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Roles of Myc and Mad in cell cycle and apoptosis

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Cover images: Examples of successfully balanced regulation of cell proliferation and apoptosis during embryogenic development.

To My Family

ABSTRACT

The Myc network proteins are key mediators in regulation of cell growth, differentiation and apoptosis. They are basic region helix-loop-helix/leucine zipper (bHLH/Zip) transcription factors that require hetero-dimerization with Max for specific DNA binding. Mad family members are expressed primarily in differentiated tissues where they recruit histone deacetylase complexes via the mSin3 interaction domain (SID) to repress transcription of target genes and prevent cell growth. In contrast, members of the Myc family activate target gene transcription by recruitment of histone acetyltransferases to their transcriptional activation domain (TAD), inducing proliferation and S phase entry. Myc activation also sensitizes cells to apoptosis in response to stress such as serum deprivation or exposure to cytotoxic drugs. However, mutations acquired during cancer therapy often block Myc-driven apoptosis, explaining the presence of activated myc in many human tumors.

We have characterized the effects of Mad1 and Myc in cell growth control and in apoptosis induced by low serum, by anticancer drugs, or by differentiating agents. Using tet-mad1 inducible cells, we emphasized the important role for Mad1 in inhibition of cell proliferation in low serum, and showed a correlation with a reduced CDK2 activity. In addition, Mad1 induction blocked cell cycle re-entry and resulted in reduced apoptosis in response to serum starvation and to the cytotoxic drug cisplatin. We demonstrated that these effects required transcriptional repression and suggest that Mad1 ensures cell survival and specialization by stabilizing quiescence and protecting against apoptosis during differentiation.

To explore the effect of Myc on the cellular response to conventional chemotherapy, routinely used to complement surgery and radiation therapy when treating cancer patients, we used tet-myc inducible cells, together with Rat1 fibroblasts with different Myc status. In these model systems, we demonstrated that c-Myc enhanced the apoptosis induced by etoposide, doxorubicin, and cisplatin. Furthermore, we found that etoposide and doxorubicin signaling involved activation of pro-apoptotic Bax and of caspase 3 and 9. In addition, etoposide required pro-apoptotic PKCδ for efficient apoptosis induction. We observed a similar Myc-dependence for efficient apoptosis induction by the chemotherapeutic agents camptothecin and paclitaxel. Apoptosis was enhanced both by c-Myc in Rat1 cells and by MYCN in neuroblastoma cells with conditional MYCN expression. While camptothecin signaling involved activation of Bax and caspases together with PKCδ, our data suggest that paclitaxel induces apoptosis through a pathway distinct from mitochondria and PKCδ signaling. Neither of the drugs affected Myc/Max DNA-binding, but camptothecin treatment reduced transactivation by several transcription factors, suggesting this as a mechanism for its effects. Taken together, our data establish the involvement of Bax, caspases, and PKCδ signaling in Myc-dependent apoptosis induced by etoposide and camptothecin, but not by cisplatin and paclitaxel.

We also analyzed cellular differentiation and apoptosis in response to treatment with all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3), used in treatment of acute myelocytic leukemia. Although the fusion protein PML-RARα is a well established target for these drugs, additional mechanisms for their induction of differentiation and/or apoptosis are poorly characterized. For this purpose, we used the PML-RARα-negative promyelocytic leukemia cell line HL60, and confirmed the connection between Myc expression and cellular differentiation status. We found that ATRA-induced terminal differentiation and apoptosis coincided with down-regulation of Myc, while the partially differentiated As_2O_3 treated cells had a repressed, but not abolished, Myc expression. Myc was also present at the promoters of its target genes human telomerase reverse transcriptase (hTERT) and carbamoyltransferase-dihydroorotase (CAD) after exposure to As_2O_3 but not ATRA, suggesting Myc as an important mediator in preventing terminal differentiation after As_2O_3 treatment, possibly through activation of hTERT and CAD.

In conclusion, characterization of the pathways for Myc-mediated apoptosis is essential in the venture to enable their re-activation in tumors overexpressing Myc and thus overcoming acquired drug-resistance. Therefore, Myc levels in human tumors should be considered for tailored treatment using anticancer drugs.

Keywords: Myc, Mad, cell cycle, apoptosis, differentiation, tumorigenesis, chemotherapy

PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following original papers and manuscripts:

I. Bejarano, M. T., **Albihn, A.**, Cornvik, T., Osterdahl Brijker, S., Asker, C., Osorio, L. M., Henriksson, M.
Inhibition of cell growth and apoptosis by inducible expression of the transcriptional repressor Mad1.
Exp Cell Res. 2000, 260(1): 61-72

II. **Albihn, A.**, Lovén, J., Ohlsson, J., Osorio. L. M., Henriksson, M.
c-Myc-dependent etoposide-induced apoptosis involves activation of Bax and caspases, and PKCdelta signaling.
J Cell Biochem. 2006, EPub ahead of print 29 Mar. PMID: 16572399

III. **Albihn, A.**[*], Mo, H.[*], Yang, Y., Henriksson, M.
Camptothecin- but not paclitaxel-induced Myc-dependent apoptosis involves PKCdelta signaling.
Manuscript. 2006
[*] Equal contribution

IV. Jiang, G., **Albihn, A.**, Tang, T., Tian, Z, Henriksson, M.
Role of Myc in differentiation and apoptosis in HL60 cells after exposure to arsenic trioxide or all-trans retinoic acid
Manuscript. 2006

c-ABL = Abelson
AIF = Apoptosis-Inducing Factor
ANT = Adenine Nucleotide Translocator
APAF-1 = Apoptotic Protease Activating Factor 1
APL = Acute Promyelocytic Leukemia
As_2O_3 = Arsenic Trioxide
ATRA = All-trans Retinoic Acid
BAX = Bcl-2 Associated protein X
BAK = Bcl-2 homologous Antagonist Killer
BCL-2 = B-Cell Lymphoma 2
bHLH/Zip = basic region Helix-Loop-Helix/ Leucine Zipper
BID = BH3-Interacting domain Death-agonist
BIK = Bcl-2-Interacting Killer
BH = Bcl-2 Homology domain
BL = Burkitt's Lymphoma
CAD = carbamoylphosphate dihydroorotase
CASPASE = Cysteinyl Aspartate Proteinase
CDK = Cyclin Dependent Kinase
CIS = Cisplatin
CKI = CDK Inhibitor
CPT = Camptothecin
DD = Death Domain
DED = Death Effector Domain
DISC = Death-Inducing Signaling Complex
DNA = Deoxyribonucleic Acid
DNA-PK = DNA-dependent Protein Kinase
DXR = Doxorubicin
E-box = Enhancer box
ER = Endoplasmic Reticulum
ERK = Extracellular signal-Regulated Kinase
ETO = Etoposide
FADD = Fas-Associated Death Domain
HAT = Histone Acetyl-Transferase
HDAC = Histone Deacetylase
HR = Homology Region
hTERT = human Telomerase Reverse Transcriptase
IAP = Inhibitor of Apoptosis
JNK/SAPK = c-Jun NH_2-terminal protein Kinase/ Stress-Activated Protein Kinase
MAPK = Mitogen-Activated Protein Kinase
MAD = Max Dimerizer (recently renamed to Mxd for Max Dimerization Portein)
MAX = Myc-Associated protein X
MB = Myc Box
MEF = Mouse Embryo Fibroblast
MEK = MAPK/ERK Kinase
MGA = Max Gene Associated
MIZ-1 = Myc-Interacting Zinc finger protein-1
MLX = Max-Like protein X
MNT = Max Binding Protein
MXI = Max-Interactor 1
MYC = Myelocytomatosis
NB = Neuroblastoma
NLS = Nuclear Localization Signal
ODC = Ornithine Decarboxylase
PARP = Poly (ADP-Ribose) Polymerase
PI3K = Phosphatidylinositol 3-Kinase
PKCδ = Protein Kinase C delta
PML-RARα = Promyelocytic Leukemia – Retinoic Acid Receptor alpha
PTP = Permeability Transition Pore
PTX = Paclitaxel/Taxol
pRb = Retinoblastoma
REF = Rat Embryo Fibroblast
RNA = Ribonucleic Acid
SID = Sin3 Interaction Domain
STS = Staurosporine
TAD = Transcriptional Activation Domain
TNF = Tumor Necrosis Factor
TRRAP = Transformation/Transcription domain-Associated Protein
VDAC = Voltage-Dependent Anion Channel
WT = Wild Type

AIMS OF THIS THESIS

The general aim of this thesis was to increase the knowledge of effects of Mad1 and c-Myc in cell growth control and in apoptosis when induced by low serum or by treatment with cytotoxic or differentiating agents. For this purpose, I intended to investigate the impact on key mediators of apoptosis and on cell cycle regulation in the presence or absence of Mad1 or Myc. The study was divided into three major parts:

Paper I

In this study we aimed to monitor the cellular response to Mad1 overexpression, analyzing effects on cell growth, cell cycle distribution, and apoptosis; and how Mad1 affected the cellular outcome when exposed to different stress inducers.

Papers II and III

The ambition in paper II was to explore the apoptosis-inducing function of c-Myc, and the interplay between c-Myc and the chemotherapeutic drugs etoposide, doxorubicin, and cisplatin in affecting the expression and/or activation of important apoptosis mediators. These drugs were chosen because they are commonly used in anticancer therapy and since they have different intracellular targets.

To extend the previous study, the objective in paper III was to analyze whether Myc potentiated apoptosis in cells treated with the cytotoxic drugs camptothecin and paclitaxel. In addition to studying drugs with different mechanisms of action compared to the previously used substances, we also wanted to evaluate the possible drug-mediated effects on transcriptional activation and of Myc/Max DNA-binding. Another purpose was to assess whether this effect was restricted to c-Myc or if other Myc family members, such as MYCN, could induce a similar response.

Paper IV

Our aim in this paper was to characterize the relation between differentiation induced by all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3), and Myc signaling. Since a high Myc level would prevent induction of differentiation, and because As_2O_3 in low doses induced only partial differentiation in the PML-RARα-negative HL60 cells, we wanted to compare the status of Myc and its target genes upon treatment with ATRA to that after As_2O_3 administration.

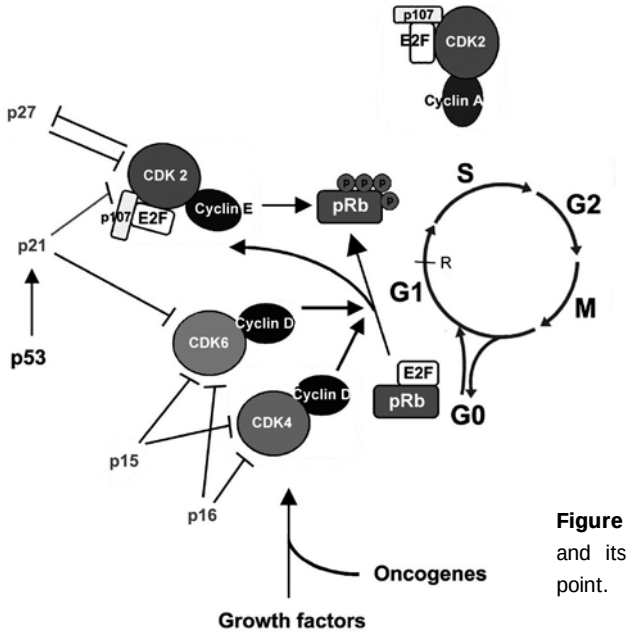


Figure 1. Overview of the cell cycle and its regulators. R = restriction point.

Introduction to cancer and the cell cycle

The definition of cancer, according to Wikipedia, the Free Encyclopedia is: "a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either through invasion or by metastasis". As such, tumorigenesis is a multistep process reflecting genetic modifications that drive transformation of normal cells into highly malignant forms. These changes can occur through lesions as subtle as point mutations, or as obvious as chromosomal translocations. Essential alterations in cell physiology that have been suggested for malignant growth include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (reviewed in (Hanahan and Weinberg, 2000)). These cellular mechanisms are tightly controlled by proto-oncogenes and tumor suppressor genes and will be aberrantly regulated in response to oncogene activation or tumor suppressor inactivation. To date, approximately one hundred potential oncogenes and twenty tumor suppressor genes have been described (Kopnin, 2000).

Oncogenes

An oncogene is a modified gene that aggravates the malignancy of a tumor cell. In early stages of cancer development, oncogene activation increases the probability that a normal cell develops into a tumor cell (reviewed in (Kopnin, 2000)). Following the discovery of the first viral oncogene (v-src for viral rous sarcoma) in 1970 (Martin, 1970), cellular counterparts have been identified, usually coding for proteins in the regulation of cell growth and differentiation. These genes, called proto-oncogenes, are converted into oncogenes by mutations such as amplifications

or chromosomal translocations (Friedrich et al., 1976; Stehelin et al., 1976). These mutations are considered dominant, since alteration in one gene copy is sufficient for induction of the oncogenic phenotype. Functionally, oncogenes can be divided into five classes; transcription factors, growth factors, receptors, signal transducers, and regulators of cell death (Pelengaris and Khan, 2006).

In addition to myc that will be described in more detail, other well characterized proto-oncogenes are ras, bcl-2, cyclin D, E2F, mdm2, hTERT and PI3K. Among these genes, ras (rat sarcoma) encodes one of the first proteins identified with the ability to regulate cell proliferation and survival (reviewed in (Downward, 2003)). Ras proteins are small membrane-bound GTPases that, when mutated, lose their ability to process GTP and thus enable a constitutively active state. The anti-apoptotic Bcl-2 (B-cell lymphoma 2) protein resides in the membranes of mitochondria, endoplasmic reticulum, and the nucleus where it regulates their permeability (Cory and Adams, 2002), and Cyclin D and E2F are central cell cycle regulators (Nahle et al., 2002). The E3 ubiquitin ligase MDM2 (mouse double minute 2) targets the p53 and pRb tumor suppressor proteins for degradation and thus, in its mutated form, MDM2 enables uncontrolled proliferation by preventing cell cycle arrest at the p53 and pRb controlled check points, as well as p53-mediated apoptosis (Sherr, 2004; Uchida et al., 2005). hTERT (human telomerase reverse transcriptase) encodes the catalytic subunit of telomerase (Poole et al., 2001), and PI3K (phosphatidylinositol 3-kinase) is a mediator in the phosphatidylinositol pathway responsible for activating the anti-apoptotic Akt kinase (Vivanco and Sawyers, 2002). One intriguing finding is that oncogenes such as c-Myc, E2F, and Ras, in addition to their ability to promote tumorigenesis, can also trigger apoptosis or cellular senescence (Kopnin, 2000; Oster et al., 2002).

Tumor suppressor genes

A tumor suppressor gene reduces the probability for a cell in a multicellular organism to enter a tumorigenic state. If such a gene is mutated or deleted, the risk for tumor formation increases. However, unlike oncogenes, tumor suppressor genes generally follow the "two-hit theory", suggesting a requirement for alterations to occur in both alleles before an effect is manifested (Knudson, 1971). An exception to this hypothesis is observed in cases of haploinsufficiency where functional loss of only one allele is required to provide a selective advantage for tumor growth (Quon and Berns, 2001). Tumor suppressor proteins function by repressing cell cycle progression at the check points or, if that fails, inducing apoptosis. They promote their activities by mechanisms such as: repression of genes essential for driving the cell cycle, coupling the cell cycle to DNA damage, initiation of apoptosis to prevent irreparable damage, and prevention of anchorage-independent growth of tumor cells (reviewed in (Sherr, 2004)).

The firstly discovered tumor suppressor gene was pRb in its mutated form, causing human retinoblastoma (Knudson, 1971), and later found altered in other malignancies (Weinberg, 1995). Among its many functions pRb is mainly recognized as an important cell cycle regulator controlling S phase entry in the G1/S transition (McLaughlin et al., 2003). Another important tumor suppressor, the p53 gene, was once dubbed "the guardian of the genome" (Lane, 1992) and is mutated or deleted in at least 50% of human cancers. In addition to inducing p21-mediated cell cycle arrest, p53 promotes apoptosis by transcriptionally activating pro-apoptotic proteins and repressing anti-apoptotic proteins (Sherr, 2004). The p19ARF (alternative reading frame, p14ARF in humans) tumor suppressor physically protects p53 from MDM2-mediated degradation by binding and sequestering MDM2 in the nucleolar compartment of the cell (Weber et al., 1999).

