and is highly expressed on monocytes. Cd11c comes later and predominates after differentiation of monocytes into tissue macrophages. CD14, the receptor for LPS and its binding protein, LBP, is almost exclusively present on monocytes and macrophages and first detectable during the promonocytic stage. It is highly expressed on monocytes, whereas in tissue macrophages the expression varies from very high to quite low depending upon where the macrophages reside. One of the more specific markers of the monocytic lineage is CD68 which is expressed on monocytes and different tissue macrophages. There are also functional assays for enzymes such as α-naphtyl acetate esterase.

The U-937 differentiation model

The U-937 cell line was established in 1976 from a patient with generalized histiocytic lymphoma [296]. The cells are arrested at the monoblastic stage and can be induced to differentiate terminally along the monocytic lineage with agents such as TPA, RA and vitD3, and these agents give rise to distinct differentiation profiles [92, 205, 283, 297] (Fig. 7). Terminal differentiation is here defined as growth inhibition and procurement of morphological and functional characteristics of mature monocytes and macrophages (Fig. 7).
Common characteristics for all three agents are induction of $G_0/G_1$-arrest, upregulation of the monocytic differentiation marker CD11c, an $\alpha$-subunit of $\beta_2$-integrins, the regulation of the Myc/Max/Mad-family, where $c-myc$ is downregulated and $mad1$ mRNA upregulated and upregulation of the CKI p21cip1/WAF1 [30, 94, 244, 297, 298]. Evidence of divergent pathways are the upregulation of the LPS receptor CD14 by vitD3 and TPA, but not by RA [205, 297]. Further, CD23, the low affinity receptor for IgE is upregulated by TPA and RA, but is rather downregulated by vitD3. The morphological appearance also differs where TPA and vitD3 induces a macrophage-like morphology with eccentric nucleus and a large vacuolized cytoplasm, while cells induced with RA are small and exhibit distinct, lobulated nuclei [297]. Other markers used to measure the differentiated phenotype are esterase activity, reduction of nitroblue tetrazolium, the integrin $\alpha$-subunits CD11a and b and the glycoprotein CD68.

U-937 cells expressing v-myc are blocked in their growth arrest and differentiation due to maintained v-myc expression during induced differentiation ([205] and addition of interferon-$\gamma$ can reestablish growth arrest and differentiation by TPA, vitamin D3 or RA [299].

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**Figure 7** The U-937 differentiation model. Cells are induced to differentiate arrest in the $G_1$ phase of the cell cycle, change their morphology, upregulate differentiation coupled surface and intracellular antigens and acquire specialized functions such as phagocytosis.
AIMS OF THE PRESENT INVESTIGATION

The general aim of this thesis was to increase the knowledge of the regulation and function of the Mad/Max/Myc-network during differentiation of neuronal and hematopoietic cells. The thesis can be divided into two parts: Paper I and II concern induced differentiation of neuroblastoma cells and how this treatment affects the expression of the Mad/Max/Myc-network, while paper III and IV describe the regulation and function of Mad1 in growth arrest and differentiation in hematopoietic cells.

The specific aims were to:

I Investigate if interferon-γ could cooperate with RA or TPA in inducing growth arrest and/or differentiation of neuroblastoma cell lines, with N-myc amplification.

II Analyze the regulation of the Mad/Max/Myc-network during neuronal differentiation in the SH-SY5Y differentiation model and after combination treatment with TPA, RA and interferon-γ in N-myc amplified neuroblastoma cells.

III Elucidate the effects of enforced Mad1 expression on cell growth, differentiation and apoptosis in U-937 monoblastic cells

IV Investigate the expression and activity of Mad1 in response to TGF-β signalling and investigate a possible cooperative effects between anti-proliferative cytokines and Mad1 in inducing cell cycle arrest in v-Myc transformed U-937 cells
RESULTS AND DISCUSSION

Interferon-\( \gamma \) cooperates with retinoic acid and the phorbol ester TPA in inducing growth arrest and differentiation of human \( N\)-myc amplified neuroblastoma cells (Paper I)

Cell lines from neuroblastoma tumors can be induced to differentiate in vitro with substances such as TPA, retinoic acid (RA) and interferon-\( \gamma \) (IFN-\( \gamma \)). Our group has earlier reported that the combination of RA or TPA with IFN-\( \gamma \) will overcome Myc-induced growth arrest and differentiation in monoblastic U-937 cells constitutively expressing v-Myc [299, 300]. We therefore chose to investigate the effect of IFN-\( \gamma \) in combination with RA or TPA on cell growth and differentiation in panel of neuroblastoma cell lines.

