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Post-translational regulation of Myc oncoprotein function

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In memory of my brother Cardo

Abstract

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The c-Myc proto-oncogene encodes a transcription factor that controls genes involved in cell cycle regulation, cell growth, apoptosis and other cellular processes together with its partner Max. c-Myc has been shown to be altered in a wide variety of human tumors.
This thesis focuses on post-translational regulation of Myc function. The aims was firstly to elucidate mechanisms of regulation of Myc degradation and components involved, and secondly to understand the mechanism(s) behind IFN-γ-induced inactivation of Myc.
The c-Myc oncoprotein is short–lived phosphoprotein. We have found that Myc is tightly regulated via ubiquitin/proteasome-mediated turnover and that mutation of the Thr-58 phosphorylation site, which is frequently mutated in both in Burkitt's lymphoma leads to stabilization of c-Myc. We have further established that the E3-ubiquitin ligase SCF^{Skp2} associates with c-Myc in late G1 and S-phase of the cell cycle, inducing not only ubiquitin-mediated degradation of c-Myc but surprisingly transcriptional activation of c-Myc target genes. Skp2 as well as proteasomal subunits were shown to be recruited to a Myc target promoter in vivo in a Myc-dependent manner. The second part of the thesis shows that the cytokine IFN-γ restore differentiation and cell cycle arrest in v-Myc expressing monocytic cells through inhibition of Myc-induced transcription, Myc DNA-binding and destabilization of Myc:Max heterodimers, correlating with dephosphorylation of Myc. We further found that IFN-γ reduces phosphorylation of Ser-62 and increases ubiquitin/proteasome-mediated degradation of Myc in a Ser-62 dependent manner.CycE/CDK2 was identified as a Ser-62 kinase in vivo and in vitro. IFN-γ-induced degradation and Ser-62 dephosphorylation correlated with inactivation of CDK2 through $p27^{Kip1}$, which was shown to be required for this process. In conclusion, the thesis suggests that ubiquitin/proteasome-mediated turnover is an essential level of regulation of Myc function that may have important future implications for treatment of tumors with deregulated Myc expression.

Key words: Myc, Skp2, $p27^{Kip1}$, CycE/CDK2, ubiquitin/proteasome pathway, transcription, cell cycle, differentiation, and IFN-γ

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Appendix

Paper I-IV

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

I Bahram F, von der Lehr N, Cetinkaya C, Larsson LG.
c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. Blood. 2000 Mar 15;95(6):2104-10.

II von der Lehr N, Johansson S, Wu S, Bahram F, Castell A, Cetinkaya C, Hydbring P, Weidung I, Nakayama K, Nakayama KI, Soderberg O, Kerppola TK, Larsson LG.
The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol. Cell. 2003 May;11(5):1189-200

III Bahram F, Wu S, Oberg F, Luscher B, Larsson LG.
Posttranslational regulations of Myc function in response to phorbol ester/interferon-gamma-induced differentiation of v-Myc-transformed U-937 monoblasts. Blood. 1999Jun1;93 (11):3900-12.

IV Fuad Bahram[1], Natalie von der Lehr[1], Richard Lilischkis[2], Sara Johansson[1] Bernhard Lüscher[2] and Lars-Gunnar Larsson[1]
Interferon-gamma inhibits CycE/Cdk2-mediated phosphorylation of c-Myc via P27Kip1 resulting in increased Myc ubiquitylation and degradation. Manuscript.

Paper I-III were reprinted with permission from the publisher.

Abbreviations

GTF	General Transcription Factor
TBP	TATA-box binding protein
IFN-γ	Interferon-γ
JAK1	Janus kinase 1
STAT1	Signal Transducer and Activator of Transcription 1
MBI, II	Myc box 1, II
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Odc	Ornithine decarboxylase
pRb	Retinoblastoma protein
TAD	Transactivation domain
CTD	C-terminal domain
bHLHzip	basic region helix-loop-helix leucine zipper
SID	Sin3-interacting domain
Cdk	Cyclin dependent kinase
CIP1	Cdk inhibitor protein 1
CK	Casein kinase
CKI	Cyclin dependent kinase inhibitor
INK4	Inhibitor of Cdk4/6
CAK	CDK activating kinase
JNK	Jun N-terminal kinase
MAPK	mitogen activated protein kinase
MEF	murine embryo fibroblast
aa	amino acid
PDGF	platelet-derived growth factor
Skp2	S-phase kinase-associated protein 2

Background

Development of cancer

The development of cancer is no longer a mystery; during the last 2-3 decades numerous researchers have made fabulous progress in their work to clarify the basis of the process.
In reality the term cancer refers to hundreds of forms of the disease. Almost all organs or tissues can be subjected to tumor development, some of which can manifest several cancer types. One common question, why a cell becomes a cancer cell?
The nearly 30 trillion cells living in the human body are regulating each another's growth and survival in a well organized but complex manner. Indeed, normal cells reproduce only when they are instructed to do so by signals from other cells in their vicinity. In contrast, cancer cells follow their own agenda for reproduction, and also eventually become more aggressive, emigrating and invading nearby tissues or forming tumor masses at sites distant form the sites of their origin.
Many years before a tumor becomes visible, usually multiple genetic alterations have occurred. Mutations in a gene can result in altered amounts or altered activity of the gene product. Two different classes of genes are important for the development of cancer, proto-oncogenes, which are involved in stimulation of cell growth and tumor suppressor genes, which inhibit it. Mutations in a proto-oncogene can result in its transformation into a carcinogenic oncogene caused either by deregulated expression or by the creation of an overly active form of the gene product, both resulting in growth stimulation. Tumor suppressor genes in contrast normally protect the organism from cancer by inhibiting cell growth, and mutations in such genes may result in inactive genes or gene products which are unable to brake cell growth, again leading in cancer (Hanahan and Weinberg, 2000, Macleod, 2000).
Genetic alterations in multiple genes affecting multiple fundamental biological processes are needed to create a cancer cell. Below some of these processes are described as summarized in figure 1.
Normal cells require growth signals before they can exit from a quiescent state into actively growing state. In adults, cells normally divide only when cells new are needed to replace damaged or worn out tissue. In contrast, tumor cells grow uncontrolled. Such external growth signals are transferred through soluble factors or cell-cell interactions. The signals are usually received by specific receptors at the cell surface, after which the signals are transmitted from the receptors into the cell interior, through signaling pathways involving G-protein-coupled receptors, GTPaes, tyrosine kinase and MAPK, etc. Numerous of oncogenes operate by mimicking such normal growth signaling pathways. Activation or overexpression of surface receptors or components of the downstream mitogenic signal pathways such as the epidermal growth factor receptor (EGF-R), (Balmer et al., 2001) and Ras, has been found in many cancers (Boettner and Van Aelst, 2002).