ARF is encoded by the same locus as the cell cycle inhibitor p16 and collectively, mutations of genes in the pRb or p53 pathways are observed in most, if not all, human cancers (Sherr, 2004). Other tumor suppressors include the cell cycle inhibitors p21 and p27 with important roles in check point regulation (Vermeulen et al., 2003), and the PTEN (phosphatase and tensin homologue) phosphatase that inhibits PI3K/Akt signalling (Vivanco and Sawyers, 2002)

The cell cycle

The cell cycle is divided into four different phases: a gap (G1), preceding the S phase where DNA is synthesized and replicated, another gap (G2), and mitosis (M) when cellular material is divided into two daughter cells. Resting cells exit the cell cycle in G1 and enter a quiescent G0 state, accounting for the majority of non-proliferating cells in the human body. The restriction point in G1 is the "point of no return" for entering the cell cycle such that cells beyond this point are unaffected by serum deprivation, while cells that have not yet reached this cell cycle stage regress into a quiescent state (G0). Two additional checkpoints are found before and after replication in the G1/S transition and in G2/M, respectively (Fig. 1). The key regulatory proteins of the cell cycle are cyclin-dependent kinases (CDKs), in their turn controlled by cyclins, CDK inhibitors (CKIs), and by phosphorylation. The best studied CDK substrate is probably the pRb tumor suppressor gene targeted by CDK4 and/or CDK6. When phosphorylated, pRb releases E2F transcription factors to activate genes such as cyclin A and cyclin E, essential for S phase entry. The cyclinE-CDK2 complex maintains the pRb hyperphosphorylation and also phosphorylates its own inhibitor p27, targeting it for degradation at the G1/S transition (Fig. 1). In response to DNA damage, cells arrest at the G1/S and G2/M checkpoints. In the p53-dependent G1/S checkpoint, kinases are recruited to the damaged DNA and enable phosphorylation and stabilization of p53, thus inducing cell cycle arrest or apoptosis, depending on the severity of the damage. DNA damage-induced G2 arrest is mediated by ATM (ataxia telangiectasia mutated)-induced protein kinases, preventing initiation of mitosis by phosphorylation of CDK1 or by sequestering the CDK1-cyclin B activator Cdc25 outside the nucleus. In addition to the p53-independent response in the G2/M checkpoint, a p53-mediated activation of CDK1-cyclin B-inhibitory proteins has also been observed in regulation of this checkpoint (reviewed in (McLaughlin et al., 2003; Vermeulen et al., 2003)).

Cell cycle regulators

Of the eleven CDKs identified to date (Loyer et al., 2005), five are involved in cell cycle regulation. CDK4 and CDK6 are active during G1, CDK2 in the G1/S transition, and CDK1 is active in late G2 and during mitosis (Fig. 1). The fifth kinase, CDK7, acts as a CDK-activating kinase (CAK) when in complex with cyclin H. Active CDKs regulate their protein targets by phosphorylation. While CDK levels remain stable during the cell cycle, the cyclin protein levels reach their peak when they are required and then decrease in a "cycling" pattern. Five out of sixteen cyclins identified thus far are cell cycle-related. The three D-type cyclins (cyclin D1, D2, and D3) bind to CDK4 and CDK6, forming complexes essential for entry into G1. Cyclin D differs from the other cyclins in that it is synthesized as long as growth factor stimulation persists. Cyclin E associates with CDK2 during late G1 to regulate the S phase entry. Upon S phase entry, CDK2 forms a complex with cyclin A, required throughout the S phase. In late G2, cyclin A instead binds CDK1 to promote mitosis where cyclin B takes over the CDK1-association (Fig. 1). The negative regulators, CKIs, are divided into the INK4 family and the Cip/Kip family. Members of the INK4 family, such as p15 and p16, specifically inactivate CDK4 and CDK6 by preventing their

association with cyclin D. The Cip/Kip family includes p21 and p27, primarily acting on CDK-cyclin complexes during G1. Other means of cell cycle regulation is promoted by the CDK-inactivating kinases Wee1 and Myt1, and can also be mediated by sequestering cell cycle proteins into distinct cellular compartments until they are needed (reviewed in (Vermeulen et al., 2003)).

Cancer-associated mutations have been observed in genes encoding most types of cell cycle regulators (reviewed in (Vermeulen et al., 2003)). While alterations in CDKs are mainly reported for CDK4 (Reifenberger et al., 1994), aberrant cyclin expression primarily involves translocations of the cyclin D1 gene resulting in its amplification (Shapiro, 2006). Overexpression of the c-Myc-regulated CDK-activating enzyme, Cdc25, has also been associated with human malignancies. In addition, many CKIs with tumor suppressor function, such as p16 and p27, are found altered or lost in a high fraction of human tumors. Since p16 is functionally interconnected with pRb and CDK-cyclin D, alterations in one of these genes should have similar consequences. In effect, abnormalities in the p16 – pRb – CDK-cyclin D axis occur in the majority of human cancers (Liu et al., 2004; Sherr, 2004).

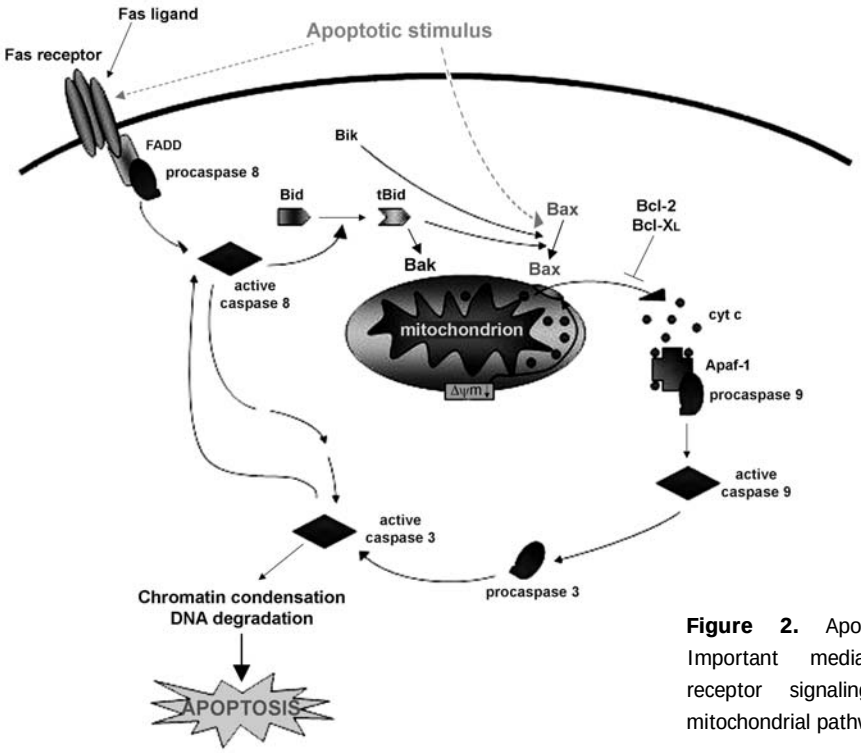


Figure 2. Apoptotic pathways. Important mediators in death receptor signaling and in the mitochondrial pathway are included.

Apoptosis

Apoptosis or programmed cell death (PCD) is an important mechanism to balance cell proliferation and to remove unwanted cells during the development and homeostasis of multicellular organisms (Evan and Littlewood, 1998). The term apoptosis was coined by Currie and colleagues and was originally used to describe the morphological characteristics in cell death different from necrosis (Eguchi et al., 1997; Kerr et al., 1972). Apoptosis is characterized by shrinkage of the cell, membrane blebbing, chromatin condensation, frequently by DNA fragmentation and the appearance of apoptotic bodies which are then engulfed by the surrounding cells. Since the apoptotic bodies are rapidly phagocytosed, there is no inflammatory response as observed during necrosis. The inflammatory response during necrosis is due to plasma membrane rupture with subsequent leakage of cellular contents into the extracellular space. The level of intracellular ATP has been suggested to determine whether a cell will die by apoptosis or necrosis since the apoptotic process is energy dependent (Eguchi et al., 1997). In a conflict situation posed by inappropriate, constitutive growth signals from activated proto-oncoproteins, the affected cell would respond by activating an apoptotic program leading to its own elimination (Evan and Littlewood, 1998).

The apoptotic process can be divided into three main phases: the initiation phase, the effector phase and the execution phase. During initiation, the cell receives signals to induce the apoptotic process. Such signals could be mediated by death receptors, DNA damage, oncogene activation, serum starvation, or other stimuli. The effector phase promotes the cellular decision to die by transducing and amplifying the death stimulatory signals. Apoptosis is then executed by the activated apoptotic machinery, through cleavage of specific cellular proteins and of DNA.

Apoptotic pathways

The two major pathways for apoptosis-induction are death receptor-signaling and signaling through mitochondria (Fig. 2). Activation of either of these pathways eventually targets caspases, the main executioners of cell death. Thus, apoptosis-induction by various stimuli converges on the activation of caspases followed by prevention of DNA repair, DNA fragmentation, and disruption of the cellular structure.

Death receptor-mediated apoptosis

The death receptor Fas/CD95/APO-1 is a member of the tumor necrosis factor (TNF) receptor family, which also includes the TNF-receptor. Upon binding to the Fas Ligand, the receptor trimerizes and recruits the adaptor protein FADD (Fas-Associated Death Domain) through their respective death domains (DD). FADD, in turn recruits procaspase 8 (and/or 10) to its death effector domain (DED), thus forming the DISC (Death-Inducing Signaling Complex) that will cleave and activate procaspase 8 to elicit a cascade of events converging on caspase 3, responsible for execution of apoptosis (Ashkenazi and Dixit, 1998; Krammer, 2000). In type I cells, caspase 8 activates caspase 3 in a mitochondria-independent fashion, whereas in cells of type II, caspase 8 is unable to directly activate downstream caspases probably due to low caspase 8 levels in these cells. In this case, the apoptotic signal is amplified by the mitochondria through caspase 8-mediated cleavage of the pro-apoptotic Bcl-2 member Bid. The truncated Bid (tBid) then translocates to the mitochondrial membrane and stimulates processes to enables cytochrome c release and subsequent events (Gross et al., 1999).

Mitochondria-mediated apoptosis

Various stress-inducing stimuli such as cytotoxic drugs, DNA-damaging agents, hypoxia, growth factor withdrawal, and death-receptor signaling converge on mitochondria. The mitochondrial events observed in response to cellular stress include membrane permeabilization followed by release of death-promoting proteins located in the intermembrane space.

The mechanism by which the mitochondrial membrane is permeabilized is not completely understood but three favored models describe proposed events in opening of the permeability transition pore (PTP), monitoring of PTP-opening by members of the Bcl-2 family, and pore formation by oligomers of the pro-apoptotic Bcl-2 proteins. The major components of the PTP complex comprise the voltage-dependent anion channel (VDAC) in the outer membrane and adenine nucleotide translocator (ANT) in the inner membrane of the mitochondria. PTP opening induces events such as ATP depletion, loss of membrane potential ($\Delta\psi m$) and influx of cytosolic components, causing mitochondrial swelling and outer membrane rupture with subsequent release of intermembrane proteins. However, since mitochondrial rupture is mainly observed in necrosis, opening of the PTP may not be the main mechanism for mitochondrial permeabilization. In the second model, Bcl-2 proteins are proposed to regulate opening of the VDAC such that pro-apoptotic members would increase its pore size to allow passage of cytochrome c and other intermembrane proteins, while anti-apoptotic members would close the channel to prevent cytochrome c release. The third model suggests that oligomerized pro-apoptotic Bcl-2 family members insert into the outer mitochondrial membrane to form channels large enough for protein passage. This theory is the most attractive and has been proven in synthetic bilayers. However, it has not yet been confirmed *in vivo* (Green and Reed, 1998).

Upon membrane permeabilization, released cytochrome c is incorporated into the apoptosome complex together with Apaf-1 (apoptotic protease activating factor 1), dATP, and procaspase 9, to mediate caspase 9-activation and downstream events. Other released pro-apoptotic factors are Smac/DIABLO and Omi/HtrA2, blocking activities of inhibitor of apoptosis proteins (IAPs), and endonuclease G and AIF (apoptosis inducing factor) induce caspase-independent DNA fragmentation (Green and Reed, 1998).

The Bcl-2 family of proteins – the regulators

The bcl-2 oncogene was isolated from the breakpoint of a t(14;18) chromosomal translocation creating the bcl-2-immunoglobulin (Ig) fusion gene (Cleary et al., 1986). To date, more than twenty Bcl-2 family members have been discovered. They share one to four Bcl-2 homology regions (BH) and, according to these, they can be subdivided into pro-apoptotic (BH1-3), anti-apoptotic (BH1-4) and BH3-only proteins. Although they are also found in the nuclear envelope and parts of the endoplasmic reticulum (ER), the outer mitochondrial membrane seems to be their primary site of action (Cory and Adams, 2002).

Pro-apoptotic Bcl-2 proteins

The pro-apoptotic members include Bax-like proteins and BH3-only proteins. The Bax-like proteins contain the BH1-3 domain but lack the BH4 domain, suggested to harbor the anti-apoptotic activity. Bax and Bak are essential for apoptosis-induction as demonstrated in cells where both genes were homozygously deleted. These cells were virtually insensitive to apoptosis induced by such distinct mediators as staurosporine, UV, growth factor deprivation, etoposide and endoplasmic reticulum (ER) stress (Wei et al., 2001), thus suggesting that neither activation of BH3-only proteins, nor suppression of pro-survival Bcl-2 is sufficient to kill cells in the absence of both Bax and Bak. In addition, the importance of Bax in preventing tumorigenesis was demonstrated in bax null mice that exhibited an increased cancer progression as a result of decreased apoptosis (Yin et al., 1997). While Bak is an integral mitochondrial protein, cytosolic Bax is restrained from the mitochondrial membrane until the cell receives a death signal. Apoptotic stimuli then induce a conformational change in Bax and/or Bak to expose the N-terminal part of the protein(s). In the case of Bax, this exposure enables insertion into the mitochondrial membrane and oligomerization, possibly responsible for pore formation and cytochrome c release. Similarly for Bak, N-terminal exposure mediates formation of an activated Bak-oligomer with the ability to permeabilize the outer mitochondrial membrane and enable release of intermembrane proteins (Cory and Adams, 2002).

BH3-only members include Bid, Bad, Noxa and Puma, responsible for transducing death signals from the cytosol to the mitochondria under certain stress conditions. For instance, Bid can be activated by caspase 8 as an amplifier in death receptor signaling; Noxa and Puma appear to be involved in DNA damage-induced cell death due to their p53-regulated expression; and Bad has been implicated in apoptosis induced by growth factor withdrawal. The truncated form of Bid (tBid) translocates to the mitochondria where it induces the activation and oligomerization of Bax and Bak. Bak and Bid are tightly interconnected, but in the case of Bax, activation can be induced by a Bid-independent mechanism. While Bid is activated by cleavage, its inactivation is promoted by phosphorylation. Bad-induced apoptosis requires the multidomain proteins Bax and/or Bak. According to the currently supported model, de-phosphorylated Bad is released from the scaffold

protein 14-3-3 and enables Bax- and Bak-induced apoptosis through binding and sequestering anti-apoptotic Bcl-2 members (reviewed in (Cory and Adams, 2002; Reed, 1998)).

Anti-apoptotic Bcl-2 proteins

Bcl-2 and Bcl-xL are the major anti-apoptotic members. They are both membrane-associated through their C-terminal transmembrane domains and contain the anti-apoptotic BH4 domain. Bcl-2 appears to be essential and its most important function may be to maintain homeostasis in adult tissues. In addition, Bcl-2 is a powerful apoptosis inhibitor with the ability to abrogate both caspase-dependent and -independent cell death, proposedly by preventing mitochondrial membrane permeabilization, and thus promote cell survival (Cory and Adams, 2002; Reed, 1998). As such, it has been implicated in tumorigenesis, and consequently protects from c-Myc-induced apoptosis. However, Bcl-2 also plays a role in regulating calcium homeostasis, modulating antioxidant pathways, and promoting glutathione sequestration to the nucleus. In addition to heterodimerization with other members of the family, Bcl-2 can bind to non-homologous proteins such as Raf-1 and calcineurin, suggested to prevent activation of pro-apoptotic Bad. Raf-1 phosphorylates Bad and calcineurin has been shown to inhibit its dephosphorylation. Negative regulation of Bcl-2 activity in turn, is conferred by pro-apoptotic members and by phosphorylation by for instance CDK1 and JNK. Bcl-xL is located in the outer mitochondrial membrane and has been ascribed a similar role as Bcl-2 in inhibiting mitochondrial membrane permeability through dimerization with Bax or Bak to prevent their oligomerization and/or insertion into the mitochondrial membrane. Another proposed model for Bcl-2/Bcl-xL-mediated inhibition of Bax and Bak activation describes sequestration of BH3-only proteins as the causal event. Furthermore, Bcl-xL has been observed to prevent caspase activation by sequestering Apaf-1 (Chao and Korsmeyer, 1998; Cory and Adams, 2002).