Specific questions:
- Will IFN-\( \gamma \) enhance growth inhibition and differentiation observed with RA or TPA treatment alone in \( N\)-myc amplified neuroblastoma cell lines?
- Will cell lines with or without \( N\)-myc amplification behave differently in their response to combination treatment?

We utilized 5 neuroblastoma cell lines in our study including LA-N-1, LA-N-2, LA-N-5 and IMR-32 containing amplified \( N\)-\emph{myc} and SH-SY5Y, lacking \( N\)-\emph{myc} amplification. These cell lines can be induced to differentiate to certain degrees with substances such as RA, TPA and IFN-\( \gamma \) [26, 89, 201, 301, 302]. To investigate the effects of the various inducers we assessed the effect on proliferation and viability by cell counting and evaluated the differentiation by studying morphology, by measuring the amount and length of neurites, and in two of the cell lines by measuring the expression of neurofilaments.

RA and IFN-\( \gamma \) cooperate in inducing growth inhibition and differentiation

RA induced growth inhibitory effects in all the cell lines where loss of viability contributed much of this effect in LA-N-1 and LA-N-2 cell lines. RA did also induce differentiation to various degrees in all cell lines investigated and this effect was most pronounced in SH-SY5Y cells, the non-\( N\)-\emph{myc} amplified cell line. The addition of IFN-\( \gamma \) to RA led to enhanced growth inhibition and/or differentiation in all the cell lines investigated. Enhanced growth inhibition by RA+IFN-\( \gamma \) was observed in LA-N-5, IMR-32 and LA-N-1 cells exhibiting \( N\)-\emph{myc} amplification, where complete growth arrest was seen after 2-4 days, in contrast to RA alone where the cell numbers still were increasing. In LA-N-2 and SH-SY5Y cells RA treatment effectively inhibited the increase of cell numbers but addition of IFN-\( \gamma \) still enhanced the rate of the process. The addition of IFN-\( \gamma \) to RA led to an enhanced differentiation in all the cell lines as observed by increased percentage of differentiated cells and in particular in the increased length of neurites.

TPA and interferon-\( \gamma \) induces growth inhibition and differentiation

TPA alone had effects on growth inhibition and differentiation to different degrees in these five cell lines. TPA had pronounced effects on inhibiting proliferation of SH-SY5Y cells and to a lesser extent in LA-N-1, LA-N-5 cells, but had only weak effects on the other two cell lines. TPA also induced pronounced morphological changes indicative of a neuronal
differentiation in SH-SY5Y, but poor or no effects in the other cell lines. The addition of IFN-γ to TPA caused enhanced growth inhibition to various degrees in all the cell lines and the effect was particularly evident in LA-N-5 and IMR-32 cells, two cell lines with very high N-Myc levels. The effect on decreased growth in IMR-32 could to a certain extent be explained by loss of viability. TPA+IFN-γ also caused pronounced effects on differentiation compared with TPA alone in four out of five cell lines where the percentage of differentiated cells and/or the neurite length were increased. Investigation of neurofilament expression, a marker of neuronal differentiation, in IMR-32 and LA-N-5, after treatment with TPA, IFN-γ or the combination corroborated the data on cell growth and differentiation since treatment with TPA+IFN-γ induced stronger intensity of neurofilament immunofluorescence than either substance alone. LA-N-2 did not respond to TPA or TPA+IFN-γ with neurite extension, but instead exhibited a more flat morphology, which was accentuated by addition of IFN-γ to TPA.

IFN-γ alone inhibited growth in SH-SY5Y, LA-N-5 and to a lesser degree in IMR-32 and induced differentiation to some extent in all of the cell lines, but the effect was most pronounced in SH-SY5Y and of LA-N-1 cells.

In summary, combination of IFN-γ with RA or TPA enhanced growth inhibition and differentiation in 5/5 neuroblastoma cell lines. Single agent treatment was in general most effective in the cell line SH-SY5Y, lacking N-myc amplification and the addition of IFN-γ had the largest impact on growth inhibition and differentiation in the N-myc amplified cell lines where single agent treatment was often not effective.

Upregulation of Mad1 during differentiation of the human neuronal differentiation model SH-SY5Y (Paper II)

As has been described earlier, Mad1 has been reported to be upregulated during in vitro and in vivo differentiation, including in the developing nervous system in mice [220, 224, 236, 241, 243]. However, so far no investigation concerning Mad1 expression in human neuronal differentiation systems has been published and we therefore initiated this study investigating the expression of the Mad/Max/Myc network during human neuronal differentiation utilizing the model system SH-SY5Y.