Insensitivity to antigrowth signals is another common genetic alteration in order for cancer cells to multiply. Normally the cell's decision whether to growth and not is determined by the balance between growth and anti-growth signals. Anti-

growth signals can block cell growth by two different ways, either by inducing cells to exit the cell cycle into a reversible G0-phase or by causing cells to move toward a more permanent postmitotic phase through differentiation or senescence. There are specific antigrowth molecules which involve signaling pathways similar to those induced by growth signals. Examples of such antigrowth molecules are the interferons including (IFN-α, β and γ) and the TGF-β family. IFNs interact with multisubunit transmembrane IFN-receptors, which associate with tyrosine-specific Janus activating kinases (JAKs) at the inner side of the plasma membrane. Following activation of JAKs by IFN-signaling. Latent transcription factors located in the cytosol called Signal Transducer and Activator of Transcription (STATs) become phosphorylated and translocate to the nucleus as a dimers where they activate transcription of genes that negatively regulate cell growth (Sangfelt et al., 2000, Brivanlou and Darnell, 2002)(see below). TGF-β bind typ I and II serine/threonine kinase receptors at the cell surface that transmit signals through another type of latent transcription factors, Smads, which translocate into the nucleus and regulate the transcription of TGF-β responsive genes. Disabling of the TGF-β pathway affecting its components by mutations thus removes a barrier that normally prevents the cells to proceed through the cell cycle (Shi and Massague, 2003).

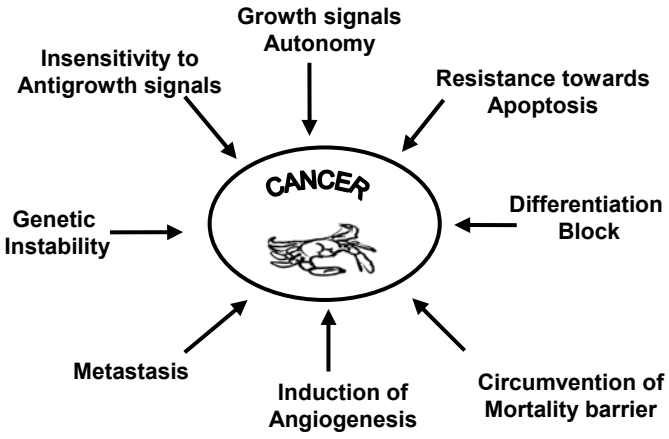


Figure 1: The cellular processes during genetics alteration in cancer cells

Cancer cells also need to block programmed cell death (apoptosis) to survive. Apoptosis is an essential regulatory mechanism that occurs in all normal cells characterized by programmed cell death. However, tumor cells develop an ability to resist apoptosis. The most common strategy is the loss of the pro-apoptotic tumor suppressor gene p53 through mutations that result in functionally inactive p53. Mutation can also occur in the MDM2 gene which encodes a negative regulator of p53. The Bcl-2 family of proteins is another component involved in apoptosis control. Those with anti-apoptotic actions include Bcl-2, Bcl-xl and Bcl-w and among those with pro-apoptotic action are Bax, Bak, Bid and Bin. Overexpression of the Bcl-2 oncogene through mutations occurs in many tumors (such as B-cell lymphoma) and leads to inhibited apoptosis. Upregulation of Bax by p53 activation can elicit apoptosis. Caspases (a group of cysteine proteases) is a group of proteins that play major role in executing the apoptosis program. Caspases can be activated by signaling through death receptors such as the FAS protein (the so called extrinsic pathway) or directly by cytochrome C release from the mitochondria (the so called intrinsic pathway) (Igney and Krammer, 2002).

Another hallmark of tumor cells is genetic instability that is manifested at the chromosomal level. Cancer cells accumulate genetic alterations, including translocations and amplifications leading to abnormal gene expression or abnormal gene products. In Burkitt's lymphoma, rearrangements involving chromosome 8 and 14 (90% of cases) lead to abnormal expression of the c-Myc oncoprotein as consequence of being translocated into vicinity of any of immunoglobulin heavy chain enhancers. Normally, DNA can be replicated only once per cell cycle. However in cancer cells, some regions undergo multiple rounds of replication called amplification. A typical example of this type of process is amplification of the myc gene in neuroblastomas (Oster et al., 2002).

Human cells have a cell autonomous program that limits their multiplication potential (to approximately 50 divisions). Cancer cells must disrupt also this program to expand and develop into immortal tumors. Telomeres, the ends of the chromosomes are composed of several thousand repeats of a six base pair sequences that appear to act as a counting tool for cell doubling. During every cell doubling, the chromosome loses 50-100 base pair of telomeric DNA due to the inability of DNA-polymerases to completely replicate the ends of the chromosomes. After reaching a critically short telomere length, the senescence machinery of the cell is activated and the cell will then undergo senescence. In almost all cancer cells telomere maintenance is achieved by activation of the telomerase enzyme which add telomeres to the ends of chromosomes after they divide, thereby which is avoiding senescence (Cech, 2004).