Bcl-2 and Bcl-xL activities are regulated by post-translational modifications such as phosphorylation, de-amidation and cleavage. Chemotherapeutic agents that cause microtubule disruption have been reported to induce their phosphorylation and abrogate their anti-apoptotic function. Caspase-dependent cleavage of Bcl-2 and Bcl-xL may occur in response to Fas ligation, etoposide treatment, or growth factor withdrawal and result in exposure of the BH3 domains, converting these anti-apoptotic proteins into cell death-promoters (Deverman et al., 2002; Fadeel et al., 1999). Deamidation has been reorted as an important mechanism for inactivation of Bcl-xL (Deverman et al., 2002).

Caspases – the executioners

Caspases are a family of cystein proteinases that specifically cleave their substrates after an aspartic acid, initiating the four-residue sequence that determines substrate specificity. The inactive procaspases are activated by proteolytic cleavage at two sites, dividing them into the large and small subunit, and removing the N-terminal pro-domain, respectively. The length of the pro-domains was used for dividing caspases into initiators and effectors.

Among the initiators, carrying long prodomains with motifs allowing interaction with adaptor proteins, caspases 2, 8, 9, and 10 are pro-apoptotic. Initiator caspases are activated by binding to adaptor proteins or by oligomerization-induced autoproteolysis. When activated, they process and activate one of the apoptosis-inducing effector caspases 3, 6, or 7. These pro-apoptotic caspases possess short pro-domains and are mainly activated by other caspases. However, cleavage by

proteinases and kinases such as calpain, granzyme B, and protein kinase C delta (PKCδ) has also been reported. The activated effector caspases then contribute to the morphological and functional changes associated with apoptosis by cleavage of substrates sch as the CAD (caspase-activated DNase) inhibitor ICAD to enable DNA fragmentation, or the DNA polymerase poly (ADP-ribose) polymerase (PARP) (Herr and Debatin, 2001; Thornberry and Lazebnik, 1998).

Stress-induced pro-apoptotic and pro-survival signaling

Apart from activating the classical apoptosis-inducing death receptor- and mitochondrial pathways, cellular stress activates signaling cascades such as pro-apoptotic and pro-survival MAP kinases (JNK and ERK, respectively), activation and cleavage of the pro-apoptotic protein kinase c isoform, PKCδ, promoting apotosis in response to death receptor-signaling and DNA damage, and the anti-apoptotic PI3K pathway in which activation of Akt will favor survival (Fig. 3).

PKCδ and apoptosis

The protein kinase C (PKC) family of serine-threonine kinases is involved in cellular growth, differentiation and apoptosis. They are activated by diacylglycerols, calcium, and/or by cleavage, releasing the catalytic subunit from its regulatory domain. Of the pro-apoptotic isoforms, the ubiquitously expressed PKCδ is important in cell growth control and can be induced by apoptotic stimuli. Activation of PKCδ can also be conferred by c-Abl (Schuler and Green, 2001). As a part of the apoptotic response, caspase 3 has been implicated in PKCδ cleavage and activation and, in turn seems to be under the control of PKCδ. Cellular stress promotes PKCδ translocation to the nucleus, the mitochondria or the Golgi apparatus, proposedly eliciting location-specific responses. For instance, its nuclear translocation in response to the cytotoxic drug etoposide seems critical for apoptosis-induction. The pro-apoptotic effects of PKCδ can be blocked by caspase inhibitors, by the PKCδ-specific inhibitor rottlerin, or by dominant negative mutants. Donwstream effectors important in the apoptotic response by PKCδ include the p53 related transcription factor p73 and DNA-PK (reviewed in (Brodie and Blumberg, 2003)).

PI3K signaling and MAP kinases

The phosphatidylinositol 3-kinase (PI3K) pathway, controlled by the Ras oncogene, is deregulated in many cancers. The PI3K downstream target Akt (or protein kinase B) directly or indirectly interferes with many pro-apoptotic genes or proteins. When activated, the serine threonine kinase Akt phosphorylates and inactivates Bad, thus allowing Bcl-2 and Bcl-xL to bind and inhibit Bax and prevent it from translocating to the mitochondria (Scheid et al., 1999). It has also been reported to inactivate caspase 9 *in vitro* and stabilize the p53 ubiquitin ligase MDM2. Indirect Akt-mediated effects include inhibiting transcription of pro-apoptotic genes, such as the Fas ligand, and down-regulating CKI expression (p21 and p27). The PI3K pathway affects both cell survival and cell growth and, depending on the cellular context, its inhibition may either protect from or promote apoptosis (reviewed in (Vivanco and Sawyers, 2002)). For instance, the PI3K inhibitor LY294002 has been shown to potentiate doxorubicin-induced apoptosis in melanoma cells (Panaretakis et al., 2002).

The mitogen-activated protein kinase (MAPK) family of serine-threonine kinases constitutes well characterized pathways in regulating cell survival and death. One of the three subfamilies, the Raf-MEK1 (MAPK/ERK kinase)-ERK module, is under the control of Ras. In response to

mitogens and growth factors, the Raf substrate MEK1 signals to extracellular signal-regulated kinase (ERK), promoting survival, proliferation, or differentiation. Among other activities, ERK mediates phosphorylation of the c-Jun and c-Fos components of the AP-1 transcription factor and induces cyclin D1. Inhibition of ERK activity correlates with activation of JNK and p38 signaling pathways as well as with induction of apoptosis. The balance between these pathways has been suggested to determine cell fate (for review, see (Chang et al., 2003)).

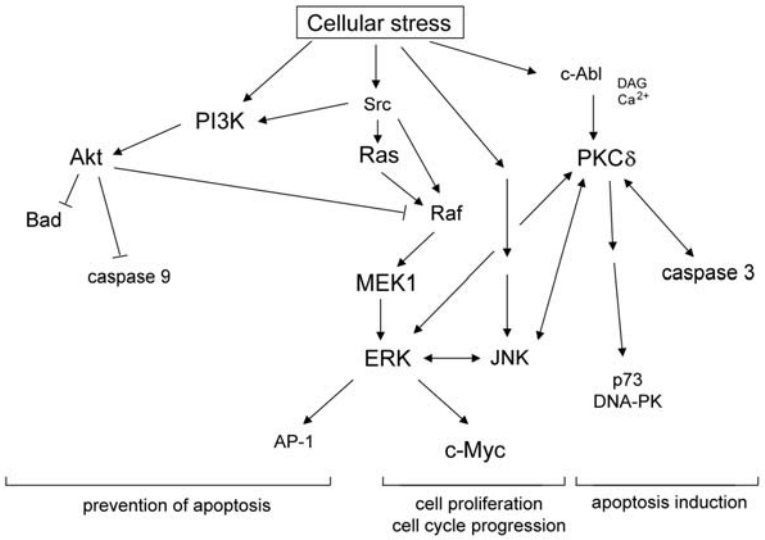
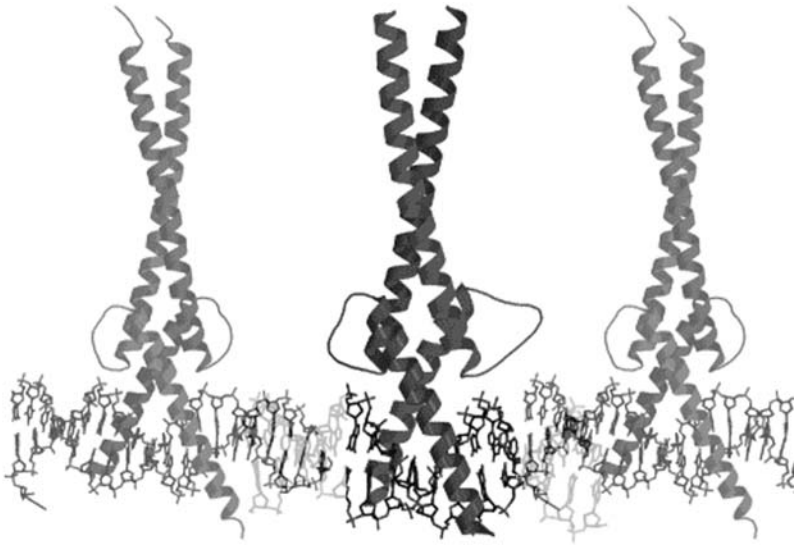


Figure 3. Stress-signaling pathways. Simplified scheme in an effort to visualize cross-talk between the pro-apoptotic and pro-survival pathways.



The Myc network

Transcription factors play an essential role in regulating gene expression. The biological consequences of transcription factor binding depend on the targeted gene and are subject to regulation by the cellular context. Co-factor complexes recruited by the transcription factors, as well as the state of the chromatin at and near the binding site, also play an important role in dictating the outcome of DNA binding. Therefore aberrant regulation of transcription factors is deleterious to cellular fate, causing too little or too much gene expression. In effect, many transcription factors have been identified as proto-oncoproteins or tumor suppressor proteins.

The Myc network of transcription factors

The transcription factors in the Myc network are basic region Helix-Loop-Helix/Leucine Zipper (bHLH/Zip) proteins important in processes such as proliferation, differentiation, cell cycle and apoptosis. The basic region (b) promotes sequence specific DNA binding and the HLH/Zip confers protein-protein interaction. The bHLH proteins recognize a common DNA sequence referred to as the E-box (CANNTG), thereby providing opportunities for regulation through competition for DNA binding. The theory is that the Zip domain functions in cooperation with the HLH to stabilize protein-protein interactions and to establish dimerization specificity. The Myc network includes proto-oncoproteins (c-Myc, MYCN, and L-Myc) as well as potential tumor suppressors (Mad1-4 and Mnt), Max, and Mga (Fig. 4). Max is the essential dimerization partner for Myc network proteins, enabling their DNA binding. Dowstream effects are partially mediated through modifications of the chromatin structure to control DNA accessibility (Oster et al., 2002; Ponzielli et al., 2005). Mouse models have revealed that Max as well as c-Myc and MYCN are essential for survival, thus placing the network in a central position in the regulation of cell growth and homeostasis (Henriksson and Luscher, 1996).

Image: Structures of Myc/Max (left and right) and Mad/Max (center) heterodimers bound to DNA. bHLH/Zip regions are outlined. Modified from: Nair and Burley, Cell (2003).

Importantly, due to recent changes in the nomenclature for Mad proteins, they should now be refered to according to their newly assigned abbreviation Mxd (Max dimerization protein). However, in this thesis I will refer to these proteins as Mad1-4 from this point onward.

The Myc family

The myc gene was originally identified in avian retroviruses as the oncogene responsible for inducing *my*elo*c*ytomatosis in birds (Sheiness and Bishop, 1979). The cellular homologue, c-myc, was found to be evolutionarily conserved. Later, MYCN and L-myc were found amplified in neuroblastoma and in small cell lung cancer, respectively (Henriksson and Luscher, 1996). Myc is a multifunctional protein with the ability to regulate activities as distinct as cell cycle, growth, differentiation, apoptosis, transformation, genomic instability and angiogenesis (Oster et al., 2002).

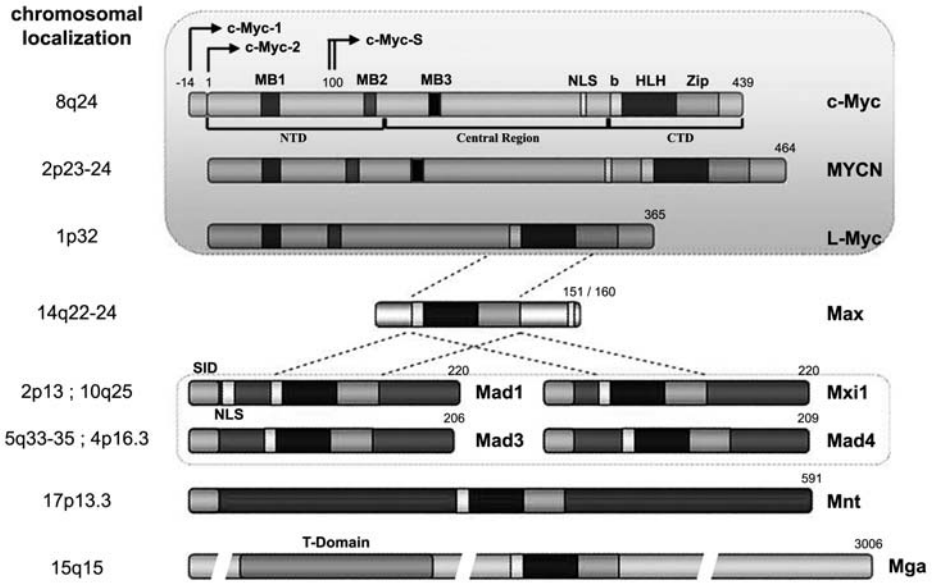


Figure 4. Proteins in the Myc network. The main protein structures are indicated, as well as chromosomal localizations of the respective genes. Heterodimerization with Max occurs through the HLH/Zip regions as outlined.

The three myc genes share the same general topography with the main open reading frame retained within the second and third exons. The highly homologous regions in these exons, the so-called myc boxes (MB), are evolutionarily conserved between different species (Ponzielli et al., 2005). L-myc is more distantly related to the other two members although it shares organizational features and several myc boxes. The Myc protein contains two nuclear localization signals (NLS). The main NLS (NLS1) at amino acids 320-328 induces complete nuclear localization and the second NLS (NLS2), spanning residues 364-374 overlapping the basic DNA-binding region, confers only a partial nuclear targeting. c-Myc and MYCN contain both NLS domains while L-Myc harbors only NLS2 (Henriksson and Luscher, 1996). The myc genes encode short-lived nuclear phosphoproteins with a half-life of 20-30 min that are subsequently ubiquitinated for proteasomal

degradation (Gregory and Hann, 2000). In addition to targeting Myc for degradation, the ubiquitination may in fact be required for its transcriptional activity (Kim et al., 2003b). Although Myc is predominantly nuclear, it has been detected in the cytoplasm, suggesting that Myc relocation occurs under certain circumstances (Oster et al., 2002).

Expression patterns of the myc family genes

During early embryogenesis, there seems to be some redundancy between c-Myc and MYCN according to the observations that c-myc -/- and MYCN -/- embryos survive until day 9-10, and day 11 respectively, before the deletion becomes lethal. Such compensatory mechanisms are proposed to be possible only until the myc expression becomes more tissue-restricted during organogenesis. c-myc expression is generally high early in embryonic development and may at this stage contribute to cell migration and/or invasiveness in addition to proliferation. In differentiated adult tissues, however, the expression is low or undetectable consistent with the virtual absence of cell proliferation. In contrast to the almost ubiquitous expression of c-myc, MYCN and L-myc expression levels are more restricted with respect to tissue and the stage of development. MYCN expression is very high early in embryogenesis in various tissues and declines dramatically during later development, generally coinciding with differentiation of these cells. The expression patterns of MYCN and L-myc are somewhat similar, but L-myc expression ceases after birth in many human tissues, except in the adult lung. Neither MYCN nor L-myc expression correlates well with proliferation, further supporting the notion that their expression is characterizing the undifferentiated state rather than promoting cell growth and division (reviewed in (Henriksson and Luscher, 1996; Oster et al., 2002; Ponzielli et al., 2005)).