Mad1 protein was studied by immunoprecipitation of 35S-methionine in vivo labelled cell lysates. The expression of the Mad1 exhibited a biphasic regulation where the synthesis of Mad1 started at 1 hour and peaked at 4 hours after TPA treatment and then decreased to near pre-induction levels at 24 hours. At the later time points 48 and 96 hours post-induction the Mad1 protein levels were again upregulated. c-Myc protein synthesis did also exhibit a biphasic pattern with an early downregulation of expression at 2 hours after TPA induction, regained levels at 12-24 hours, and thereafter reduced levels at later time points. Comparing the protein levels of Mad1 and c-Myc with levels observed in monoblastic U-937 cells used as reference cells showed that the levels were quite low. Max protein levels were not regulated to any major extent throughout the differentiation process.

In summary, Mad1 is upregulated and c-Myc downregulated during TPA-induced differentiation of SH-SY5Y cells.

Mad1 has been observed to be upregulated during in vitro and in vivo differentiation of several cell types including cells of the developing murine nervous system [220, 224, 236, 241, 243, 244]. We here show that Mad1 is also upregulated in human neuronal cells during in...
vitro differentiation induced by TPA. Mad1 was upregulated early after TPA treatment and thus preceeded the differentiation process. This is in agreement with studies during differentiation of myeloid and keratinocytic cells where early upregulation of mad1 is observed [220,243,244]. Unlike the myeloid and keratinocytic cells, Mad1 expression in SH-SY5Y cells was biphasic and exhibited an early and late upregulation. The murine erythroleukemia cell line on the other hand also exhibited a similar biphasic pattern during induced differentiation [230]. Late upregulation of mad1, as is also observed in SH-SY5Y cells, was observed in the teratocarcinoma cell line p19 and in adipoblasts induced to differentiate [234,241]. These differences in Mad1 expression can have several explanations such as differences in cell type and stage of differentiation, differential effects on Mad1 expression of the inducers. The need of proliferation for commitment and differentiation to take place could also be a factor explaining the differences. In the adipoblast system, for example, it is suggested that ectopic mad1 expression inhibits differentiation by way of inhibiting the proliferative burst needed for the differentiation to proceed. In these adipoblasts mad1 was also upregulated late during induced differentiation possibly to allow for the proliferative phase [234]. The levels of Mad1 was quite low in SH-SY5Y cells when comparing with U-937 monoblasts, but since c-myc levels were also very low the observed upregulation of Mad1 could still be enough to cause a switch towards Mad1:Max heterodimers.

Regulation of the Mad/Max/Myc network after combination treatment with interferon-γ and RA or TPA (Paper I and II)

In paper I we show that interferon-γ can cooperate with RA or TPA in enhancing growth arrest and differentiation in several neuroblastoma cell lines with or without N-myc amplification. This raises the question whether these signals counteract N-Myc expression and/or activity and we therefore extended the study to investigate the regulation of the Mad/Max/Myc-network in response to the combined treatments.

Specific questions:
- Will the biological effects on differentiation and growth arrest after treatment with RA,TPA and IFN-γ correlate with regulation of the components of the Mad/Max/Myc-network?
Since combination therapy was effective, in particular in N-myc amplified cell lines, will N-Myc be downregulated and/or Mad upregulated?

Regulation of the Mad/Max/Myc network in response to combination treatment

We chose to investigate the regulation of the Mad/Max/Myc network in response to combination treatment in LA-N-5 and IMR-32,two N-myc amplified cell lines, which both exhibited growth arrest in response to the combination of IFN-γ with TPA or RA. The protein expression of Mad1, N-Myc and Max was investigated by immunoprecipitation of cell lysates from 35S-methionine labelled cell lysates from IMR-32 and LA-N-5 cells with specific antibodies of after three days of treatment.

Treatment of the two cell lines with TPA alone showed weak upregulations of Mad1 expression. This is in contrast to the non-N-myc amplified cell line SH-SY5Y where induction of Mad1 was much stronger after TPA treatment alone. Addition of IFN-γ led to a pronounced upregulation of Mad1 in LA-N-5 cells and to a lesser extent in IMR-32 cells, thus correlating
with cooperative effect of TPA+IFN-γ on growth inhibition and differentiation. N-Myc protein levels, on the other hand, were not at all or weakly reduced after TPA or TPA+IFN-γ treatment.

In contrast, RA+IFN-γ did not affect Mad1 levels, instead RA+IFN-γ treatment led to downregulation of N-Myc protein expression in both cell lines. RA alone had small effects on N-Myc expression in LA-N-5 cells, in repeated experiments the reduction of N-Myc synthesis was around 2-fold, or less in both cell lines.

Protein levels of Mad1 and N-Myc in general reflected mad1 and N-myc mRNA levels thus pointing towards a transcriptional regulation of the network by these treatments. One exception was the maintained protein levels of N-Myc protein in IMR-32 cells after TPA+IFN-γ treatment where N-myc mRNA levels are reduced. We have not investigated this any further, but hypothetical explanations for this discrepancy are regulation of protein stability, post-translational changes or different kinetics.