Proliferating cells require both oxygen and nutrients to survive and grow. Cells do not normally have the ability to initiate and induce blood vessel formation, a process knows as angiogenesis; hence tumor cells have to develop this ability to survive. Activation of angiogenesis inducers, such as vascular endothelial growth factor (VEGF) has been found in many tumors (Ferrara, 2002). In addition to angiogenesis, cancer cells also develop the ability to move out and invade nearby tissues by metastasation. Numerous proteins have found to be involved in this

process such as E-cadherin and β-catenin which has been inactive in many types of tumors.

The Eukaryotic Cell Cycle

The Eukaryotic cell cycle is divided into four phases, G1 (gap 1), S (DNA Synthesis), G2 (gap 2) and M (mitosis), see Figure2. In G1 the cell prepares itself for DNA replication that occurs during S-phase. The G2-phase allows preparation for chromosome segregation and cell division during mitosis. In addition to these phases, the cells can also enter an inactivate phase (G0) during development, differentiation or growth factor depletion. To ensure that cells pass accurate copies of their genomes on to the next generation, evolution has overlaid the core cell-cycle machinery with a series of surveillance pathways termed cell-cycle checkpoints. These checkpoints of the cell cycle ensure faithful DNA replication and separation of the chromosomes at cell division thereby sustaining genetic stability. Any alteration or failure of these checkpoints to arrest the cells can result in cancer development (Malumbres and Barbacid, 2001) During G1, cells typically respond to proliferative or antiproliferative signals, which determine whether the cell should continue cell cycle progression or arrest at G1/G0-phase. Cell cycle progression is regulated by two classes of cell cycle regulatory proteins, the cyclins (Cyc) and the cyclin dependent-kinases (CDKs). The cyclins can be divided into G1 cyclins (CycD and E), S-phase cyclins (CycA), and mitotic cyclins (CycA and B). CycB and A bind and activate CDK1 (cdc2) during G2 and M-phase. The CycD family consisting of D1, D2 and D3 bind and activate CDK4 and CDK6, while the CycE family (CycE1 and E2) interact with and activate CDK2. Both CycD/CDK4 or 6 and CycE/CDK2 acts as positive regulators of G1 to S-phase transition through phosphorylation of the retinoblastoma tumor suppressor protein (pRb). The active unphosphorylated form of pRb arrest the cells at the restriction point in the late G1-phase of the cell cycle, at least in part through interaction with the E2F-family (including E2F1-3) of DNA binding transcription factors, (Harbour and Dean, 2000). The ability of pRb to arrest cells in G1-phase of the cell cycle is seems to be dependent upon the transcriptional repression of E2F target genes by the Rb/E2F complexes through requirement of histone deacetylases (Magnaghi-Jaulin et al., 2000). Progressive phosphorylation of pRb through G1-phase leads to release of E2F from pRb. This switches E2F from a repressor to an activator and allows activation of the transcription of E2F target genes, (Lavia and Jansen-Durr, 1999), involved in DNA replication.
On the other hand, a diverse set of negative cell cycle regulatory proteins including cyclin-dependent kinase inhibitors (CKIs), many of which act as tumor suppressors, have been identified. The CKIs consist of two families, the Cip/Kip family including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and the INK family including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. The Cip/Kip families negatively regulate the cell cycle through binding to an inactivation of CDK2 complexes. Formations of heterotrimeric CDK4 or 6-CycD-Cip/Kip complexes contribute to G1 progression by sequestration of these inhibitors, preventing them from binding and inactivating CycE/CDK2 complexes (Sherr and Roberts, 1999). Formation and activation of CycD/CDK4 or 6 complexes is however, potentiated by Cip1/Kip1, for review see (Swanton, 2004). The activity of CDK4/6 is negatively regulated by the INK4 family by preventing CycD binding, see Fig2. Many

antiproliferative signals such as IFNs and TGFβ upregulate members of the two CKI families resulting in inactivation of CDK4/6 by Ink4 families leading to disruption of CycD/CDK/Cip/Kip complexes and release Cip/Kip from the complex. Cip/Kip can in turn interact with and inactivate CycE/CDK2 complexes thereby causing cell cycle arrest in G1/G0. CycE/CDK2 once activated phosphorylates $p27^{Kip1}$ on Thr-187, after which it is recognized by SCF^{Skp2}, which induces its ubiquitin-mediated degradation by the proteasome. In addition to cyclins and CKIs, CDKs can also be regulated by modifications. The amount of the CDKs remains constant throughout the cell cycle but the activity can be regulated via phosphorylation or dephosphorylation. Phosphorylations on threonine 14 or tyrosine 15 by the wee1/mik1 protein kinases inactivate CDKs. The phosphatases Cdc25A-C activate CDKs by dephosphorylation of these residues. Furthermore, CDKs can also be activated by phosphorylation of a conserved threonine residue at position 160 by CDK-activating kinase (CAK). The steady state levels of both cyclins and Cip/Kip proteins are not only regulated by through gene expression but also by turnover via the ubiquitin-proteasome pathway, for review see (Hershko and Ciechanover, 1998).