Myc is continuously expressed throughout the cell cycle but the expression level is rapidly affected by agents interfering with proliferation. In nonproliferating or growth-arrested cells myc mRNA and protein are virtually undetectable but the Myc levels increase rapidly after serum stimulation followed by a relatively slow decline initiated before the onset of S phase (Henriksson and Luscher, 1996). Bearing features of an "early response" gene, no protein synthesis is required for the rapid and transient myc induction during the G0/G1 transition. However, in contrast to many genes of this class, myc levels are maintained at a constant intermediate level in continuously proliferating cells. Hyperphosphorylation of c-Myc during mitosis, together with nuclear envelope breakdown, causes its redistribution into the cytoplasm. Upstream regulators of myc expression are not well characterized. In the proposed platelet-derived growth factor (PDGF) – src – myc cascade, the Src tyrosine kinase would in response to PDGF stimulation, mediate c-myc up-regulation via GTPase-signaling. Another proposed Myc regulator is the transcription factor Miz-1 (Oster et al., 2002).

c-Myc – a multifunctional protein

c-myc is one of the most widely studied proto-oncogenes and it is localized at the chromosomal region that is translocated in Burkitt's lymphoma cells (Dalla-Favera et al., 1982). As the best characterized myc gene, human c-myc encodes the two major isoforms p67 (Myc-1) and p64 (Myc-2), with different expression patterns and biologically distinct functions (Hann et al., 1994). Transcription of Myc-1 is initiated at a cryptic start codon at the end of exon 1, whereas the more abundant Myc-2 protein is transcribed from an ATG start codon in exon 2, yielding a 439 residue protein (Fig. 4). Studies of the c-myc gene revealed that the bHLH/Zip region, as well as Max heterodimerization, and specific DNA binding to the E-box are critical for all known Myc

functions (Conzen et al., 2000; Luscher and Larsson, 1999). In addition to the C-terminal bHLH/Zip, the transcriptional activation domain (TAD) in its N-terminus is required for Myc function. Within the TAD, the two highly conserved Myc boxes (MB), span aa's 47-62 (MB1) in the proline rich region and 128-143 (MB2) harboring a potential ubiquitination site, respectively (Conzen et al., 2000; Gregory and Hann, 2000). Myc box 2 seems to be essential for cell transformation (Conzen et al., 2000). Recently, a third conserved region (MB3) was described, spanning aa's 188-199 within the central region, and also found to be important for cellular transformation (Herbst et al., 2005; Herbst et al., 2004). Several factors have been found to block Myc's transactivation properties by interaction through the HLH/Zip, thus inhibiting Max dimerization (Oster et al., 2002). Phosphorylation has been implicated in regulation of Myc turnover as well as in Myc-mediated transformation. At least three sites (Thr-58, Ser-62, and Ser-71) in the Myc N-terminus are subject to *in vitro* phosphorylation by several kinases. Since, the Thr-58 residue within MB1 is frequently mutated in Burkitt's lymphoma, phosphorylation at this site may be important for Myc function (Gregory and Hann, 2000). Indeed, mutations of Thr-58 and Ser-62 alter the transforming potential of Myc (Henriksson et al., 1993; Oster et al., 2002).

Protein interaction and target genes

Potential Myc-interacting proteins, include the pRb-like p107 protein, the co-activator transformation/transcription domain-associated protein (TRRAP), and the transcriptional repressors TFII-I, MM-1 and Miz-1 (for review see (Oster et al., 2002)). There seems to be a regulatory loop between Myc and p107 since the growth-inhibitory effects of p107 are counteracted by Myc while p107 significantly inhibits Myc-mediated transcriptional activation. However, p107 is unable to repress mutant c-Myc in Burkitt's lymphoma (Gu et al., 1994), possibly due to N-terminal alterations, preventing CDK1-cyclin A-mediated c-Myc phosphorylation. Such c-Myc mutations may be one way for the protein to escape regulation and contribute to the oncogenesis in Burkitt's lymphoma (Hoang et al., 1995). Myc also associates with histone acetyltransferases (HATs) to acetylate histones and enable a transcription-permissive state of the chromatin at its target (Fig. 5). The coactivator TRRAP is a component of the HAT complex and associates with Myc by binding to MB2 (Bouchard et al., 2001). Observed interactions with other HAT complexes and TRRAP-independent molecules with chromatin remodeling capacity raise the possibility that Myc can also recruit other complexes for controlling target gene transcription (Oster et al., 2002). For instance, c-Myc-mediated inhibition of transcription can be conferred through interaction with TFII-I in the transcription machinery, binding at initiator elements. Together with observations that Myc-mediated repression by Miz-1 also started from the initiator element (Staller et al., 2001), it was originally believed that the initiator element was a prerequisite for Myc-mediated transcriptional repression. However, Myc was later found to repress genes, such as p21, without initiator elements, through interaction with the Sp1 transcription factor (Gartel et al., 2001). Another mechanism for Myc-mediated repression of target genes is recruitment of an an mSin3/HDAC-complex, when associated with MM-1 (Satou et al., 2001).

Many Myc target genes have been proposed and most of them have been documented in the Myc Target Gene Database: http://www.myccancergene.org/site/mycTargetDB.asp. Some examples are ornithine decarboxylase (ODC), p53, carbamoylphosphate dihydroorotase (CAD), hTERT, and genes encoding cell cycle regulators (for reviews, see (Henriksson and Luscher, 1996; Oster et al., 2002; Ponzielli et al., 2005)). The ODC enzyme controls polyamine biosynthesis and is essential for progression into S phase. Myc-mediated ODC up-regulation may contribute to the oncogenic phenotype since ODC overexpression in mouse fibroblasts results in transformation

(Moshier et al., 1993). The tumor suppressor protein p53, as previously described, is important in the cellular response to DNA damage with the ability to induce cell cycle arrest or apoptosis (Sherr and Weber, 2000). Myc may activate p53 as a safeguard mechanism to prevent transformation by inducing apoptosis in Myc overexpressing cells (Hermeking and Eick, 1994). Transcriptional induction of the CAD enzyme, required for *de novo* pyrimidine-synthesis in the G1/S transition, may be partially conferred by Myc (Boyd and Farnham, 1997; Bush et al., 1998; Miltenberger et al., 1995). The catalytic subunit of telomerase (hTERT) also harbors E-box-elements to which Myc/Max as well as Mad1/Max complexes have been shown to bind (Oh et al., 2000; Wang et al., 1998a; Xu et al., 2001). In addition to targeting other proteins, the myc gene itself harbors Myc-responsive elements and has been shown to regulate its own expression (Facchini et al., 1997). Taken together, these findings delineate a role for Myc both in activating and repressing target gene transcription.

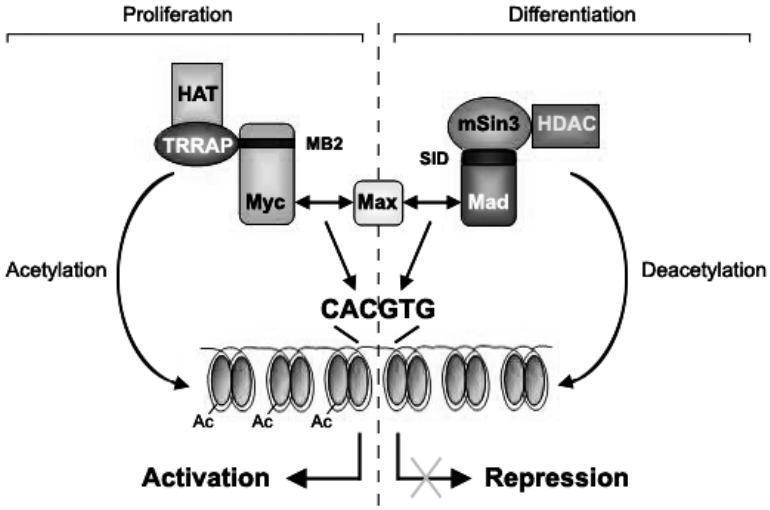


Figure 5. Effects of Myc and Mad on gene transcription. Generally, Myc recruits a histone acetyltransferase complex to activate transcription and Mad represses transcription through histone deacetylation. They both heterodimerize with Max and bind DNA at E-box sequences (CACGTG).

Myc in cell cycle control and growth

c-Myc plays a critical role in normal cell cycle progression, especially during transition from G0 to S phase but it is also suggested to play a role in G2 (Mateyak et al., 1997; Spencer and Groudine, 1991). These effects can be mediated by c-Myc-induced transcription of genes such as cdc25A, E2F, cyclins D1, D2, E, and A, and CDKs 1, 2, and 4. Particularly, the Myc-mediated promotion of E2F release as a result of pRb phosphorylation is proposedly induced by transcriptional activation of cyclins A, E and D1 (Pelengaris et al., 2002). Another means of Myc-induced inactivation of pRb is proposedly promoted by stimulating expression of the pRb-inhibiting Id2 protein (Lasorella et al., 2000). In addition, c-Myc is suggested to indirectly modulate the rapid decrease in p27 levels by ubiquitination (for review, see (Oster et al., 2002; Pelengaris et al., 2002)). Other check point genes that are suppressed by c-Myc include: growth arrest and DNA-damage-inducible (gadd) 45, gadd153, and the CKI genes p15 and p21 (Pelengaris et al., 2002). In quiescent cells, c-Myc is sufficient to stimulate the DNA synthesis required for progression through G1 and for S phase

entry, and also seems functionally important in the S and G2 phases (Jones and Kazlauskas, 2001; Mateyak et al., 1997). Cells with constitutively high Myc levels have reduced growth factor requirements, increased growth rate, spend less time in G1, and can in some cases circumvent growth arrest (Henriksson and Luscher, 1996; Pelengaris et al., 2002). The tight control of cell cycle regulators is important for Myc function, as c-myc +/- cells display a slower growth rate and delayed serum-induced S phase entry, most likely as a consequence of their reduced c-myc expression (Shichiri et al., 1993). Taken together, Myc coordinates the molecular events required for G1/S transition by regulating both D and E CDK-cyclin kinase complexes as well as the E2F transcription factors (Beier et al., 2000; Obaya et al., 1999). In addition to driving cell cycle progression, Myc can also regulate cell growth (increase in cellular mass). These two events have been shown to be uncoupled, by blocking cell cycle progression without affecting cell growth and vice versa (Beier et al., 2000; Iritani and Eisenman, 1999).

Effects on differentiation and apoptosis

When cells are induced to differentiate, myc expression is rapidly down-regulated (Larsson et al., 1994). In proliferating cells, myc down-regulation induces growth arrest and terminal differentiation. However, c-Myc-enabled proliferation can be uncoupled from its ability to block differentiation, which makes Myc an important mediator in the decision-making process between these two mutually exclusive events (Henriksson and Luscher, 1996; Pelengaris et al., 2002).

Another important function of Myc is the potentiation of apoptosis in response to cellular stress (reviewed in (Nilsson and Cleveland, 2003)). Cyclin A and ODC are potential mediators of Myc-induced apoptosis since ODC-blockage inhibits apoptosis in Myc-overexpressing cells and forced expression of cyclin A is sufficient to induce apoptosis under low serum conditions (Hoang et al., 1994; Packham and Cleveland, 1994). Ectopic expression of Cyclin A could also restore apoptosis in c-myc null cells treated with etoposide (Adachi et al., 2001). Induction of apoptosis by c-Myc has also been correlated with regulation of the Fas receptor and its ligand as well as pro-apoptotic Bax (Fulda et al., 1998; Mitchell et al., 2000; Soucie et al., 2001) (Paper II). Even though there are few reports describing changes in Bax levels in response to c-Myc overexpression, this molecule appears to be essential for signaling c-Myc-induced apoptosis (Mitchell et al., 2000) (Papers II and III). The effect on Bax may be indirect by regulating upstream molecules such as caspase 8, which is frequently inactivated in childhood neuroblastomas with amplified MYCN (Teitz et al., 2000). There is also the possibility of direct protein-protein interaction, since Myc under some circumstances can localize to the cytoplasm (Oster et al., 2002). The tumor suppressor protein p53 is important but not required for c-Myc-induced apoptosis although a number of tumor cell lines with deregulated c-myc carry p53 mutations or deletions (Gaidano et al., 1991; Wagner et al., 1994). In response to c-Myc activation and myc/ras-induced transformation, p53 is up-regulated and stabilized to induce cell cycle arrest or, if the cell cycle blockade is overcome by c-Myc, apoptosis (Wagner et al., 1994).

The many and diverse effects of c-Myc in promoting pathways as distinct as proliferation and apoptosis, has brought forth the proposal of a model where activated Myc promotes apoptosis as the the preferred physiological response. In case of excessive amouts of survival factors or mutations in the apoptotic pathway, the cellular Myc response would then instead be uncontrolled proliferation. This model has been coined "the dual signal model" (Harrington et al., 1994; Hueber and Evan, 1998), and is supported by the observation that different regions of the c-Myc N-

terminal domain can control distinct biological functions, including apoptosis (Chang et al., 2000; Conzen et al., 2000).

Oncogenic properties of Myc

Genetic alterations at the c-myc locus are common in many tumors, proposedly derived from cells that were initially unable to down-regulate c-myc expression in response to differentiating agents (DePinho et al., 1991; Spencer and Groudine, 1991), thus enabling an induced capacity to cycle and making these cells perfect targets for mutations. A major fraction of all human cancers display deregulated Myc activity (Nilsson and Cleveland, 2003; Ponzielli et al., 2005). Alterations include chromosomal translocations exemplified by the c-myc-immunoglobulin (Ig) fusion gene in Burkitt's lymphoma (Hecht and Aster, 2000), and increased c-myc expression due to gene amplification (Hogarty, 2003), or protein stabilization (Shindo et al., 1993). In mouse models with conditional Myc expression, ectopic activation of c-Myc in localized cellular compartments gave rise to tumors that regressed upon Myc deinduction (Felsher and Bishop, 1999a; Pelengaris et al., 2002). Other oncogenic features of Myc are induction of genomic destabilization (Felsher and Bishop, 1999b; Mai et al., 1999), and of vascularization and angiogenesis in tumor development (Oster et al., 2002).

Oncogenes found to frequently synergize with c-myc in transformation include bcl-2, ras, raf, and c-abl (reviewed in (Oster et al., 2002; Pelengaris et al., 2002)). c-Abl and Bcl-2 have been proposed to negatively regulate Myc-induced apoptosis. Consequently, a large proportion of tumors with deregulated c-Myc expression overexpress Bcl-2 (Cory and Adams, 2002). There are also reports on Myc regulation of cyclins D1 and D2, both of which seem essential for Myc-driven proliferation, the latter being a direct Myc target, activated in response to growth factor stimulation (Bouchard et al., 2001). The nuclear zinc-finger protein encoded by bmi-1 synergizes with c-Myc in lymphomagenesis, possibly by negative regulation of p19ARF in the ARF-p53-MDM2 pathway (Jacobs et al., 1999). Pro-survival molecules that protect cells from c-Myc-induced apoptosis, such as the insulin-like growth factors (IGFs) and platelet-derived growth factor (PDGF), may also facilitate transformation (Harrington et al., 1994). A recently indentified mediator of the IGF-1-anti-apoptotic effect, the bHLH family member Twist, promotes oncogenesis by inhibiting the apoptotic function of p19ARF (Dupont et al., 2001).

Max – the obligate heterodimerization partner

Max was identified in 1991 and found to be an essential heterodimerization partner for all known c-Myc functions (Blackwood and Eisenman, 1991; Shen-Li et al., 2000). In addition, it has an impotant role in embryonic development as mice with homozygously deleted max die at day 5-6 of gestation (Gilladoga et al., 1992; Shen-Li et al., 2000). Max is highly conserved in the evolution of vertebrates and, with a half-life longer than 14 hours, it is constitutively expressed in a number of different cell types. The two major splice variants of Max, p21 and p22, are both dimerization competent and, in contrast to Myc and Mad proteins, form homodimers as well as Myc/Max and Mad/Max heterodimers. However, the homodimers possess less affinity to DNA, seem less discriminating compared to Max heterodimer complexes and their DNA binding properties may be negatively affected by phosphorylation. In addition to the bHLH/Zip, Max contains an acidic region and a C-terminal NLS (Fig. 4) (reviewed in (Henriksson and Luscher, 1996)). During differentiation, max expression is subject to transcriptional regulation but the long half-life of the protein makes it difficult to see effects in short-term experiments (Larsson et al., 1994).

Overexpression of max reduces growth and delays differentiation. Some of these effects were counteracted by introducing a basic region mutant of Max (dMax), proposed to sequester Myc into DNA binding-incompetent Myc/dMax complexes (Billaud et al., 1993; Borre et al., 1996). To fully understand the different effects of Max on cell growth and differentiation, it should be remembered that Max overexpression not only influences Myc function but most likely also interferes with the effects of Mad family proteins (Henriksson and Luscher, 1996).