In summary, the cooperative effect of TPA+IFN-γ or RA+IFN-γ on growth inhibition and differentiation correlated with increased Mad1 protein levels and decreased N-Myc expression, respectively. This suggests that different pathways affect the components of the network in distinct ways, but with similar outcomes.

Differentiation and/or growth inhibition also take place at certain instances where no or very small changes in Mad1 or N-Myc expression are observed, for example after TPA treatment of LA-N-5 cells. There might be several explanations for this; (i) even though N-Myc is highly expressed, the differentiation inducers might have downregulated N-Myc activity as have been shown for U-937 monoblastic cells after TPA+IFN-γ treatment [300], (ii) the different agents might affect pathways downstream of N-Myc, thereby circumventing the negative effect of Myc on differentiation and (iii) small differences in expression levels of components of the Mad/Max/Myc network might still be important, since the Mad-proteins could for example cooperate with other factors negatively affecting growth and/or inducing differentiation such as cyclin dependent kinases inhibitors. Further, small upregulations of Mad1 expression levels could be accompanied by enhanced Mad1 activity by for example phosphorylations potentially affecting for instance interaction with Sin3 or other interacting proteins.

**Ectopic expression of Mad1 induces growth inhibition, but does not enhance differentiation in U-937 cells (Paper III)**

Mad1 is, as mentioned previously, expressed mainly in differentiated tissues and is upregulated during induced differentiation of many cell lines in vitro. We used the parental differentiation model U-937 and the v-Myc transformed U-937 cell line to investigate the role of ectopically expressed Mad1 in growth inhibition and differentiation in these cells.

**Specific questions:**
- Will ectopic expression of Mad1 inhibit growth in U-937 cells?
- Can Mad1 induce differentiation of U-937 cells in the absence of differentiation signals or can Mad1 enhance differentiation induced by optimal or suboptimal levels of RA, TPA or VitD₃?
- Can Mad1 reestablish growth arrest and differentiation blocked by v-myc in the v-Myc transformed U-937 cells?

The inducible mad1 expression systems

We created stable cell lines in both U-937 and v-Myc transformed U-937 cells by using the inducible lac- operator repressor or tet-operator activator systems [303, 304]. In short, the lac operator system consists of two constructs, one containing the lac repressor and the other containing the gene of interest controlled by the lac operator in front of a minimal CMV promoter. In the absence of isopropyl-β-D-thiogalactoside (IPTG), a nonhydrolyzable galactose analog, the lac repressor bind the lac operator sequences as a homotetramer, thereby inhibiting expression of the lac operator-controlled gene, in this case mad1. Addition of IPTG induces a conformational change in the lac repressor complex which decreases the repressor’s affinity for the operator thereby alleviating the repressing effect and the gene of interest can be transcribed. The tet activator system, in analogy with the lac repressor system, also utilizes two constructs; one harboring the artificial tet activator, that is derived from fusing a VP16 activation domain with a mutant Tet repressor thereby creating an activator, and the other construct containing several tet operators upstream of a minimal CMV IE promoter sequence regulating the expression of the gene of interest. Addition of doxycycline, a tetracycline analog, induces binding of the tet-activator to the tet operator sequences thereby inducing expression of the gene, i.e. mad1 in this case. The tet operator system exists in two variants; the “tet-off” system where addition of tetracycline inhibits the tet activator from binding to the tet operator thereby stopping transcription, while addition of tetracycline in the “tet-on” system activates the tet activator and the gene controlled by the tet operator is transcribed. We have chosen to use the “tet-on” system.

Characterization of Mad1-expressing U-937-1 clones

We have created several U-937 clones with inducible mad1 expression. U-937-rtTA cells, containing a stably integrated tet activator, were transfected with the plasmid containing the tet operator upstream of mad1 by electroporation. We selected a number of clones and investigated the mad1 protein and mRNA levels by immunoprecipitation of 35S-methionine labelled cell lysates with specific Mad1 antibodies by and northern blot analysis, respectively, in the presence or absence of doxycycline. The clones exhibited variable basal and inducible levels of Mad1. Kinetic studies of the Mad1 protein expression showed that Mad1 expression was upregulated by 6 hours after induction with doxycycline and protein levels were maintained at least up to 48 hours. Coimmunoprecipitation studies showed very small amounts of endogenous Mad1 in complex with Max in a control clone containing the luciferase gene instead of Mad. In the Mad1-expressing clone, Mad1 protein was readily detected in complex with Max already without induction with doxycycline. Addition of doxycycline further induced the amount of Mad1:Max complexes. At all times, the expression of basal levels of Mad1 were higher in the Mad1-expressing clones than in the control clones and in U-937 cells induced to differentiate with TPA.