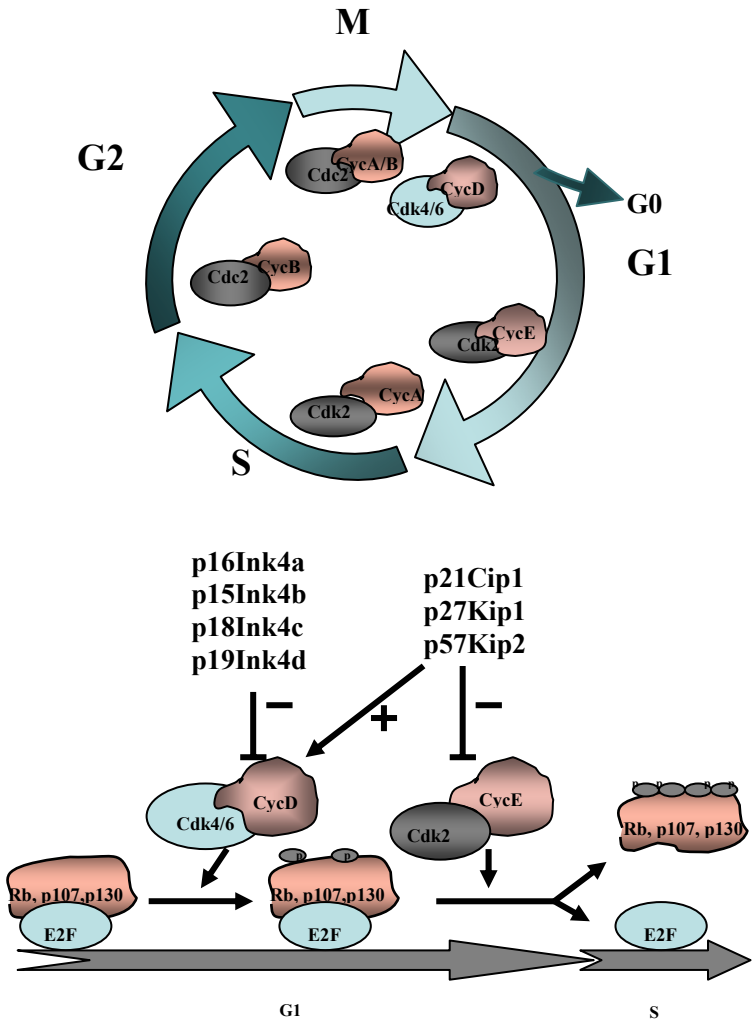


Figure 2: Cell cycle regulators
Schematic presentation of the mammalian cell cycle. *A,* mammalian cell cycle is divided into four phases, G1, S, G2, and M. Progression from one phase to the next is regulated by Cdks associated with their cyclin subunits. Inhibition of the cyclin-Cdk complexes is mediated by two families of cdk inhibitors, Ink4 proteins (p16Ink4a, p15Ink4b, p18Ink4c, and p19Ink4d) and Cip/Kip proteins (p21Cip1, p27Kip1, and p57Kip2). Whereas Ink4 proteins specifically bind to and inhibit cdk4/6, Cip/Kip family members can inhibit cyclin/Cdk complexes including cyclin E-Cdk2, cyclin A-cdk2, and cyclinB-cdk1 Cip/Kip proteins can also positively regulate cyclin D-Cdk4/6 by promoting their assembly. CycD-cdk4/6 and cyclin E-Cdk2 complexes phosphorylate (*P*) pRb, result in release from the transcription factor E2F. Free E2Fs (1, 2, or 3) bind to their DP partners and activate the transcription of genes necessary for S phase entry.

Transcriptional regulation

The regulation of gene expression of protein-encoding genes is a complicated multi step process that starts with initiation of the transcription by RNA polymerase II at specific DNA sequences called promoters. RNA pol II binding to DNA can not specifically start transcription, additional factors, so called general transcription factors (GTFs), interacting directly or indirectly with RNA pol II are required for this process, and include enzymes with the ability to open up the DNA doublehelix and to initiate and elongate transcription. The GTFs include TFIIA, TFIID, TFIIB, TFIIE, TFIIF and TFIIH (Burley and Roeder, 1996). TFIID is a multi complex of proteins including TBP (TATA-box Binding Protein) (Comai et al., 1992), which directs binding to TATA-boxes A TATA-box is a T/A-rich promoter sequence located 25-30 base pairs upstream the 5`end of a gene, which determines the transcriptional start site. In addition to TBP, TFIID contains other components known as TAFs (TBP associated factors) (Hernandez, 1993). The binding of TBP to the DNA, bents the DNA and creates a platform for the interaction with other transcription factors (Hoffman et al., 1990). TFIID binding to DNA is followed by TFIIA binding to the amino terminus of TBP, thereby protecting TFIID from inhibition by transcription repressors. In contrast, TFIIF interacts with carboxy-terminus of TBP, acting as a bridge between TBP and RNA pol II, in association with another factor TFIIB. This binding is essential step in pre-initiation complex formation since TFIIB in addition to TFIID can also bind to the RNA-pol II it self. TFIIB further recruits the TFIIH complex. Following TFIIB-mediated binding of RNA polymerase II to the promoter, TFIIE, IIH and IIJ associate rapidly to the pre-initiation complex. The TFIIH complex, which contains helicase and kinase activities, catalyzes the ATP-dependent melting of the promoter at the transcriptional start site. The DNA doublehelix opens and thereby allowing it to be transcribed into RNA, (Wu et al., 1998). The kinase activity of TFIIH consists of the cyclin H and CDK7 complexes which phosphorylates the carboxy-terminal domain (CTD) of RNA-pol-II. This phosphorylation stimulates promoter escape and elongation, probably by preventing the binding of negative elongation factors such as DSIF and NELF (Conaway et al., 2000). TFIIH activity is dependent on TFIIE binding to the pre-initiation complex. Both TFIIE and TFIIH are required for promoter escape and transcription elongation (Reese, 2003). The positive transcription elongation factor b (P-TEFb) complexes contain a common catalytic subunit (CDK9) and the unique regulatory cyclins Cyc T1, CycT2a and Cyc2b. P-TEFb has been suggested to phosphorylate the CTD of RNA pol II and allowing the elongation of transcription to proceed (Peng et al., 1998, Wei et al., 1998).