The Mad family

Mad1 and Mxi1 (Mad2) were found by protein-protein interaction screens, using the Max protein as bait. Subsequently, Mad3 and Mad4 were identified as Mad1- and Mxi1-related bHLH/Zip proteins highly homologous in the bHLH/Zip and two additional homology regions (HR1 and HR2). The Mad proteins, of around 30-35 kDa in size, have relatively short half-lives, readily heterodimerize with Max and bind to the same CACGTG E-box sequence as the Myc/Max complex. The two homology regions shared in addition to the centrally located bHLH/Zip, include 28 aa's at the N-terminus (HR1) and 69 residues C-terminal of the Zip domain (HR2) (Fig. 4). The N-terminal HR1 mediates interaction with mammalian Sin3A and B, homologues to the yeast transcriptional repressor Sin3. Since these proteins are DNA binding-incompetent, they are suggested to mediate Mad/Max-promoted recruitment of co-repressors and histone deacetylases (HDACs) (Laherty et al., 1997; Sommer et al., 1997). Coincidently, Mad proteins have been shown to efficiently repress CACGTG-mediated transcription through *in vivo* interaction with Sin3. The other homology region (HR2) in the C-terminus may also interact with an as yet unidentified co-repressor. The four Mad proteins are highly homologous in sequence and there is also evidence for functional redundancy. Mnt and Mga are larger members of the Mad family (Fig. 4). Apart from the bHLH/Zip domains, the ability to heterodimerize with Max and bind to E-box sequences, these two proteins do not share a high homology with the other four Mad family members. The Mnt protein also harbors an N-terminal mSin3-interaction domain (SID), but since MntΔSID enables transformation, it is proposed that Mnt also possesses a Myc-TAD-like activity. Mga contains a T-box domain which, together with its expression pattern suggests involvement in embryonic development. Transcriptional activation at the T-box binding sites seems to depend on Max presence at the bHLH/Zip domain. Some of the Mad proteins interact with the bHLH/Zip protein Mlx, suggested to be in the center of a network existing in parallel with the Myc network (for reviews, see (Baudino and Cleveland, 2001; Henriksson and Luscher, 1996; Zhou and Hurlin, 2001)).

Expression patterns of the mad family genes

The expression pattern of separate mad family members is tissue specific and quite complex (Queva et al., 1998). Mad1 expression varies throughout development and is associated with differentiated cells. Up-regulation of mad1 follows down-regulation of myc genes such that mad1 levels are very low or undetectable in proliferating cells, but are rapidly induced upon induction of differentiation (Larsson et al., 1994). Expression of mxi1 increases with progressive development and growth arrest in many organs of the mouse, with a preference for tissues in which the cells are terminally differentiated. In analogy with mad1, mxi1 expression is induced with differentiation in hematopoietic cells, but in contrast mxi1 is also expressed in proliferating cells (Larsson et al., 1994). Mad3 is clearly distinct from other Mad family proteins in that its expression is S phase specific and virtually undetectable in most adult tissues. Despite this fact, overexpression of Mad3

still inhibits proliferation and transformation (Hurlin et al., 1995). Mnt is rather ubiquitously expressed (Hurlin et al., 1997). It is currently believed that c-Myc/Max complexes are replaced by Mad1/Max or Mxi1/Max complexes during the differentiation process and/or inhibition of cell growth. It has been indicated that Mad1 and probably also Mxi1 are potent inhibitors of cell growth which may be a prerequisite for differentiation (Cerni et al., 1995; Chen et al., 1995).

Presently, there are no identified Mad regulators. However, there is an E2F binding site in the mad3 promoter, suggesting that E2Fs are responsible for the S phase specific expression mad3 expression. In addition, the Miz-1/c-Myc complex has been demonstrated to associate with the mad4 promoter (Fox and Wright, 2003; Kime and Wright, 2003). Based on findings that Mad proteins bind E-box sequences in genes overlapping with the targets for c-Myc, it is also proposed that c-Myc has a part in regulating their expression (O'Connell et al., 2003). The mechanisms for induction of mad expression by differentiation-promoting substances such as tetradecanoylphorbol acetate (TPA), retinoic acid, and certain cytokines also remain to be elucidated (Larsson et al., 1994).

Transcriptional repression by Mad

Mad1 and Mxi1 have been found to repress c-Myc/Ras-promoted transformation of primary rat embryo fibroblasts (REFs) (Baudino and Cleveland, 2001; Cerni et al., 2002), provided that their bHLH/Zip and interaction with Max remained intact. Studies of mouse Mxi1 isoforms revealed the importance of the N-terminal mSin3 interacting region for repression, thus suggesting that Mad-mediated regulation of Myc activity is an active process mediated by transcriptional repression, and not a passive effect by sequestering Max or occupying Myc target sequences (Fig. 5) (Schreiber-Agus et al., 1995). Considering that Myc and Mad promote their transcriptional activities at least partially through activation of HATs or HDACs respectively, it is intriguing to note that aberrant acetylation is observed in many tumors (Marks et al., 2001). In addition to repressing Myc/Ras transformation, Mad can inhibit transformation of REFs when promoted by Ras in cooperation with other mediators such as E1A, and mutant p53. Since Mad-mediated inhibition of transformation was not abrogated by Bcl-2, it seemed that Mad would influence the growth rather than the viability of cells, as observed in human astrocytoma cells (Chen et al., 1995). Given these functions, it is tempting to consider Mad proteins as tumor suppressors, but despite their localization to regions frequently altered in human tumors, there is no conclusive evidence of tumor-specific changes in their expression (reviewed in (Baudino and Cleveland, 2001)).

Mad targets, and involvement in cell cycle control

Mad1 target genes include hTERT and cyclin D2, both originally identified as c-Myc targets (Bouchard et al., 2001; Wu et al., 1999). hTERT is found reactivated or amplified in most human cancers. Mad1 can counteract this activity by binding to the hTERT promoter and, probably in cooperation with other repressors, silence the hTERT gene through histone deacetylase (HDAC) activity (Lin and Elledge, 2003; Xu et al., 2001). Several studies have been undertaken to identify Mad target genes with varying results. In a microarray screen for Mad1 target genes, the positive hits mainly included genes involved in cell growth control. Many of these genes had previously been identified as Myc targets, indicating that Mad proteins also control general aspects of cell proliferation (reviewed in (Oster et al., 2002; Zhou and Hurlin, 2001)). It is suggested that Mad regulates a set of genes partially, but not entirely, overlapping with that of Myc family genes. Mxi1 has been found to block serum-induced c-Myc expression in quiescent cells and was therefore

suggested to directly repress the c-myc gene. However, the mechanism for this repression needs to be further defined since it does not require the basic region or the SID of Mxi1 and is antagonized by Max (Lee and Ziff, 1999). Mga has been speculated to associate with E2F since it has been isolated in an E2F-containing complex (Ogawa et al., 2002).

In controlling the cell cycle, it is hypothesized that Mad proteins have much the same target genes as Myc proteins and that there is a switch from Myc/Max to Mad/Max complexes when cells exit the cycling stage and start to differentiate (Ayer and Eisenman, 1993). In support of this, studies have shown that Mad1 can bind E-boxes of Myc responsive genes and repress their transcription (Bouchard et al., 2001; Xu et al., 2001). Specifically, Mad1 is a potent inhibitor of the G1/S-transition, imposed by histone deacetylase-mediated repression of cyclin D2 gene expression (Bouchard et al., 2001).

Effects on proliferation, differentiation and apoptosis

Observations that Mad protein expression was associated with differentiated cells led to the suggestion that they might regulate or even induce differentiation. In support of this, Mad1 and Mxi1 were demonstrated to interfere with proliferation, resulting in the accumulation of cells in the G0/G1 phase of the cell cycle (Chen et al., 1995). However, this was not a complete blockage, suggesting that additional signals were required to efficiently arrest cycling cells. In growth arrested cells on the other hand, Mad1 is sufficient to prevent reentry into the cell cycle (Sommer et al., 1997). The importance of Mad in modulating proliferation and/or differentiation was further analyzed in transgenic mouse models with conditional mad1 expression, as well as in mad1, mxi1, and mad3 knock-out mice (reviewed in (Baudino and Cleveland, 2001; Foley and Eisenman, 1999)). Except for a reduced size of the mice and cell-type specific hyperplastic changes, no obvious correlation with altered cell proliferation was observed, suggesting that Mad proteins are not essential for this function. In contrast, p27/mad1 double knock-outs are partially embryonic lethal, have an impaired granulocyte differentiation and fail to down-regulate cyclin E/CDK2 (McArthur et al., 2002). Mice transgenic for mnt die at embryonic day 9-10, coinciding with the death of c-myc -/- mice and with a very similar phenotype, suggesting that mnt may play a role in controlling proliferation (Hurlin et al., 1997). Later, several lines of evidence have been gathered to support a role for Mnt as a Myc modulator, and a potential tumor suppressor (reviewed in (Hooker and Hurlin, 2006)). When interpreting these data, it is important to consider limitations in the experimental setup. In the transgenic mice, the Mad1 gene and protein expression does not coincide with that of endogenous Mad1 and homozygous deletions of only one Mad family gene at a time may allow other Mad proteins to compensate for this loss (Baudino and Cleveland, 2001).

In line with the notion that Mad proteins oppose Myc functions, Mad was expected to antagonize the apoptosis-promoting capacity of Myc. From the studies of mad1 transgenic and knock-out mice, the effect on proliferation and apoptosis indeed suggested an apoptosis-inhibitory role for mad1 (Foley and Eisenman, 1999; Queva et al., 1999). This hypothesis is supported in *in vitro* studies where Mad1 was found to reduce apoptosis induced by serum starvation, oncoprotein expression, and by apoptosis-inducing stimuli such as the cytotoxic drug cisplatin and Fas receptor signaling (Gehring et al., 2000) (Paper I). The inhibition of Fas-induced apoptosis may, at least in part, be mediated by reducing caspase 8 activity (Gehring et al., 2000). A functional and regulatory interaction between the Myc network and caspase 8 is supported by data obtained from neuroblastoma cells where MYCN amplifications often coincided with deletion or silencing of the caspase 8 gene (Teitz et al., 2000). While Mad3 has been suggested to regulate apoptosis during S

phase (Queva et al., 2001), the involvement of the other Mad family proteins in apoptosis-control has not yet been studied.

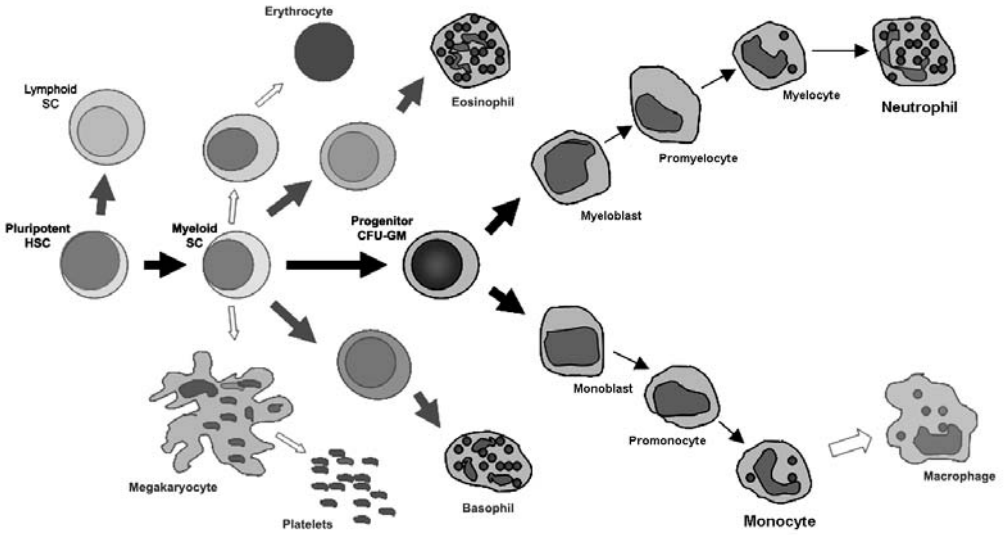


Figure 6. Outline of cellular differentiation stages during hematopoiesis. Myc has been associated with malignancies in cells of lymphoid and myeloid origin.

Myc-associated cancers

Cancers associated with Myc are mostly developed as a consequence of a deregulated Myc expression, either through translocations as that seen in Burkitt's lymphoma or through amplifications as observed in neuroblastoma. In leukemias, arising from hematopoietic cells unable to differentiate, the mechanism by which Myc is activated may differ from case to case. The highly malignant leukemia subtype acute promyelocytic leukemia readily responds to treatment with differentiating agents, thus providing a good model for studying pathways activated in cellular differentiation and/or apoptosis.

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is a non-Hodgkin's lymphoma originally detected as an endemic form as B-cells were latently infected by the Epstein-Barr virus (EBV). Later, sporadic forms were found, not associated with viral infection. The common trait for BL forms is the development of tumors in extranodal sites in adolescents or young adults. The disease is classified as a distinct category of peripheral B-cell lymphomas, and comprises a heterogenous group of highly aggressive B-cell malignancies (reviewed in (Hecht and Aster, 2000)). It is invariably associated with chromosomal translocations, preferentially the t(8:14)(q24:q32) translocation, bringing the c-myc proto-oncogene in proximity with the immunoglobulin heavy chain promoter (Dalla-Favera et al., 1982; Taub et al., 1982). Even though the chromosomal breakpoints are widely dispersed along the genes, the end-result is a fusion gene where c-Myc is constitutively active. Because of the c-Myc overexpression, BL cells have the highest cell division rate observed in any human tumor. This malignancy differs from other B-cell lymphomas in the specific response to chemotherapy. Although c-myc rearrangements are observed in the majority of BL cases, this is not a BL-specific phenomenon as they are also observed in other types of lymphoma (reviewed in (Hecht and Aster, 2000)).

Neuroblastoma

As the most common solid childhood malignancy, neuroblastoma (NB) arises in the peripheral nervous system, usually before the age of five. Most cases are diagnosed during the first year of life at the peak of disease incidence (reviewed in (Hogarty, 2003)). The clinical outcome is heterogenous, ranging from spontaneously regressing tumors, to differentiating tumors or those that can be cured with chemotherapy, but also includes cases of aggressive metastatic tumors, often associated with a lethal outcome (Weinstein et al., 2003). For clinical purposes NB is classified into four different stages where stages I and II have a good prognosis while the outcome is more uncertain in stages III and IV. There is also the special case, stage IVs, which is a benign NB form (Brodeur et al., 1993). For prognostic purposes, clinical stage, together with histopathological assessment and age at diagnosis are the most informative parameters (Brodeur et al., 1997a). Chromosomal abnormalities frequently associated with NB are chromosomal deletions in 1p, 11q, and 14q, gain of genetic material at 17q, and MYCN amplification (Bown, 2001). Furthermore, ploidy is a strong prognostic marker, triploid tumors being associated with low stage disease in younger children with favorable outcome, whereas diploid and tetraploid tumors are associated with unfavorable prognostic markers such as MYCN amplification and 1p deletion (Look et al., 1984). Evaluation of tyrosine kinase receptor gene (trk) expression is another means to predict the biology and clinical behavior of NBs (Brodeur et al., 1997b). High trk A expression indicates a favorable outcome in the absence of MYCN amplification, regardless of age or stage (Nakagawara et al., 1993). Similarly, trk C is expressed primarily in lower stage tumors with favorable outcome (Ryden et al., 1996). trk B on the other hand, is preferentially expressed in tumors with MYCN amplification (Nakagawara et al., 1994). Presently, MYCN amplification is the most important biological marker in clinical assessment of NB, as it is well known to correlate with advanced stage and poor outcome independently of clinical stage and age (Schmidt et al., 2000). However, the exact molecular mechanism by which MYCN amplification and other chromosomal alterations contribute to the aggressive behavior of NB remains unclear (reviewed in (Westermann and Schwab, 2002)). Neuroblastoma treatment is based on surgical resection that is, depending on the disease stage, complemented with chemotherapy and/or radiation. The use of camptothecin derivatives, together with differentiating agents such as retinoids, are promising new approaches in NB therapy (Weinstein et al., 2003)

Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a highly malignant form of acute myelocytic leukemia (AML), often associated with infection and hemorrhage. Characteristic traits include a predominance of abnormal promyelocytes with a hypergranular appearance residing in the bone marrow, and a predisposition for the t(15:17)(q22:q21) translocation giving rise to the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) fusion protein (de The et al., 1990; Kakizuka et al., 1991). Under normal conditions, RARα plays an important role in myeloid differentiation and maturation, maintaining a condensed chromatin conformation and preventing transcription in the absence of retinoic acid (RA). When the PML-RARα fusion protein replaces RARα, chromatin condensation can only be relieved in response to supraphysiological doses of RA, thus efficiently blocking differentiation. The PML protein is mainly localized to nuclear bodies, called PML oncogenic domains (POD), where it plays an important role in transcriptional regulation and has been found to suppress oncogenic transformation (for reviews, see (Melnick and Licht, 1999; Soignet and Maslak, 2004)). Due to the high incidence of the PML-RARα protein in