The effect of Mad1 on proliferative capacity

When studying the effect of Mad1 on growth we observed that the Mad-clones grew slower in suspension and also formed fewer colonies than control clones in semi-solid medium. This effect was evident already before the addition of doxycycline suggesting that the
basal levels of Mad1 were sufficient to affect the proliferation rate. Further induction of Mad1 with doxycycline only gave small additional growth inhibiting effects. Cell cycle analysis showed that the Mad-clones exhibited increased accumulation of cells in the G1-phase of the cell cycle. Addition of nocodazole, which blocks cells in the G2/M phase of the cell cycle, also showed that a higher percentage of Mad1-expressing cells accumulated in the G1 than in the G2/M phase compared with the control cells. The effect of Mad1 in our system therefore corroborates data from other groups in establishing the role of Mad1 as a growth inhibitor.

The effect of Mad1 on differentiation

We investigated whether induced Mad1 expression would influence the state of differentiation of U-937 cells by measuring CD11c and CD14, surface antigens specific for monocyte differentiation, by flow cytometry at different time points after addition of doxycycline. We could not detect any changes in the expression of these surface markers on the mad1-containing clones compared with the control clones in the presence or absence of doxycycline. Further, we could neither detect any enhancing effects of Mad1 on differentiation induced with optimal and suboptimal levels of RA, vitD3 or TPA when analyzing the expression of the differentiation markers CD11c and CD14. In contrast, the RA induced differentiation was rather inhibited in the Mad-expressing clones at 1 and 3 days post-treatment at both optimal and suboptimal concentrations of RA as indicated by a lower percentage of Mad1-expressing cells expressing CD11c. At 5 days post treatment the percentage of cells expressing CD11c was similar in the control and the Mad-expressing cells, thus indicating a delayed, rather than inhibited differentiation. In summary, Mad1 induced growth inhibition of U-937 cells, but did not induce or enhance differentiation. Rather delayed RA-induced differentiation was observed in Mad1-expressing cells.

We also investigated the effect of Mad1 expression in the v-Myc expressing U-937 cells using the inducible lac repressor system. We created several mad1-containing clones by co-electroporation of the lac repressor construct together with the lac operator/mad1. A lac operator/luciferase construct was used as a control. Clones were selected and characterised regarding mad1 protein and mRNA expression by immunoprecipitation of 35S-methionine labelled cell lysates from v-Myc transformed Mad1-expressing U-937 cells with specific Mad1 antibodies and by northern blot analysis, respectively, before and after the addition of IPTG. The clones contained variable amounts of basal and induced levels of Mad1. The Mad1-expressing cells had elevated basal levels of Mad1 as compared to parental v-Myc transformed U-937 cells. Induction of Mad1 expression with IPTG increased the levels of Mad1 high above the TPA-induced level of Mad1.

Since v-Myc transformed U-937 cells are blocked in their ability to differentiate, we speculated that Mad1 could reestablish this differentiation together with TPA. We first investigated the ability of these clones to differentiate after induction with TPA in the absence or presence of IPTG by looking at morphological features characteristic of TPA-induced differentiation. Cells differentiated by TPA adhere to the plastic of the flask and also to each other, forming large cell clusters. When investigating the morphological appearance the development of adherence was inhibited in the parental v-Myc transformed cells and in the v-Myc transformed lacO/luciferase containing control cells as expected. Surprisingly, no improvement of adherence could be observed in the Mad1-expressing clones. We also investigated the surface expression of CD11c as an additional marker of differentiation, after 3
days of TPA treatment in the absence or presence of IPTG. No or very small upregulation could be observed in CD11c expression in the parental v-Myc transformed U-937 cells, as expected, in contrast to the U-937-GBT cell line, lacking v-Myc expression which exhibited high levels of CD11c expression 3 days after TPA treatment. Despite the high level of Mad1 expression no or very small increases in CD11c expression could be observed in mad1-containing U-937 cells in the absence or presence of IPTG.

We next investigated the cell cycle distribution after TPA treatment for three days in v-Myc transformed parental, Mad1-expressing and luciferase containing U-937-myc-6 cells. We only observed modest increases in G1 accumulation in the wild type and luciferase expressing clones after TPA treatment. In contrast, a pronounced increase in G1 cells and a corresponding decrease of S phase cells was observed in the Mad1-expressing cells. In summary, the G1 arrest was reestablished in Mad1-expressing clones together with TPA, but the differentiation block still prevailed.