DNA binding transcription regulators

In order to activate or repress transcript for instance in response to cellular signaling, DNA binding transcription factors that specifically recognize promoter or enhancer elements within the DNA control regions of genes are required to recruit RNA polymerase II to the promoter. The DNA-binding transcription factors can act as activators or repressors of transcription (reviewed in Lemon and Tjian), and possess at least two essential domains: a DNA-binding domain (DBD)

and a transactivation domain (TAD). The negative regulating transcription factors possess a domain that is capable of repressing recruitment of Pol II or through binding to corepressors. The DNA binding transcription factors have been classified according to their structural motifs. One of these is the homoeodomain, which is a highly conserved domain of 60 amino acids found in a large family of transcription factors. The homeodomain itself forms a structure highly similar to the bacterial helix-turn-helix proteins. The DNA binding can further be mediated by the helix-loop-helix (HLH), domain which is involved in protein dimerization. The HLH motif is composed of two regions of a-helix separated by a region of variable length which forms a loop between the 2 alpha-helices. This class of transcription factor most often contains a region of basic amino acids located on the N-terminal side of the HLH domain (termed bHLH proteins) that is necessary in order for the protein to bind DNA at specific sequences. The HLH domain is necessary for homo- and heterodimerization. Examples of bHLH proteins include c-Myc. Another DNA-binding motif is the zinc finger domain consisting of specific sequences of cysteine and histidine residues that allow the protein to bind zinc atoms. The finger domains can penetrate into the major groove of the DNA helix. The spacing of the zinc finger domain in this class of transcription factor coincides with a half-turn of the double helix. The classic example is the RNA pol III transcription factor, TFIIIA. Proteins of the steroid/thyroid hormone family of transcription factors also contain zinc fingers. POU domain is a domain that is a hybrid between a domain related to the homeobox and conserved domain, the POU-specific domain (for review see Ryan and Rosenfeld). The leucine zipper is another domain essential for protein dimerization. These leucine residues end up with their R-groups protruding from the a-helical domain in which the leucine residues reside. The protruding R-groups are thought to interact with leucine R groups of another leucine zipper domain, thus stabilizing homo- or heterodimerization. The leucine zipper domain is present in many DNA-binding proteins, such as c-Myc, and C/EBP.

Coactivators and corepressors

The understanding of transcription factor function requires knowledge of the nature and effect of interaction between 2000-3000 different transcriptions regulators that animal cells make use of. The direct interactions between activators and the basal machinery is necessary for activation of transcription stimulation, and an additional class of factors, referred to as cofactors, adaptors and mediators are also considered to be fundamental to this process. Studying the role of these co-activators and co-repressors is essential to understanding transcriptional regulation in eukaryotes. Transcriptional cofactors can be classified into five classes according to the structure of their DNA binding domains. The TAFs of the initiation factor TFIID fits into the first class. The cofactors that is associated with activator or repressor molecules at the promoter fits into second class. Such as cellular factors OCA-B, Groucho, Notch, CtBP, HCF and viral coregulators E1A and VP16. summarized in (Lemon and Tjian, 2000).
The cofactor that is multisubunit coactivators are classified as the third class. One example is the yeast mediator (Thompson et al 1993., Kim et al., 1994). The yeast mediator was originally purified as an activity that was believed to facilitate the

stimulation of activator-dependent transcriptional activity in reconstituted transcription reactions. Additionally the TFIIH dependent phosphorylation of CTD was also found to be stimulated by the yeast mediator interacts with the CTD of PolII. The mediator consists of components such as a subset of SRB polypeptides and several previously uncharacterized proteins (Med1-Med7) (Thompson et al 1993., Kim et al., 1994, Koleske and Young, 1994, Myers et al., 1998). Furthermore, the yeast mediator was found to copurify with a proposed subcomplex containing the Srb8 Srb9, Srb10/Cdk8 and Srb11/cyclin C polypeptides. These polypeptides have been suggested to be involved in negative regulation of gene activity.

Metazoan mediator-like complexes have been subsequently identified: (i) CRSP and PC2 (Ryu and Tjian, 1999, Malik et al., 2000), (ii) ARC/DRIP/TRAP (Gu et al., 1999, Ito et al., 1999, Naar et al1999) and (iii) NAT/SMCC/Srb-mediator (Sun et al., 1998, Gu et al., 1999) Many components of mediator-like complexes have been found to interact with transcriptions factors as corepressors or coactivators, which implicates inhibition or enhance transcription. Class IV includes those cofactors that are covalently modified nucleosomes, see below. The cofactors that hydrolyze ATP in catalytic reactions to reorganize are classified as class V.

Chromatin and transcriptional regulation

Eukaryotic DNA in contrast to prokaryotic is packaged by association with histones to form a structure known as chromatin, (Latchman, 1996). The fundamental unit of this structure is the nucleosome in which the DNA is wrapped twice around an octamer of histone molecules (two of each histone H2A, H2B, H3 and H4). This structure is compacted further in genes which are transcriptionally not active. Transcriptional activation of these such genes requires modulation of chromatin structure by altering the position and conformation of nucleosomes thereby facilitating the transcription factors to bind to the promoter or enhancer region of the genes. During the last decade, at least two mechanisms behind the alteration of the chromatin structure have been recognized involving at least two different classes of enzymes that can be targeted to the promoters by interaction with transcription coactivators. The first class includes ATP-dependent chromatin remodeling complexes such as SWI/SNF, ISWI, RSC and Mi-2/NuRD complexes. These complexes change the chromatin structure by altering the nucleosome location without involving any covalent modifications. One example is the proteolytical member of chromatin-remodeling complexes, SWI/SNF that alters chromatin structure by changing the location or conformation of the nucleosome without covalent modification. Some types of activators have been shown to physically or functionally interact with SWI/SNF activities. Such as the C/EBPβ activator and the c-Myc proto-oncoprotein that binds directly to subunits of mammalian SWI/SNF complexes, reviewed in (Naar et al., 2001).

Histone modification

The second class of enzymes alters the chromatin structure through modification. The histones can be subjected to a number of post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitylation (Strahl and Allis,

2000, Wu and Grunstein, 2000). Acetylated forms of histones have been found preferentially in active genes where the chromatin is less tightly packed. This suggests that hyperacetylation of histones could play causal role in opening the chromatin structure, A number of enzymes with histone acetyltransferase (HAT). HAT activity modifying lysine residues at the histone tails have been found, among these GCN5, Esa1, MOF in yeast, and GCN5/PCAF, Tip60 and CBP/p300 in mammalian (Bannister and Kouzarides, 1996, Ogryzko et al., 1996, Cheung et al., 2000b). The connection between transcription activation and acetylation has been confirmed by demonstration that yeast GCN5 protein which is a positive regulator of transcription of many genes, has HAT activity, (Georgakopoulos and Thireos, 1992, Brownell et al., 1996). Mutation of the catalytic domain of GCN5, results in inactivation of the transcription, and abolish the acetylation of histones, (Kuo et al., 1998, Zhang et al., 1998). In addition to GCN5, many other coactivators such as TAFII250, SRC-1 ACTR have intrinsic HAT activity that is important for their transcriptional role (Schiltz and Nakatani, 2000, Sterner and Berger, 2000, Wu and Grunstein, 2000). HATs often function as part of multisubunit complexes such as 2 MDa SAGA and 1 MDa ADA complexes, (Grant et al., 1997). In addition, the SAGA complex contains several serine palmitoyltransferase (SPT) proteins which act as a bridge with TBP, the ATM/PI-3 kinase related protein Tra1, TBP associated factor TAFII250 and Ada proteins (Ogryzko et al., 1998).