APL (approximately 90%), its presence is used as a diagnostic marker and enables initiation of treatment at an early stage with a minimal leukemia burden. This distinguishes APL from other types of AML where diagnosis still relies on less precise techniques, such as morphological assessment. Untreated, APL is rapidly fatal, but given the appropriate therapy, it has the highest rate of curability among the subtypes of adult AML (Parmar and Tallman, 2003; Zhou et al., 2005a). It is worth mentioning however, that out of the other three RARα fusion partners associated with APL pathogenesis (promyelocytic leukemia zinc finger, PLZF; nucleophosmin, NPM; and nuclear matrix associated, NuMA), the PLZF-RARa fusion protein is associated with a bad prognosis as it does not respond to therapy (Melnick and Licht, 1999). Historically, treatment of APL had the apparent drawback that it induced coagulopathy, often causing death by cerebral hemorrhage, and generated a relatively low frequency of disease-free survival. Even so, the complete remission (CR) rate and long-term disease free survival was superior to that for AML as a whole, with the best results observed in response to treatment with anthracyclines (reviewed in (Soignet and Maslak, 2004)). As the patient response to conventional chemotherapy was not satisfactory, other therapy approaches were sought for. In that search, efforts to induce differentiation of APL cells brought forth the introduction of all-trans retinoic acid (ATRA) in 1987 (Huang et al., 1987), and arsenic trioxide (As_2O_3) in the mid- to late 1990's (Shen et al., 1997; Soignet et al., 1998), revolutionizing APL therapy. These differentiating agents target different sites in the PML-RARα fusion protein, eventually inducing its degradation. As ATRA treatment does not completely eliminate the fusion protein, post-remission therapy is required to eliminate minimal residual disease, thus preventing its reappearance and favoring long-term survival (Lo Coco et al., 1999). For such consolidation therapy, anthracycline analogues belong to the most commonly used drugs. With present treatment strategies, high complete complete remission rates are achieved in newly diagnosed APL patients (more than 90%) (Fenaux et al., 2000), as well as in relapsed patients (85-90%) (Niu et al., 1999; Soignet et al., 1998). As a result, APL is now a curable disease for 70-80% of patients (Parmar and Tallman, 2003), showing that targeted therapy and induction of differentiation can be effective strategies also in the treatment of other subtypes of leukemias (Zhou et al., 2005a).

Myeloid differentiation and markers

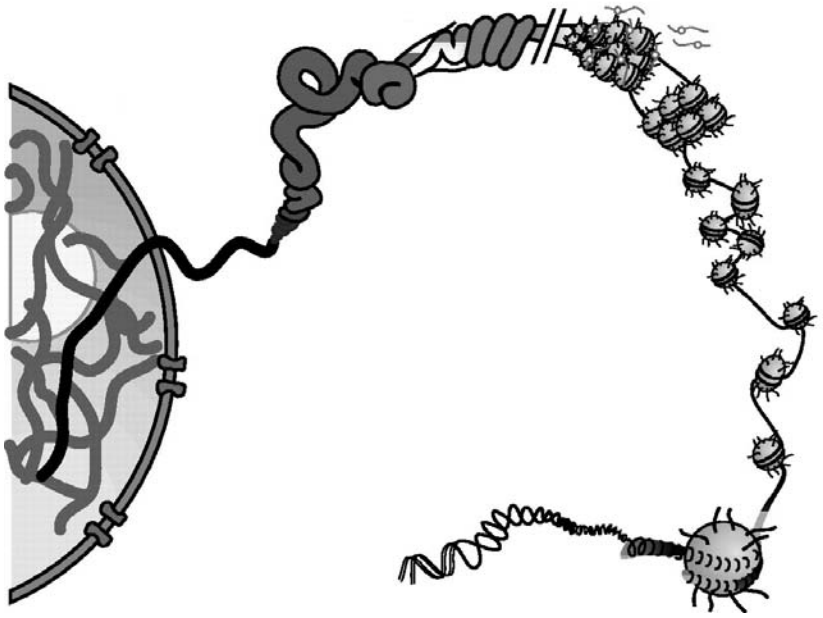
In myelocyte development, morphological changes together with stage-specific alterations in the cell surface antigen profile and cell metabolism features reflect the cellular differentiation status, and can be used for analysis. The myeloid precursor cell originates from a pluripotent hematopoetic stem cell and gives rise to platelets, erythrocytes, monocytes, and granulocytes. Neutrophils comprise the largest pool of granulocytes in the human body (around 90%), while eosinophils and basophils are much less common. Neutrophil development progresses through myeloblasts into promyelocytes, and finally myelocytes before developing into a fully differentiated granulocyte (Fig. 6). The developmental stages in monocyte differentiation are initiated by proliferating monoblasts, giving rise to promonocytes before entering the stage of mature circulating monocytes. When required, the monocyte can also develop into a macrophage for a better immune response (Roitt et al., 1996).

One cell surface antigen that is present on all granulocytes is the complement receptor CR3 or CD11b, required for cellular chemotaxis. Since its expression is restricted to differentiated cells, the cellular differentiation status can be determined by monitoring CD11 surface expression, for instance by flow cytometry, using fluorescently labeled antibodies.

Another granulocyte-specific feature is their ability to generate superoxide when stimulated, as part of their phagocytizing process. Superoxide generation can be artificially induced by for instance TPA stimulation, and monitored in the nitroblue tetrazolium (NBT) reduction assay, where the tetrazolium dye is converted from soluble yellow to insoluble intracellular blue formazan. The abundance of intracellular precipitates thus indicates the cellular differentiation status (Baehner et al., 1976; Segal, 1974).

Myc in APL

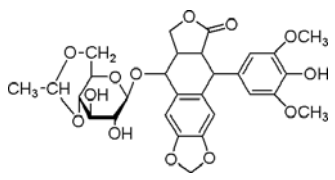
Since down-regulation of Myc is required for cellular differentiation (Coppola and Cole, 1986; Nguyen et al., 1995), it is conceivable that the resistance to differentiating agents sometimes observed in APL cells, may be due to a deregulated Myc expression (Ponzielli et al., 2005). Publications on the relationship between Myc and differentiation in APL indicate complete down-regulation of Myc in response to ATRA (Liu et al., 2000; Mitchell et al., 1992), but only partial down-regulation in As_2O_3 treated cells (Chen et al., 1996). Our observations (paper IV), together with results from previous studies (Chen et al., 1996; Dimberg et al., 2002; Liu et al., 2000), suggest that Myc blocks terminal differentiation in response to As_2O_3 treatment by activation of target genes important in cell cycle regulation and and cell turnover. This hypothesis, together with implications that Mad1 is involved in controlling differentiation (McArthur et al., 2002) (paper IV), provides a starting point for investigating more specific involvement of molecular mechanism coupling Myc to induction of differentiation (reviewed in (Fang et al., 2002)).



Anticancer treatment

Chemotherapy is usually not the first line of treatment in cancer therapy, but is rather used as a complement to surgical tumor resection and/or radiation therapy. Among the different types of anticancer drugs, the most commonly used compounds are conventional chemotherapeutics such as those targeting topoisomerases, DNA-damaging agents, mitotic inhibitors, antimetabolites, and nucleotide analogues (Herr and Debatin, 2001). However, despite the induction of complete remission often observed when used as an adjuvant treatment to kill undetectable residual cells, conventional chemotherapy suffers the disadvantages induced by adverse effects together with resistance development, particularly when treating metastatic disease (Luqmani, 2005). Side-effects associated with most conventional chemotherapy include myelotoxicity, nausea, vomiting, diarrhea, and fatigue (Nieboer et al., 2005).To circumvent this problem, the relatively novel use of differentiating agents has been introduced, predominantly for treatment of acute promyelocytic leukemia (Fang et al., 2002; Zhou et al., 2005b), but their applications are being expanded to treatment of other cancer forms (Okuno et al., 2004). As the search for improved anticancer agents is constantly ongoing, I have also included a section discussing some of the promising new derivatives obtained from drug screenings.

Conventional chemotherapy

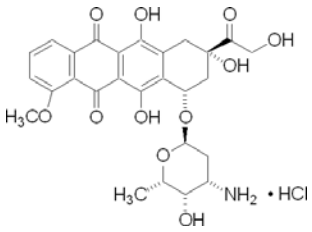


Etoposide

Etoposide (eto) was identified in 1967 as a derivative from the plant *podophyllum*, and possessing significant antitumor activity (Hande, 1998). As such, the eto podophyllotoxin was the

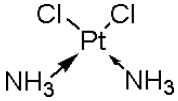
Image: Chromatin structure. A cartoon showing higher order structuring of DNA, being wound around histones that are in turn tightly folded to enable packing into the nucleus. Printed with permission from Prof. J. Hansen, CO, USA.

first anticancer drug demonstrated to inhibit topoisomerase-II. It does so by stabilizing the formed topoisomerase-II-DNA complexes, thus causing permanent double strand breaks. In addition, eto causes p53 phosphorylation, possibly mediated by the DNA damage sensor DNA-PK, with subsequent up-regulation of pro-apoptotic Bax and promotion of apoptosis through cytochrome c release (Karpinich et al., 2002). At lower etoposide concentrations however, caspase activity seems required for efficient apoptosis induction (Robertson et al., 2002). Activation of caspase 2 is of particular importance due to its early activation during apoptosis and its nuclear localization. Regarding the conflicting reports on the involvement of death receptor signaling (Friesen et al., 1999; Robertson et al., 2002), our work supports the model where eto induces apoptosis independently of the Fas receptor (Paper II). Although poorly soluble in water and thus difficult to administer in effective doses, eto together with other topoisomerase-II-inhibiting agents are among the most effective chemotherapeutic drugs available for cancer therapy (Baldwin and Osheroff, 2005).



Doxorubicin

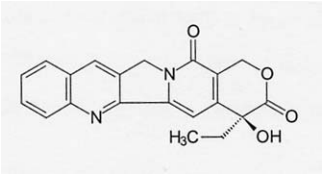
Doxorubicin (dxr) is an anthracycline antibiotic, analogous to daunorobucin, with broad-spectrum anti-tumor effects. The apoptosis promoting effects of dxr are diverse and include DNA damage and DNA damage-induced signaling, generation of reactive oxygen species, binding to cellular membranes, disruption of the mitochondrial membrane, generation of cellular diacylglycerol and Fas up-regulation (Friesen et al., 1999; Gewirtz, 1999). DNA damage is probably the most important mechanism for *in vivo* cytotoxicity of dxr treatment, mainly achieved by inhibition of topoisomerase-II, but also caused by intercalation and adduct formation. The cellular response to doxorubicin treatment is cell cycle arrest in the G1 and G2 phases, followed by apoptosis due to irreparable DNA lesions. Signaling from DNA damage-sensitive kinases indirectly activates p53 and c-Abl (Rich et al., 2000). Doxorubicin also activates pro-apoptotic PKCδ through diacylglycerol signaling. Support for Fas receptor and ligand involvement in dxr-induced apoptosis comes from studies in MYCN-inducible neuroblastoma cells where overexpressed MYCN was shown to enhance this event (Fulda et al., 1998). However, several lines of evidence including our findings (Paper II), imply that despite the up-regulation of Fas receptor expression in response to dxr, apoptosis-induction is not dependent on the Fas pathway. The most severe side-effect of dxr treatment is the risk of developing cardiomyopathy (Gewirtz, 1999).



Cisplatin

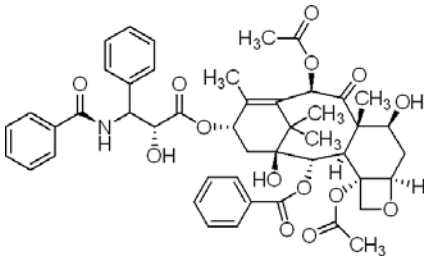
Cisplatin (cis) was the first platinum-derived anticancer drug introduced in the clinic and is still one of the most potent chemotherapeutic drugs in use (Eastman, 1991). Upon cellular uptake, alterations in the cis molecule generate a positively charged nucleophil, highly reactive with cellular proteins, membrane phospholipids, RNA and DNA. DNA-cis-interaction yields intra- and inter-strand crosslinks and DNA-protein crosslinks (Eastman, 1991). Cisplatin confers inhibition of DNA replication, G1 and G2 cell cycle arrest, and apoptosis induction. However, the mechanism

by which cis induces apoptosis remains poorly defined. Although DNA is proposed to be the main target, observations that cis induces caspase activation in enucleated cells suggest additional activation of a DNA damage-independent pathway (Mandic et al., 2003; Mandic et al., 2002). From these studies, two distinct cis-promoted apoptosis pathways have emerged. The MAP kinase pathway where MEKK1 is proposed to promote the DNA-dependent signal by Bak activation, and induction of endoplasmic reticulum (ER)-stress where calpain-mediated Bid cleavage occurs independently of DNA damage (Mandic et al., 2003; Mandic et al., 2002). Cisplatin-induced DNA damage has been observed in the absence of an apoptotic morphology, suggesting necrosis as an alternative response to cis-treatment (reviewed in (Gonzalez et al., 2001)) (paper II). p53 and c-Abl are two of the additional mediators activated in cis-induced apoptosis. Overexpression of c-Myc has been shown to sensitize certain cells to cis, whereas c-Myc down-regulation increases cis-resistance (Kim et al., 2003a). Side-effects of cis treatment include nephrotoxicity, neurotoxicity, ototoxicity (Nieboer et al., 2005; Schweitzer, 1993), the most severe one being ototoxicity since it can be irreversible and sometimes develops years after treatment (Knoll et al., 2006).



Camptothecin

The S phase-specific plant-derived alkaloid camptothecin (cpt) belongs to the first identified topoisomerase-I inhibitors (Hsiang and Liu, 1988). Camptothecin induces irreversible DNA damage by stabilizing the topoisomerase-I-DNA complex and preventing religation of single strand breaks. As cpt itself is poorly soluble and highly toxic, less toxic and more water soluble derivatives have been developed. As such, Topotecan and Irinotecan with extensive antitumor effects, have been approved for treatment of various human cancers (Lorence and Nessler, 2004). Apoptosis-induction by cpt most likely involves the mitochondrial pathway and caspase activation, but if caspase activation is blocked, cells death is induced by necrosis (Sane and Bertrand, 1999). Tumor cells are often more susceptible to cpt-mediated cytotoxicity compared to normal cells due to dissociation-deficient topoisomerase-I-DNA-complexes, resulting in increased topoisomerase-I activity. In general, side-effects from using cpt-derivatives seem transient and non-cumulative, the most severe effect being diarrhea. However, their use may be counteracted by increased renal clearance (reviewed in (Pizzolato and Saltz, 2003)).

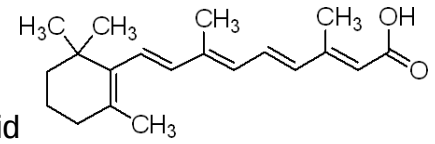


Paclitaxel

Another plant-derived alkaloid, paclitaxel (ptx), was discovered in 1967 when it was isolated from the bark of the tree *taxus brevifolia* (Wani et al., 1971). It binds to tubulin structures in the cell and prevents their dissociation. Paclitaxel, thus considered a polymerizing anti-microtubule agent, prevents cell division and arrests cells in the mitotic phase of the cell cycle, resulting in induction of cell death by apoptosis. One defined pro-apoptotic mechanism of ptx is the binding and inhibition of the anti-apoptotic Bcl-2 protein (Wang et al., 2000). As ptx targets dividing cells in general, it can be applied in the treatment of a wide range of cancer forms such as breast, lung, and ovarian

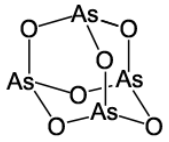
cancers. Neurotoxicity and cardiotoxicity are adverse effects of prolonged ptx treatment (Wang et al., 2000).