The lack of or negative effects of Mad1 expression on spontaneous or induced differentiation that we observe in U-937 cells, contrast with the data from Cultraro et al. [230] where Mad1 induces differentiation, while our results agree with a recent report from Pulverer et al. [234] suggesting inhibitory effects of Mad1 on adipocyte differentiation. The differences between these systems regarding the effect of Mad1 on differentiation may have several explanations. The three reports, as well as others, all agree on Mad1 as a growth inhibitor. This anti-proliferative effect of Mad1 might affect the differentiation process differently depending upon cell type. Growth inhibition and differentiation are usually linked, but in several cell systems one has observed a need for an initial proliferative phase possibly allowing for transcription and translation of genes necessary for the differentiated phenotype and also expansion of the committed cells to receive larger numbers of terminally differentiated effector cells. The link between proliferation and differentiation has been observed in several cell systems, for example in resting B-chronic lymphocytic leukemic cells where treatments inducing proliferation and subsequent differentiation induced a higher degree of differentiation than a protocol only inducing differentiation and no DNA synthesis [210]. In murine erythroleukemia cells, c-myc exhibited a biphasic pattern during the induction of differentiation. Ectopically expressed c-myc, which still exhibited the normal downregulation during differentiation was reexpressed earlier, correlating with accelerated differentiation [209]. In human primary keratinocytes, ectopic expression of c-myc also stimulated differentiation [211]. Also in U-937 cells, the cells keep proliferating for some time after induction of differentiation, before arresting in the G1 phase of the cell cycle. There are therefore several examples of a proliferative phase being necessary for differentiation and the inhibitory effect of Mad1 on growth could therefore explain the negative effect observed on RA-induced differentiation in our U-937 cells.

It may seem surprising that Mad1 did not reestablish TPA-induced differentiation in v-Myc transformed cells, in particular since the TPA-induced G1 arrest indeed was regained. One explanation could be that Mad1 is "overdoing the job" resulting in too much growth inhibition, which may be negative for differentiation as discussed above. Another possible explanation is that Mad and Myc control common and distinct sets of target genes, as is implicated by a recent report from O’Hagan et al [197]. One scenario is therefore that Mad1 could counteract the expression of Myc-regulated proliferative genes, but not counteract Myc-induced repression of genes involved in differentiation. As mentioned above, Myc has been observed to inhibit genes via Inr elements in their promoters by inhibiting Inr-binding transactivational
factors such as Miz-1, TFII-I and YY-1 [171-173, 175, 197]. No reports so far has implicated Mad-members in relief of Myc-repression at these sites, even if Max has been found in complex with Myc at these sites. [174, 175]. Myc can also promote cell growth and inhibit differentiation by induction of genes such as id2, a HLH protein that inhibits bHLH proteins necessary for differentiation [305]. A third explanation for the lack of reestablishment of differentiation by Mad1 in v-Myc transformed U-937 cells could be that different threshold levels of Mad1 are required for counteracting v-Myc induced proliferation and v-Myc repression of differentiation. The levels of Mad1 may therefore not be high enough to relieve the v-Myc block of differentiation, but sufficient to reestablish G1 arrest. We are currently investigating if the overexpression of Mad1 reduces common target genes for the Myc- and Mad-family, such as hTERT, cyclin D2 and ODC. We are also investigating the complex-formation of Myc:Max and Mad:Max heterodimers in v-Myc transformed Mad1-expressing U-937 cells to clarify if there really is a switch into Mad:Max dimers or if Myc still could predominate.

**Mad1 reestablishes or enhances growth arrest induced by TPA or cytokines in v-myc expressing U-937 cells (Paper III and IV)**

The growth inhibitory effects of TGF-β has been suggested to be at least partly mediated by downregulation of c-myc at the transcriptional level [73] and constitutive c-Myc expression has been reported to abrogate TGF-β induced cell cycle arrest in fibroblasts and epithelial cells [53, 74]. It has recently been reported that the induction of p15INK4b by TGF-β is mediated by relieved c-Myc induced repression of the Miz-1 activator through downregulation of Myc [176]. IFN-γ treatment has also been reported to induce downregulation of Myc [30, 48]. However, TGF-β and IFN-γ, have been observed to have growth inhibitory effects on v-Myc expressing U-937 cells despite the maintained expression of the v-Myc protein indicating that these cytokines induce signals which counteract Myc activity [299, 306]paper IV). This suggests that inhibiting the expression and/or activity of Myc may be important for the growth inhibitory effects of molecules such as TGF-β and IFN-γ. Regulation of the Mad-family of repressors could be one way of inhibiting Myc function. We therefore investigated if Mad1 expression was regulated by TGF-β and if growth inhibition induced by TGF-β or IFN-γ was enhanced in Mad1-expressing v-Myc transformed cells.

**Specific questions:**
- Does TGF-β affect the expression of Mad1?
- Is growth inhibition induced by TGF-β and IFN-γ enhanced by Mad1?