There are also histone deacetylases (HDACs) that remove acetyl groups from lysines in N-terminal tails of histones. While hyperacetylation of histones have been correlated to a transcriptionally active state, hypoacetylation or deacetylation correlates with a transcriptionally repressed state. The connection between deacetylation and repression of the transcription was clarified by the isolation of human HDAC1 or in yeast Rpd3, (Struhl, 1998). Other members of deacetylase complexes were also identified such as the Rb associated proteins RbAp46 and p48 which required deacetylases and chromatin remodeling complexes to repress the transcription. Several transcription factors and nuclear receptors have also found to interact with complexes which contain HDAC activity such as NCoR/SMART and Sin3. The Myc antagonist, Mad proteins has found to interact with Sin3/ NCoR complexes to repress the expression of myc target genes and controlling the cell proliferation and differentiation. The mammalian transcription factor YY1 can also act as a repressor through its directly binding with histone deacetylase and NCoR/ Sin3 complexes, (Alland et al., 1997).

Additionally, histones can also be modified by phosphorylation. Phosphorylation of Ser-14 is correlated with cells undergoing apoptosis (Cheung et al., 2000a, Cheung et al., 2003). Methylation of histones is another type of modification and found linked to transcription activation. Studies have shown that H3 and H4 are frequently methylated at lysine residues by methyltransferase (Chen et al., 1999).

Interferon-γ and STAT/JAK pathway

Interferons (IFN) constitute a family of cytokines that includes three major classes called IFN-α, –β and-γ. IFN-α and β belong to type I, while IFN-γ is more distantly related and belongs to type II. Type II interferon has been widely studied and has been shown to carry out many biological functions, including antiviral and antiproliferative activities, expression of HLA I and II in transplantation antigens, activation of macrophages and regulation of apoptosis and cell differentiation (Boehm et al., 1997, Hu et al., 2002b). IFN-γ has in fact been considered as antitumor curative and has been used in treatment of certain human cancer, (Gleave et al., 1998). IFN-γ action is regulated in two different ways; control of IFN-γ production and modulation of IFN-γ signaling. The production if IFN-γ is regulated by T-cells receptor engagement in T-helper cells and by interleukin 12 (IL-12,) and 18 (IL-18) in natural killer cells. Upon binding of IFN-γ to its receptor, the receptor associated tyrosine kinases Janus kinase 1 and 2 (JAK1, JAK2) become activated through auto phosphorylation of a specific tyrosine residue (Y440). This leads to activation of STAT1, by phosphorylation of specific tyrosine residue (Tyr-701) located in the Src-Homolgy 2 domain (SH2). This is common structural motif of STAT proteins and other signaling molecules that has shown to mediate protein-protein interaction. N-terminal of the SH2 domain is the linker domain (LD) which is also highly conserved among other STAT families and shown to be important for transcriptional response to IFN-γ but not IFN-α. Transcriptional activation is mediated by C-terminal domain and may further regulated by phosphorylation of conserved serine residue present in STAT1, STAT3, STAT4, STAT5α and STAT5β (Stark et al., 1998, Decker and Kovarik, 2000). A coiled-coil domain in the N-terminus is required for protein-protein interaction, while the DNA binding domain is essential for binding to specific sites in the promoters of target genes STAT1 forms homodimer leading in translocation to the nucleus and binding to gamma activated sequences (GAS) IFN-γ target genes (Muller et al., 1993, Watling et al., 1993), see figure 3. Other studies have shown that STAT proteins may be activated through additional pathways including growth factor receptors and non-receptor with tyrosine kinases activity such as Src and Abl (Bowman et al., 2000). Other groups of proteins can negatively regulate cytokine signaling (Greenhalgh and Hilton, 2001). Among these are phosphatases such as SHP-1, which have shown to suppress cytokines receptor-mediated signals. The suppressor of cytokine signaling (SOCS) family can also inhibit cytokine signaling either through directly binding to JAK or through competitive binding to the activated receptors. In addition, the protein inhibitor of activated STATs (PIAS) family of proteins, suppress STAT signaling by directly interacting to STATs. In addition to the STAT-dependent pathway, IFN-γR can also signal through a STAT-independent pathway through activation of Ras/Raf pathway, also mediates biological responses, for review see (Stark et al., 1998).