Differentiating agents



All-trans retinoic acid

The differentiating agent all-trans retinoic acid (ATRA) is a vitamin A analogue that was introduced in the treatment of APL in 1987 (Huang et al., 1987) and found to successfully induce complete remission in a high proportion of patients (Fang et al., 2002; Fenaux et al., 2000). ATRA targets the RARα part of the PML-RARα fusion protein often present in APL cells, and initiates its degradation. While high doses of ATRA induce apoptosis, low doses (≤ 1 μM) induce terminal differentiation of myelocytic leukemia cells (Carpentier et al., 1998). In spite of the well characterized connection to and targeting of the PML-RARα protein, other mechanisms for ATRA-induced differentiation or apoptosis are less well understood. However, reports on the interaction with pro-apoptotic proteins such as caspases, mitochondria-associated proteins, and others are beginning to unveil other molecular targets and pathways involved in the response to ATRA treatment (Gianni et al., 2000; Liu et al., 2000). Lately, ATRA has been successfully used in the treatment of malignancies other than APL. In neuroblastoma treatment (Reynolds and Lemons, 2001), ATRA was found to decrease MYCN mRNA expression and prevent cell proliferation (Reynolds et al., 1991), and has been proposed to be one of the most potent inducers of differentiation (Pahlman et al., 1984). An important note in the use of ATRA is that combination with chemotherapy is advisable to prevent adverse effects and reduce the frequency of relapse. The most serious side-effect of ATRA treatment is generation of the life-threatening retinoic acid syndrome (RAS), initiated by hyperleucocytosis (reviewed in (Fang et al., 2002; Soignet and Maslak, 2004)). Other side-effects such as cheilosis, headache, joint-pain and liver-dysfunction are milder and tolerable.



Arsenic trioxide

Arsenic trioxide (As_2O_3) is another differentiating agent initially used in ancient Chinese medicine but, abandoned due to its toxicity, the drug was almost forgotten for decades (Hu et al., 2005). However, in the mid-50's, As_2O_3 was rediscovered as a highly potent treatment specimen in several cases of acute promyelocytic leukemia (APL), yielding an almost complete remission rate. Since the late 1990's, As_2O_3 is the treatment of choice for relapsed APL patients with acquired resistance to all-trans retinoic acid (ATRA) (Shen et al., 1997; Soignet et al., 1998). In clinical trials, the possiblility of using As_2O_3 in combination with ATRA as a first line of treatment is being explored (Shen et al., 2004). Similarly to ATRA, As_2O_3 is known to target the PML-RARα fusion protein for degradation, but the downstream mechanisms are not very well defined (Cai et al., 2000; Liu et al., 2003). One interesting feature of As_2O_3 is that it induces only partial differentiation of myelocytic leukemia cells in low doses, ranging from 0.1 μM – 0.5 μM, while apoptosis is induced at higher doses (Chen et al., 1997). Side-effects are often moderate and tolerable in relapsed patients and include skin reaction, gastrointestinal disturbances, nausea and fatigue. More alarming are

observations of electrocardiographic changes with the rare but worrying outcome of sudden cardiac death (Unnikrishnan et al., 2001; Westervelt et al., 2001). In addition, more than 50% of patients have hyperleucocytosis and, when used as a fist line of treatment in newly diagnosed patients, there have been reports on development of severe and sometimes lethal hepatic lesions (reviewed in (Fang et al., 2002; Soignet and Maslak, 2004)).

Novel therapies

In the search for new cancer therapeutic drugs, a recently developed strategy is to enhance the anticancer activity of a target selected for its importance in promoting or preventing tumor progression. Some of the approaches to this task include screening of low molecular compound libraries in search for those eliciting target-specific anti-proliferative or pro-apoptotic effects. This widely used method was successfully applied in generating the compound PRIMA (p53-reactivation and induction of massive apoptosis), which is currently undergoing evaluation for entering clinical phase I trials (Bykov et al., 2002). Similarly, molecular screens for compounds interfering with MYCN transactivation or with Myc/Max heterodimerization have been successful, yielding candidates awaiting further investigation (Hueber and Evan, 1998; Lu et al., 2003). Recently, our lab employed a screening strategy in search for Myc-responsive agents (MYRAs) and identified two compounds, promising for future development into clinically useful substances (Mo and Henriksson, 2006). More specific screenings aimed to find molecules targeting the kinase domain of tumor-associated proteins. This approach yielded the Gleevec™ (STI-571/Imantinib mesylate) compound targeting the kinase domain of the fusion protein Bcr-Abl (Druker, 2002), and later found to target other kinases, such as the stem cell factor receptor c-kit (Nadal and Olavarria, 2004). Gleevec is currently used in treatment of chronic myeloid leukemia (CML) (Nicolini et al., 2006) and gastorointestinal stromal tumors (GIST) (von Mehren, 2006). In a more direct approach, the three-dimensional structure of crystallized protein(s) is used for modelling site-specific compounds by computer-based predictions. These compounds are then synthesized for analysis of their biological activity (Kontopidis et al., 2003; McClue et al., 2002). The highly specific CDK2-cyclin E-targeting compound R-roscovitine (Seliciclib/CYC202), currently undergoing clinical trials (Raynaud et al., 2005; Shapiro, 2006), was identified using this approach (De Azevedo et al., 1997; Meijer et al., 1997) and has been found to significantly reduce tumor size in nude mice (McClue et al., 2002; McLaughlin et al., 2003). However, in spite of these exact measures to engineer the perfect anticancer drug, it remains difficult to find a compound for which the mechanisms of action can be exclusively specified. Another approach at the stage of clinical trials is the use of tumor-specific antibodies designed to be unharmful for normal cells. By conjugating these antibodies with isotopes or toxins, specific killing of tumor cells is enabled without inducing major side-effects. Examples of such antibodies are rituximab, targeting the B cell-specific antigen CD20, and trastuzumab (Herceptin) that targets Her-2, a receptor tyrosine kinase that is often overexpressed in breast cancer (Stern and Herrmann, 2005).

Since many of these new strategies still require validation, the majority of cancer patients are still treated with conventional methods such as surgery and/or radiation complemented with chemotherapeutic drug treatment. Drug resistance, whether inherent or acquired, is most likely due to a deficient apoptotic program in tumor cells together with increased efflux and decreased influx of the drug, and increased DNA repair (Luqmani, 2005). In order to improve the therapeutic response to some of these drugs, it is necessary to elucidate the molecular mechanisms by which they induce cell death.

RESULTS AND DISCUSSION

In this section I provide an overview of the separate studies compiled in this thesis and discuss potential applications for the obtained results.

Paper I

The focus in this first study was to explore the function of Mad1 in cell growth control by analysis of Mad1 effects on proliferation, cell cycle distribution, and apoptosis in NIH3T3 fibroblasts with a tetracycline-inducible mad1 expression (tet-mad1). We conducted these studies to evaluate the proposed c-Myc-antagonizing role ascribed to Mad1, suggesting that it represses transcription and inhibits Myc-induced proliferation (Baudino and Cleveland, 2001; Cerni et al., 2002).

In logarithmically growing cells, we found that Mad1 overexpression resulted in a density-dependent reduction in proliferation and growth as observed in a colony formation assay and by quantification of [^{3}H]thymidine incorporation. The finding that mad1 could inhibit colony formation in cells seeded at low densities is in agreement with previous observations that Mad1 inhibited proliferation of serum starved cells as they were stimulated to re-enter the cell cycle (Sommer et al., 1997). Although Mad1 did not entirely block cell growth in our system, its effect was potentiated by serum starvation paralleled by an increased accumulation of cells in the G1 and the G2/M phases of the cell cycle. These data, together with findings in other cell types (Cerni et al., 2002; Chin et al., 1995; Sommer et al., 1997), indicate that Mad1 inhibits proliferation under restrictive growth conditions, whereas it is less efficient in exponentially growing cells.

Image: Commonly used lab consumables: eppendorf-tubes.

We observed that Mad1 expression reduced apoptosis when induced by serum-starvation, accompanied by an increase in G1 and G2/M populations. We also found a Mad1-mediated reduction of the apoptosis induced by the cytotoxic drug cisplatin. The apoptosis inhibitory effect of Mad1 is supported by findings that Mad1 blocked death receptor-induced as well as oncogene-induced apoptosis in human tumor cells and mouse fibroblasts, respectively (Gehring et al., 2000).

Enforced Mad1 expression reduced the CDK2 activity while we did not observe significant changes in the expression levels of cyclin D1, CDK2, or CDK4 upon Mad1 overexpression in the tet-mad1 cells. We did however, observe a slightly increased expression of the CKIs p21 and p27 in response to Mad1 overexpression. The p21 level was increased in the mad1 clone with the strongest effect on proliferation, suggesting a requirement for p21 to enable efficient reduction of proliferation. The observed increase in p27 may be due to direct or indirect inhibition of Myc-mediated p27-ubiquitination (Oster et al., 2002). This is supported by findings that the slowly growing phenotype found in myc -/- cells correlated with increased p27 levels (Mateyak et al., 1999). Since p27 is a potent CDK2-inhibitor, this may explain the observed reduction in CDK2 activity upon mad1 overexpression. From our study and studies by others (Berns et al., 1997; Queva et al., 1999), it can be suggested that Mad1 elicits some of its effects through negative regulation of CDKs (Rottmann et al., 2005).

In search for the mechanism by which Mad1 mediated the reduction in cell growth and apoptosis, we found that it was dependent on the N-terminal mSin3 interaction domain (SID), since a SID truncated Mad1 protein had no such effect. Considering that Mad1 recruits mSin3-HDAC corepressor complexes via the SID, it is tempting to speculate that Mad1 represses a factor/factors important in promoting apoptosis and/or cell cycle progression (Laherty et al., 1997). We propose that Mad1-mediated repression may be enabled by inhibition of Myc target genes, thus counteracting Myc function.

The different expression patterns of Mad1 and Myc may explain why the effects of Mad1 overexpression are low in proliferating cells and potentiated during starvation when Myc levels are low. Similarly, Mad1 is up-regulated during differentiation, while Myc is down-regulated. Taken together, these data suggest that Mad1 may have a critical role in stabilizing a quiescent state, and to ensure cell survival and specialization by protecting against apoptosis during differentiation.

Paper II

Here, we have explored the cellular response to c-Myc expression and/or cytotoxic drug treatment by analyzing cell death, expression and activation of Bcl-2 family members, caspase activation and involvement of the Fas receptor/ligand system.

In mouse and rat fibroblasts with different c-Myc status we found that c-Myc potentiated apoptosis induced by the drugs etoposide (eto), doxorubicin (dxr), and cisplatin (cis). This effect was dose-dependent with the most obvious impact of Myc elicited at low drug concentrations. Apoptosis, as induced by all three drugs, was higher in c-Myc overexpressing cells compared to cells with wild type (wt) c-myc. Furthermore, the drug-induced apoptosis was significantly reduced in rat cells with a c-myc null phenotype (HO15.19). In eto and dxr treated samples, there was a good correlation between cell death and apoptosis. In cis treated cells however, cell death was high also in the c-myc null cells, emphasizing the importance of necrosis in the cellular response to cis treatment in the absence of c-Myc. These findings were corroborated by analyzing cisplatin-induced nuclear condensation after Hoechst staining. In contrast to our observations, Sedivy's group

reported that c-myc null cells were virtually resistant to eto-induced apoptosis but readily died by apoptosis when treated with cis (Adachi et al., 2001). Possible reasons for these discrepancies may be the use of different techniques, drug concentrations, and timepoints for analysis. Our results are supported by observations that cis induces necrosis as well as apoptosis, in part depending on effects on the tumor suppressor p53 (Gonzalez et al., 2001; Pestell et al., 2000). It is not clear however, whether c-Myc can act as an important "switch" in discriminating between the distinct effects induced by cisplatin. A direct interaction with Myc in activating transcription is unlikely, judging from our results in luciferase-based reporter assays where no effects on c-Myc-mediated transactivation were observed in response to treatment with any of the drugs analyzed (not shown).

The three anticancer drugs used in this study have all been reported to target mitochondria through activation of Bax or Bak, as a mechanism for caspase-mediated apoptosis-induction. We therefore analyzed the expression and activation of Bax in response to drug treatment. In the rat cells with wt c-myc (TGR-1), Bax was activated by eto- and dxr treatment but not by cis. In contrast, none of the drugs induced Bax activation in the c-myc null cells. All three drugs have previously been associated with Bax activation, but in the case of cis-induced Bax activation, it was observed in c-Myc overexpressing Rat1 cells (Desbiens et al., 2003; Karpinich et al., 2002; Rebbaa et al., 2001). Together with our observations that Bax was activated in response to all three drugs in c-Myc-overexpressing tet-myc cells, we suggest that c-Myc overexpression is required for cis-induced Bax activation. Our findings that Bax was not activated in c-myc null cells, in spite of similar protein levels as in cells with wt c-Myc, support the theory that c-Myc is important for activation but not transcription/degradation or translocation of Bax (Soucie et al., 2001). One potential mediator of drug-induced Bax-activation is the pro-apoptotic BH3-only protein Bid (Hueber et al., 1997; Iaccarino et al., 2003). We observed down-regulation of Bid in response to drug treatment in c-Myc expressing but not in c-myc null cells. As we did not observe the truncated tBid form in response to any treatment, we considered the decrease in full-length Bid as cleavage (Cartier et al., 2003). In contrast to suggestions that c-Myc-mediated cytochrome c release occurs by direct effects on the mitochondrial permeability and does not necessarily require Bcl-2 family members for apoptosis induction (Iaccarino et al., 2003), our data strongly indicate that activation of Bid and Bax is a major route through which these drugs activate the intrinsic pathway of apoptosis in the presence of Myc.

We found that eto- and dxr-induced activation of the initiator caspases 8 and 9, and the effector, caspase 3 was higher in c-Myc expressing than in c-myc null cells. While activation of caspase 3 was higher in c-Myc expressing cells also in response to cis treatment, there were no obvious differences between c-Myc expressing cells and c-myc null cells when comparing cis-induced caspase 8 and 9 activation. Importantly, the extent of caspase 3 activation was much higher than that of caspase 8 and 9 at all time points. The higher caspase activation seen in c-Myc expressing cells compared to c-myc null cells correlated well with our findings of a more pronounced apoptosis in these cells. Interestingly, even though the general caspase inhibitor zVAD-FMK efficiently inhibited eto-induced caspase activation in wt c-myc cells, it could only partially block apoptosis.

In tet-myc cells, dxr and eto treatment increased Fas receptor expression, and antibody-mediated stimulation of Fas enhanced drug-induced apoptosis. These effects were not enhanced by c-Myc overexpression, supporting theories of parallel pathways for Fas- and c-Myc-induced apoptosis (Evan and Littlewood, 1998; Juin et al., 1999). However, these theories have been challenged (Fulda et al., 1998) suggesting that the involvement of of Fas receptor/ligand signaling

in promoting the apoptotic response to chemotherapeutic drugs could differ depending on the cell type (Friesen et al., 1999; Teitz et al., 2000). We also evaluated the effect of stimulation with soluble Fas ligand and found that c-Myc expressing rat cells were sensitized to killing by the Fas ligand while c-myc null cells were resistant. This data, together with the observed caspase 8 activation in wt c-myc cells and a previous report that c-Myc overexpression sensitizes Rat1 fibroblasts to killing by soluble Fas Ligand (Hueber et al., 1997), suggests that the Fas pathway is important in drug-induced apoptosis in c-Myc expressing rat cells.

To establish whether the apoptosis pathway was disrupted in the c-myc null cells, we analyzed the effect of the non-specific protein kinase-inhibitor staurosporine (sts), selected for its reported ability to induce apoptosis without the involvement of caspases (Belmokhtar et al., 2001). In effect, treatment with sts resulted in apoptosis in c-myc null cells, even though it was to a lower extent compared with c-Myc expressing cells. Since it was possible to trigger c-myc null cells to death by apoptosis, our results indicate that these cells have a functional, but probably not intact, apoptosis pathway. However, this hypothesis needs to be verified by further experiments. It is conceivable that cyclin A-expression/activity is affected by sts treatment, since it was demonstrated that eto-induced apoptosis in c-myc null cells could be reconstituted by overexpressing cyclin A (Adachi et al., 2001).