We first investigated the effect of TGF-β treatment on cell growth in v-Myc expressing U-937 cells. As shown earlier, TGF-β, but not TPA, induced growth arrest as seen by an accumulation of 86% of the cells in G1 phase of the cell cycle and a concomitant decrease of cells in S phase [205, 306]. U-937-GTB parental and U-937-neo-6 control cells lacking v-myc expression were cell cycle arrested in response to both TGF-β and TPA. To investigate the effect of TGF-β treatment on the synthesis of the Mad/Max/Myc network proteins, the respective proteins were immunoprecipitated with specific antibodies from 35S-methionine labelled v-Myc expressing U-937 cell lysates. The synthesis of Mad1 increased substantially, while that of c-Myc decreased in response to TGF-β treatment. v-Myc expression was
somewhat decreased, while Max levels remained unchanged. To further investigate the hypothesis that Mad1 is involved in TGF-β's antiproliferative effect on v-Myc expressing cells, a kinetic study of Mad1 upregulation after TGF-β treatment was carried out. Mad1 synthesis started to increased 8 hours after TGF-β treatment and increased gradually to reach a maximum level at 72 hours. Cell cycle analysis show that TGF-β induced G1-arrest is a quite slow process and cells started to accumulate in G1 first at 72 hours posttreatment leading to cell cycle arrest by 96 hours. Titration of TGF-β concentrations did also correlate with cell cycle distribution where already very low levels of TGF-β led to Mad1 upregulation and an increased G1/S ratio. This shows that Mad1 upregulation correlated with and preceeded the G1-cell cycle arrest. The amount of Mad1 and Myc in complex with Max after TGF-β treatment was assessed by coimmunoprecipitation of 35S-labelled v-Myc expressing cell lysates with antibodies against Max under low stringency conditions. The proteins in complex with Max were released by high stringency wash and the amount of Mad1 and Myc could be investigated using specific Mad1 and Myc antibodies. The upregulation of Mad1 during TGF-β treatment was followed by an increased amount of Mad1 in complex with Max, its partner and in contrast, the amount of c-Myc, and to a lesser degree v-Myc, in complex with Max decreased. TGF-β treatment also led to decreased Myc dependent transactivation assayed by using an artificial Myc-responsive promoter/reporter system, m4mintk-Luc, driven by four copies of E-box binding sites upstream of a minimal tk promoter. TSA, a histone deacetylase inhibitor, partly relieved this repressive effect of TGF-β. This result is consistent with a role of Mad1 or some other repressor protein dependent upon histone deacetylase activity for its repressional effect (for review [307] in inhibiting v-Myc activity in response to TGF-β. Next, v-myc expressing U-937 cells with a stably integrated inducible mad1 was utilized to investigate whether the responsiveness to TGF-β was altered in such cells. Kinetic experiments showed that the accumulation of cells in G1 was evident already at 48 hours in response to TGF-β in the Mad1-expressing cells, while the corresponding increase of cells in G1 in control cells was seen at 72 hours. And at 72 hours the number of cells in G1 in the Mad1-expressing clones was even larger and a further decrease of cells in S phase was also seen suggesting that Mad1 somehow enhances TGF-β's growth inhibitory effect.

IFN-γ has also been observed to induce partial growth arrest in v-Myc expressing U-937 cells [299]. A larger accumulation of cells in the G1 phase was observed in Mad1-expressing v-Myc transformed U-937 cells in response to IFN-γ in comparison with parental and control luciferase containing cells (Paper III).

In summary, upregulation of Mad1 expression preceeds growth arrest by TGF-β treatment and results in increased Mad1:Max heterodimer formation. The observed repression of Myc-induced transactivation by TGF-β is partly relieved by histone deacetylase inhibition implicating Mad1 or some other HDAC-dependent repressor in counteracting Myc activity. The growth arrest induced by TGF-β and IFN-γ occurred with faster kinetics and/or was more pronounced in Mad1-expressing cells.