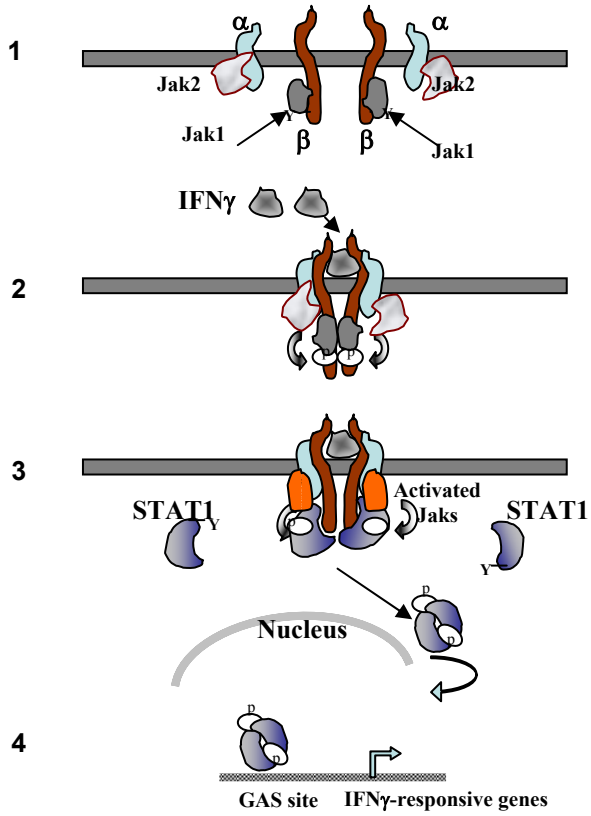


Figure 3: IFN-γ signaling: 1) Component of IFN-γ Receptor α and β chains in absence of IFN-γ. 2) Aggregation of receptors component, Jaks are activated through auto-phosphorylation activated Jak phosphorylate IFN-γ-Rα. 3) STAT1 binds to phosphorylated receptor and are then phosphorylated by Jaks. 4) Phosphorylated STAT1 form homodimer and translocate to nucleus, binds the DNA and activate transcription of target genes.

The ubiquitin-proteasome system

Eukaryotic cells use a variety of mechanisms to regulate gene expression at different level. Regulation at the posttranslational level can occur through various modifications such as phosphorylation, glycosylation, acetylation, sumolyation and ubiquitylation, etc thereby regulating protein activity or turnover. The ubiquitin-proteasome system provides a mechanism for regulating protein degradation. This involves two different and successive steps, attachment chain of 76 a a protein molecule ubiquitin to the substrate and secondly degradation of the ubiquitylated substrate by the 26S-proteasome. The ubiquitin conjugation process proceeds through three enzymatic steps. The C-terminal Gly residue of ubiquitin is first activated in an ATP-requiring step by a specific activating enzyme, E1. In this step consists of formation of an intermediate ubiquitin adenylate, followed by the binding of ubiquitin to Cys residues of E1 in a thiolester linkage, with AMP

release. In the second step the activated ubiquitin molecular is next transferred to an active site of Cys residue of an ubiquitin carrier/conjugating enzyme E2 which transfers ubiquitin molecules from E1 either to one of the E3 enzymes which are called ubiquitin-ligases, or directly to the substrate with or without help of an E3 ligase. The E3-ligases catalyze the conjugation process, resulting in covalent attachment of ubiquitin to a specific substrate (Hershko and Ciechanover, 1998, Weissman, 2001). Prior to the ubiquitylation process, the E3-ligase recognizes the substrate directly through specific recognition surfaces or indirectly through a secondary protein such as a molecular chaperone. In many cases, recognition by the E3 ligase requires posttranslational modification of the substrate. Ubiquitin-E3-ligases are generally divided into three classes based on the specific structure motif they have. HECT-based E3-ligases such as E6-AP, RING-finger-based E3-ligases including MDM2 and Cb1 and finally multi-protein subunit E3 ligase families. These are Cullin-based E3-ligases, including the anaphase promoting complex, the (von Hippel Landau tumor suppressor BC complex) VBC and the (**S**kp1-**C**ullin-**F**-box proteins) SCF complex. SCF represents a large family of modular complexes consisting of the Cul1, Rbx1, Skp1, and F-box protein subunits. The Cul1 and Rbx1 subunits form a catalytic core that recruits the upstream E2 enzyme, the variable F-box subunit binds the substrate, and Skp1 links the F-box subunit to Cul1. There are six cullin proteins and more than 50 F box proteins in the human genome (Ou et al., 2002) and this presumably allows the SCF to specify the ubiquitylation of numerous, diverse substrates (Deshaies, 1999, Jackson and Eldridge, 2002, Zheng et al., 2002). One of the most studied F-Box protein is Skp2 which has been shown to be interact with and promote degradation of p27, CycE, E2F, Myc, p130, and Cdk9 (Yu et al., 1998, Carrano et al., 1999, Tsvetkov et al., 1999, Carrano and Pagano, 2001, Yeh et al., 2001, Mendez et al., 2002, Kim et al., 2003, von der Lehr et al., 2003, Wei et al., 2004). Originally Skp2 was identified as S-phase kinase-associated protein 2 and plays an important role in cell cycle progression. Skp2 expression can be detected first near the G1/S transition and declines in G2. Overexpression of Skp2 in serum starved fibroblast has shown to induce $p27^{Kip1}$degradation and S-phase transition through induced expression and activation of CycA/CDK2, Cyc/Cdk2 (Mongay et al., 2001). Moreover, Skp2 has been suggested to be an oncogene since it's overexpressed in transformed cells (Gstaiger et al., 2001, Zhang and Wang, 2003).

Connection between ubiquitylation and transcription

Transcription activators are often short-lived proteins regulated by the ubiquitin-proteasome pathway (Molinari et al., 1999). Recent studies have suggested a connection between the functional activities of many transcription factors and their ubiquitylation/degradation. The domains mediating degradation termed "degrons" and transactivation domains (TAD) have often found to be overlapping and functionally equivalent (Molinari et al., 1999, Salghetti et al., 2001). Recent reports has demonstrated that TAD-dependent ubiquitylation of transcription factor are also required for their function in transcription activation and not only for degradation (Salghetti et al., 2001). More direct evidence demonstrated that strains deficient in the F-box protein Met30 in yeast failed not only to ubiquitylate and degrade VP16, but also to function as a transcription activator, indicating that VP16 ubiquitylation by Met30-E3 ligase is needed not only for destruction but

also for activation (Salghetti et al., 2001). Several studies have also suggested that proteins involved in ubiquitylation of transcription factors or histones are crucial parts of preinitiation complexes and of the RNA polII holoenzyme, further implying a role of ubiquitylation in regulation of the transcription (Pham and Sauer, 2000, Chi et al., 2001). Recent evidence suggests that ubiquitylation and proteasomal subunits can also have a non-proteolytic role in RNA PolII transcriptional initiation and elongation. The later has been proposed to act as "remodeling factors" as has been suggested for the APIS (AAA proteins independent of 20S) complex of AAA ATPases of the 19S regulatory subunit of the 26S proteasome, which have been shown to bound directly with promoters in yeast (Ferdous et al., 2001, Gonzalez et al., 2002), for review see (Conaway et al., 2002, Ottosen et al., 2002).