We next explored the impact of PKCδ signaling by specifically inhibiting PKCδ using rottlerin. We found that rottlerin pre-treatment reduced eto-induced apoptosis in c-Myc expressing cells, but had no effect in c-myc null cells. Furthermore, eto-induced apoptosis was reduced in cells expressing dominant negative PKCδ (PKCδ-DN), compared with vector transfected, or wt PKCδ expressing cells. As the pro-apoptotic function of PKCδ has been ascribed to the catalytic subunit (Brodie and Blumberg, 2003), we assessed its activation by monitoring eto-induced proteolytic processing. We observed PKCδ cleavage in c-Myc expressing but not c-myc null cells, suggesting that defects in PKCδ signaling may be partially responsible for the reduced apoptotic response to eto, and possibly dxr, treatment of c-myc null cells. We next investigated the connection between PKCδ and Bax, using mouse embryo fibroblasts (MEFs) with endogenous bax together with bax -/- MEFs. As we observed eto-induced PKCδ cleavage independently of bax-status, we concluded that it occurred upstream of Bax. This observation was verified by flow cytometry, showing reduced eto-induced Bax activation in cells that were pre-treated with rottlerin.

To conclude, our observations strongly implicate Bax activation together with activations of caspases and PKCδ signaling in c-Myc-dependent eto-induced apoptosis.

Paper III

To further characterize the role of Myc in drug-induced apoptosis, we used the chemotherapeutic agents camptothecin (cpt) and paclitaxel (ptx) to induce apoptosis in the previously used Rat1 cells with different c-Myc status and in neuroblastoma cells with conditional MYCN expression (Tet21N). Camptothecin and ptx are both plant-derived alkaloids, but while cpt is a topoisomerase-I (topo-I) inhibitor mainly targeting cells in S phase, ptx is a polymerizing anti-microtubule agent preventing dissociation of tubulin structures in the cell and thereby blocking cell division. Both drugs are used for treatment of tumors, but since their relation to Myc has been debated, we found it important to clarify this issue and possibly enable a broader spectrum for their clinical applications. In spite of the distinct mechanisms by which cpt and ptx elicit their

cytotoxicity, we found that c-Myc as well as MYCN enhanced the apoptosis induced by both drugs. Together with our previous data (paper II), this observation led to the suggestion that Myc is capable of enhancing apoptosis when induced by drugs with a wide range of cellular targets, and that this function may not be restricted to c-Myc. In support of our findings, MYCN was previously shown to trigger apoptosis in response to drug-treatment ((Fulda et al., 1999) and reviewed in (Hogarty, 2003)).

In assessing the signaling pathways used in Myc-enhanced apoptosis by cpt and ptx, we found indications of mitochondrial involvement in c-Myc expressing cells when treated with cpt, but not in response to ptx treatment. Our initial data pointing in this direction were the much lower activation of caspase 9 elicited in response to ptx compared with that induced by cpt. Activation of caspases 3 and 8 were similar in response to both drugs. Observed differences in caspase 9 activities were followed up by analysis of events preceding mitochondrial activation. Here, we observed cleavage of pro-apoptotic Bid in response to treatment with cpt, but not ptx, thus prompting us to study whether cpt would induce activation of the mitochondria-associated Bax protein. As expected, Bax was activated in response to cpt treatment. In the case of ptx, we did not assess Bax activation, mainly because of the negative results obtained in analysis of events upstream and downstream of the mitochondria. However, as Bax activation has been observed in response to ptx treatment of cells overexpressing c-Myc (Soucie et al., 2001), it will be important to evaluate this further. One explanation for this discrepancy could be that the observations by Soucie *et al.* were made in HOmyc3 Rat1 cells that overexpress Myc and that cells were treated with a higher ptx dose than the one used in our study (0.5 μM compared with 0.1 μM). Our preliminary data, showing Bid cleavage in HOmyc3 cells when treated with 0.1 μM as well as 0.5 μM ptx, and in TGR-1 (wt Myc expressing) cells in response to 0.5 μM ptx confirm this hypothesis (H. Mo, data not shown).

Since the pro-apoptotic effects of cpt and ptx were more pronounced in presence of the Myc transcription factor, we investigated whether these drugs would directly interfere with Myc-mediated transcriptional activation or DNA-binding. In a luciferase reporter assay, we found that cpt, but not ptx, reduced Myc-mediated transactivation. This effect by cpt was not promoted through inhibition of DNA-binding by the Myc/Max complex, as visualized in electrophoretic mobility shift assays where neither of the drugs was effective, even at very high concentrations. These results suggested that cpt-mediated effects on transcription might not be restricted to Myc, even though cpt-induced apoptosis is enhanced in a Myc-dependent manner. Using other reporters, we confirmed that cpt represses transactivation both from p53 and from the CMV promoter. In contrast, ptx resulted in a small but reproducible increase in luciferase activity. In contrast to our analysis using reporter gene assays, previously conducted studies on effects on gene transcription were more indirect by analyzing drug-mediated up- or down-regulation of selected targets on the level of protein, mRNA, or gene expression (Jiang et al., 2000; Liu and Stein, 1997; Srivastava et al., 1998; Stahl et al., 1997). While the genes analyzed in those studies were distinct from the ones we used, their results were either in contrast with (Jiang et al., 2000; Liu and Stein, 1997), or supportive of (Jiang et al., 2000; Srivastava et al., 1998) our findings. We exclude the possibility that the cpt-mediated reduction in transactivation is inflicted through DNA-damage, since the DNA-damaging drugs eto, dxr, and cis do not affect Myc-mediated transactivation. Therefore we suggest that cpt interferes with transactivation by interacting with the general transcription machinery.

To investigate whether cpt and ptx activated similar pathways as observed in response to eto-treatment in the presence of Myc (paper II), we monitored PKCδ signaling. By doing so, we demonstrated that PKCδ signaling was important in Myc-enhanced apoptosis induced by cpt.

Camptothecin treatment induced proteolytic processing of PKCδ, and cpt-induced aoptosis was reduced when blocking PKCδ with the specific inhibitor rottlerin or by expression of dominant negative PKCδ (PKCδ-DN). Neither of these effects was observed in response to ptx treatment, using doses equitoxic to the employed cpt concentration, or even at a concentration eliciting extreme toxicity. Our finding that cpt treatment induced PKCδ cleavage also in bax -/- MEFs suggested that this event preceded Bax activation. Furthermore, the observed reduction in cpt-mediated caspase activation when cells were pre-treated with rottlerin suggests that PKCδ is of significant importance for caspase activation.

From our data we conclude that the two cytotoxic drugs analyzed in this study activate separate sets of apoptosis mediators, given that we did not observe involvement of the mitochondrial pathway or of signaling through PKCδ in ptx-induced apoptosis. However, as caspases 3 and 8 were activated to a similar extent as in response to cpt, it is possible that ptx signaling is partially mediated by death receptors. It has been proposed that ptx acts independently of p53 (Debernardis et al., 1997; Vasey et al., 1996), but when it comes to the relation between ptx and Myc, further investigation is required. In evaluating cpt, we have here begun to discern a mechanism by which to enhance Myc-driven apoptosis in response to cpt treatment. We provide evidence that PKCδ signaling is important for these effects and suggest that potentiating this pathway might be the way to go in re-activating apoptosis in tumors with retained or activated Myc.

Paper IV

We next focused on the differentiating agents all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3), and how Myc affected their impact on the promyelocytic leukemia cell line HL60. These agents are currently used in treatment of acute myelocytic leukemia with promising results ((Huang et al., 1988; Niu et al., 1999) and reviewed in (Shen et al., 2004)). However, although it is well established which parts of the fusion protein PML-RARα are targeted by these drugs, the mechanisms for their induction of differentiation and/or apoptosis are poorly understood (Gianni et al., 2000; Mathews et al., 2001). As HL60 cells lack the PML-RARα fusion protein (Wang et al., 1998b), these cells provide an optimal model system for studying such downstream effects.

The drugs were titrated to obtain the optimal dose at which they affected cell proliferation without inducing excessive apoptosis. Evaluation of the titration readout prompted us to use 1.0 μM ATRA and 0.5 μM As_2O_3 for additional experiments. These doses are well in the range of the plasma levels reached when treating APL patients (Zhou et al., 2005b). In differentiation assays, we verified previous observations that ATRA induces terminal differentiation (Breitman et al., 1980), while the response to As_2O_3 was not indicative of differentiation, judging from morphological observations, NBT reduction capacity, or CD11b expression. From these results together with the observed effects on cell cycle distribution, we deduce that As_2O_3 most likely induces partial differentiation, as previously suggested (Cai et al., 2000).

Interestingly, ATRA induced strong activation of caspases already after two days of incubation, suggesting that apoptosis is initiated, even at this low concentration. It has been established that both ATRA and As_2O_3 induce apoptosis when administered at high concentrations (Carpentier et al., 1998; Chen et al., 1997), and activation of caspases is a step of the apoptosis-promoting process (Cai et al., 2000; Gianni et al., 2000; Liu et al., 2003). In view of this, it was intriguing to observe that the As_2O_3-induced caspase activation was always lower than that induced by ATRA, even in response to the high concentration 4.0 μM. In addition, we found that the activation of caspase 8,

reported to be induced in a death receptor-independent manner in response to As_2O_3 (Kitamura et al., 2000), was only moderately higher in response to 4.0 μM as compared to 0.5 μM As_2O_3.

Because the HL60 cell line harbors amplified c-myc (Eckhardt et al., 1994), it is a useful tool for conducting studies of the role of Myc in controlling the outcome of treatment with ATRA and As_2O_3. Down-regulation of Myc is a prerequisite for cellular differentiation (Pelengaris et al., 2002), and we therefore analyzed how Myc expression varied over time when cells were incubated with drug. As expected from our observations in the differentiation assays, Myc was almost completely down-regulated in response to ATRA treatment, both on the mRNA level and the protein level, enabling differentiation (Larsson et al., 1994). Another requirement for cellular differentiation is up-regulation of the Myc antagonist Mad (Larsson et al., 1994), which we also observed within two days of incubation with ATRA in our study. In contrast to the expression pattern in ATRA treated cells, Myc expression persisted after treatment with As_2O_3, and Mad1 mRNA remained at the level observed in control cells, concurrent with the inability of the cells to differentiate properly.

Having established that ATRA and As_2O_3 differentially affected expression of Myc network proteins, we further charaterized their effects on Myc/Max DNA-binding to the promoters of the well characterized Myc target genes human telomerase reverse transcriptase (hTERT) (Oh et al., 2000; Wang et al., 1998a; Xu et al., 2001) and carbamoyltransferase-dihydroorotase (CAD) (Boyd and Farnham, 1997; Miltenberger et al., 1995), important in controlling the cell turnover and maturation (Grayson et al., 1985; Poole et al., 2001; Wang et al., 1998a). In chromatin immuno-precipitation assays, we observed Myc at these promoters after exposure to As_2O_3 but not after ATRA treatment. These observations were paralleled by a cell cycle modulator expression pattern indicative of differentiation in response to ATRA, such as down-regulation of CDK2 and CDK4, cyclin E and cyclin A, together with up-regulation of p21 and p27, while the effects were weaker or not at all observed in As_2O_3-treated cells.

The results obtained in this study provide the basic structure in a model characterizing the pathways used for differentiation and apoptosis induced by ATRA and As_2O_3. Disregarding the fact that additional experiments could be performed for obtaining a more complete model, we suggest that Myc is important in preventing terminal differentiation after As_2O_3 treatment, in part through activation of hTERT and CAD.

FUTURE PROSPECTS

The results presented in this thesis provide new insights regarding the involvement of Myc and Mad in regulation of cell cycle and apoptosis, but in addition, they give rise to new questions. For instance, it would be important to assess how the cellular response to drugs would be affected by Mad1 overexpression. As we demonstrated in paper I, Mad1 overexpression protects cells from apoptosis induced by serum starvation and cisplatin, but it is not known whether this is a general effect or if it only applies to a select set of insults. Using the drugs employed in papers II, III, and IV, we would also investigate the status of Mad in response to treatment. In case we observe a protection, several additional experiments are required to pinpoint the mechanism by which apoptosis is prevented. To begin with, we would analyze the mediators found to be involved in Myc-mediated apoptosis.

One of many additional trails that could be followed for increasing the knowledge of effectors in Myc-enhanced apoptosis is evaluation of involvement of the Fas pathway. The importance of such a study is emphasized by implications that Fas signaling enhances apoptosis when induced by Myc (Fulda et al., 1999; Hueber et al., 1997), as well as by many of the drugs used in our investigations (Fulda and Debatin, 2004). We initiated this analysis in paper II and will continue to evaluate the role of Fas in response to the remaining drugs (papers III and IV). Such analysis could be conducted by introduction of dominant negative FADD, which would also provide insights into the unresolved question whether Fas is required for c-Myc-induced apoptosis or acts in a parallel pathway to potentiate the apoptotic response.

In analyzing expression and activation of Bcl-2 family members, there was no suitable antibody for assessing activation of the pro-apoptotic Bak protein in rat cells. However, in view of reports on cis- as well as dxr-induced cytochrome c release through Bak activation (Mandic et al., 2001; Panaretakis et al., 2002), together with our data on cis-induced apoptosis in wt c-Myc cells, we

 Image: Safety equipment for diving into the unknown and exploring new exciting theories.

anticipate involvement of Bak in cases where we do not observe Bax activation and will pursue this analysis in Myc-inducible cells originating from human or mouse.

Due to the involvement of PI3K signaling in promoting the anti-apoptotic effects of Bcl-2 and Bcl-xL (Zha et al., 1996) and because we observed that Bcl-xL was up-regulated in the c-Myc null cells (Adachi et al., 2001) (paper III), we investigated whether inhibition of the PI3K-Akt cascade would stimulate apoptosis. For this, we used the PI3K inhibitor LY294002, previously found to potentiate dxr-induced apoptosis (Panaretakis et al., 2002). However, this inhibitor did not potentiate eto-induced apoptosis in the c-myc null cells, nor did it prevent cis-induced cell death (A. Albihn, data not shown), suggesting additional alterations in the cell physiology of these cells, such that the indirect inhibition of Bcl-xL is not enough to alter their response to stress. Another approach to overcome the Bcl-xL mediated block of apoptosis in c-myc null cells would be to overexpress Bax and thus tilt the balance of pro-apoptotic versus anti-apoptotic effects.

The analysis of drug-mediated effects on expression and/or activation of p53 was beyond the scope of this thesis. However, in view of the central role of p53 in apoptosis signaling (Sherr, 2004), and established interactions with Myc proteins (Hermeking and Eick, 1994; Nilsson and Cleveland, 2003), these studies will provide an important continuation of our work.

Another important intermediary is the cystein aspartate proteinase, caspase 2. Activation of caspase 2 has been reported to be directly associated with, and initiating the apoptosis process in response to, DNA damage (Robertson et al., 2002). In addition, a connection between caspase 2 and PKCδ in activation of apoptosis has been established (Panaretakis et al., 2005), indicating caspase 2 as a possible upstream inducer of PKCδ signaling in response to eto and cpt treatment in our cells (papers II and III). This might also explain the absence of PKCδ involvement in response to ptx, which is not a DNA-damaging agent (paper III). Therefore, much information would be gained by analyzing caspase 2 activation in response to drug treatment.

Last and maybe most importantly, the Myc-dependency in dictating drug impact on these effectors should also be analyzed in human cell lines with conditional Myc expression. A comparison of the apoptosis pathways induced by c-Myc and MYCN would be highly informative and could be performed in neuroblastoma cells with conditional MYCN expression together with the B-cell line p493-6 harboring an ER-fused c-Myc protein and a regulatable EBNA2 gene expression, thus possessing the ability to mimick either a lymphoblastiod cell line or a Burkitt's lymphoma cell line (Schuhmacher et al., 1999). The targets identified to be consistently regulated in response to myc ovrexpression and drug treatment, expression/activation of these mediators would be evaluated in tumor samples to establish whether assays for their analysis could be developed for use in clinical assessment. Our hope is that such markers, together with Myc status, will be used for predicting the cellular response to certain treatment regimens and thus enable the design of specific and individualized therapy.

TO CONCLUDE

With this thesis, I would like to emphasize the importance in characterization of the pathways activated by Myc in the interaction with cytotoxic drugs for induction of apoptosis. Because drug-resistant tumors frequently harbor deregulated myc expression, expected to induce apoptosis, the most likely explanation for their resistance would be an acquired blockage in the apoptosis pathways activated by Myc. Since chemotherapeutic drugs are often necessary as a complement to radiation therapy and surgery to enable tumor eradication, a possibility to reactivate Myc-induced apoptosis in these tumors could enhance the drug efficacy and circumvent the resistance problem. The results gathered in the presented original works collectively provide a starting point in gaining insights required to formulate strategies for future clinical implications.

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