This work suggests that Mad1 can cooperate with several different substances to overcome the proliferative effect of Myc and to induce growth arrest. The mechanism for this is at present unclear. As mentioned above, inhibition of Myc activity seems important for inducing differentiation and/or growth arrest by several inducers such as TPA, TGF-β and interferon-γ. There are several possible mechanisms for interfering with Myc activity. (i) by increasing the expression of Mad family members, (ii) by directly inhibiting Myc activity or decreasing Myc
expression and (iii) affecting factors acting downstream of Myc, for example CKIs. As we show here TGF-β is apparently capable of inducing Mad1 and this upregulation correlates with TGF-β:s inhibition of Myc expression and activity. TGF-β also upregulates the expression of the CKIs p21Cip1, p27Kip1 and p15Ink4b in many cell types. TPA differ from TGF-β and IFN-γ in that it cannot induce growth inhibition of v-Myc expressing U-937 cells. Although TPA normally induces both Mad1 and p21Cip1 expression in U-937 cells, this is blocked in v-Myc expressing cells. As mentioned above Myc represses the p21 promoter and obviously influences the expression of Mad1 directly or indirectly. TGF-β-induced signals apparently have the capacity to induce both Mad1 and p21Cip1(data not shown) in v-Myc transformed cells. It is tempting to speculate that the combined action of Mad1 and p27 or some other CKI contribute to the inhibition of v-Myc activity. The combination of TPA+IFN-γ has been shown to reestablish growth arrest and differentiation and to inhibit Myc activity, as evidenced by decreased Myc-dependent transactivation, specific reduction of v-Myc;Max complexes and associated DNA-binding activity [300] TPA and IFN-γ also reestablishes TPA-induced expression of p21Cip1. Thus, several examples point towards the importance of inhibiting c-Myc activity for growth arrest and differentiation to proceed. The Mad-family seems to be one important player in this process.
CONCLUSIONS

I Mad1 is upregulated during human neuronal differentiation of SH-SY5Y cells

II Interferon-γ cooperates with retinoic acid and TPA in inducing growth arrest and differentiation of N-myc amplified neuroblastoma cell lines

III Regulation of the Mad/Max/Myc network by combination therapy with interferon-γ and TPA or RA correlates with enhanced differentiation and growth arrest

VI Ectopic expression of Mad1 induces growth inhibition, but does not enhance induced differentiation of monoblastic U-937 cells

V Mad1 reestablishes TPA-induced growth arrest, but not differentiation in v-Myc expressing U-937 cells

VI Mad1 is upregulated during TGF-β induced growth inhibition of v-Myc expressing U-937 cells
The Mad/Max/Myc network is important in regulating several cellular mechanisms and the function of the Mad/Max/Myc network probably mainly lies in their ability to regulate certain sets of target genes. A plethora of putative Myc target genes have recently been identified by micro array technique and also some putative Mad-family targets. The knowledge about how this network regulate transcription has also increased the last few years with the identification of their involvement in chromatin modification (for review [144]. In short, Mad-family members could repress their genes by recruiting histone deacetylase activity to DNA sequences of specific genes. The Myc-proteins, on the other hand, interact with complexes containing histone acetyltransferase activity and also with chromatin remodelling complexes. The role of these histone modification complexes in Myc mediated repression is at the moment unknown, although knowledge concerning the overall mechanism of Myc repression is growing.

However, upstream signals negatively regulating the activity and expression of the Mad/Max/Myc network is still an enigma. Although several pathways have been suggested in regulating Myc, both positively and negatively, the mechanism for inhibition of Myc activity or downregulation of Myc expression or how Mad-family members are positively regulated still remains unclear [183, 184, 300, 308, 309]. Since deregulated Myc has been found in many tumors and high Myc expression and activity is correlated with more aggressive tumors, finding ways of interfering with Myc activity or expression could be important for treatment of these tumors. We have therefore chosen to investigate growth inhibitory pathways or factors, such as TGF-β, retinoic acid, IFN-γ, TPA and the Mad-family members, to elucidate if these could be potential candidates for inhibiting Myc. We have utilized neuronal and hematopoietic model systems for studying the effects on Myc since Myc deregulation is found in tumors of neuronal and hematopoietic origin. We show here that induced Mad1 expression and/or inhibited Myc expression or activity correlate with induced growth arrest and differentiation. The studies of neuroblastoma cells show that combination of interferon-γ with TPA or RA enhances growth inhibition and differentiation correlating with induction of Mad1 or downregulation of N-Myc. In hematopoietic cells our investigation further establishes Mad1, in agreement with other reports, as a growth inhibitor and as a component in inducing growth arrest in v-Myc transformed cells [184, 226, 228-231]. We also show that one way of TGF-β to counteract Myc and exert its growth inhibitory actions could be through the observed upregulation of Mad1. Growth inhibition by TGF-β is an obstacle many tumor cells need to overcome, since mutations in the TGF-β pathway is found in many cancers [6]. Myc can also overcome cell cycle arrest by repressing CKIs and by inducing id2 which inhibits the Retinoblastoma protein, [32, 174-176, 190, 305]. These examples highlight the central role the Mad/Max/Myc network play in regulation of growth/growth arrest or growth/differentiation. The results presented in this thesis suggest that cytokine-induced signals and the intracellular Myc-antagonist Mad1 seem to cooperate in counteracting Myc activity, a finding with potential therapeutic relevance for tumors with deregulated Myc.
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