The oncoprotein c-Myc

The c-Myc protein is a transcription factor of the basic helix-loop-helix-leucin zipper (bHLH Zip) family that was discovered more than two decades ago. Originally c-Myc was identified as cellular homologue to the viral oncogene (v-Myc), which induces myeloid leukemia, sarcomas, liver, kidney and other type of tumors in chickens. (Gonda et al., 1982). It was also observed that c-Myc was activated by translocation in human Burkitt's lymphoma, murine plasmocytoma and rat immunocytoma (Spencer et al., 1990, Spencer et al., 1992).
The members of the Myc oncogene family involved in human cancer include c-Myc, N-Myc and L-Myc, have all been shown to promote proliferation, growth, apoptosis and to inhibit terminal differentiation (Grandori et al., 2000). Several studies have also suggested that c-Myc plays an important role in embryonic growth and development. (Dang et al., 1999, Levens, 2002). c-Myc is expressed at very low level in quiescent G0 cells but rapidly induced in response to various mitogenic signals or stimuli, and expressed continually in cycling cells (Oster et al., 2002).
The molecular function of Myc was still unclear until 1989 when Murre et al demonstrated that Myc belongs to the bHLHZip family of transcription factors and Blackwell et al the year after identified the E-box (CACGTG) as a six base pair core of DNA binding motif of Myc (Murre et al., 1989, Blackwell et al., 1990). The identification of Myc binding site was quickly followed by the landmark discovery of the Myc bHLHZip partner protein Max and its requirement for Myc DNA binding activity in vitro and in vivo. These findings established the function of c-Myc as a transcription regulator (Blackwood and Eisenman, 1991, Blackwood et al., 1992, Prendergast and Ziff, 1992).

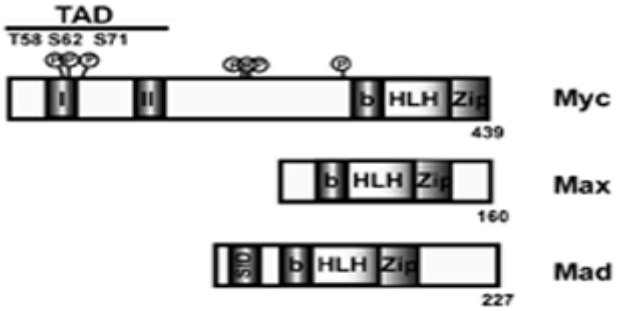


Figure 4: Schematic illustration of c-Myc, Max and Mad Structure.
Transactivation domain, TAD. MBI and MBII conserved homology regions. Nuclear localization signal, NLS. Basic-helix-loop-helix/leucine zipper region, bHLHZip. Sin3 interaction domain SID.

Myc structure and Myc/Max/ Mad network

The Myc family genes are conserved in organisms from sea urchin and Drosophila to humans. The vertebrate c-myc genes consist of three exons including noncoding exon 1 which functions as a target site for several transcription factors, whereas exon 2 and 3 contain the coding region for the major Myc proteins Two promoters located at the 5`- end of exon 1 account for 90-95% of the transcripts and give rise to mRNAs of about 2,2 and 2,4 kb. From these mRNAs c-Myc proteins of about 64 and 67 kDa are translated. Several functional domains of the resulting Myc proteins have been mapped to the amino terminal, central and carboxy-terminal region of the protein (Watson et al., 1983, DePinho et al., 1987, Katoh et al., 1988, Sawai et al., 1990).
The N-terminus of c-Myc (1-143) harbours the transactivation domain (TAD) which is involved in Myc-regulated transcription, and contains two highly conserved regions called as Myc box 1 and 2 (MBI and MBII). Different studies have suggested that MBII is essential for all biological functions of Myc. Significantly both MBI and MBII are required for cell transformation. (Chang et al., 2000, Grandori et al., 2000).
The C-terminal domain of Myc contains the basic region/helix-loop-helix/leucine zipper (bHLHZip) motif, which is required for all known biological functions of the Myc protein. The bHLHZip domain mediates dimerization with Max, and binding to the E-box, DNA recognition sequence (See figure 4).
The central region contains a nuclear localization signal (NLS) which has been suggested to be important for import into the nucleus and a central acidic domain which includes several phosphorylation sites with unclear function. (Luscher et al., 1989, Hagiwara et al., 1992).
In addition to Myc, Max can also form dimers with the Mad family of bHLHZip proteins, including Mad1, Mxi1 (Mad2), Mad3, Mad4, and with Mnt which all are Myc antagonists and mediate transcription silencing or repression by binding to similar if not identical E-boxes as Myc:Max. Indeed, Myc and Mad/Mnt proteins compete for Max binding within the Myc-Max-Mad network. In contrast to c-

Myc, the expression levels of several Mad genes increase during differentiation, (Ayer et al., 1993, Amati and Land, 1994, Foley and Eisenman, 1999). In addition to Mad and Mnt proteins, Max can also interact with Mga (Billin et al., 1999, Hurlin et al., 1999, Sommer et al., 1999, Meroni et al., 2000).
The Mad proteins and Mnt contain a Sin3-interacting domain (SID) motif, which recruits the adaptor protein, Sin3 which is part of transcriptional complexes containing HDAC1/2 (histone deacetylases), (Ayer et al., 1995, Alland et al., 1997, Heinzel et al., 1997). In contrast to Mad, Myc activates transcription through interaction with the coactivator Transformation-transactivation domain-Associated Protein (TRRAP) through the TAD of Myc, involving MBII. TRRAP associates with several histone acetyltransferase complexes containing the HATs GCN5, PCAF or Tip60 (McMahon et al., 2000, Fuchs et al., 2001, Flinn et al., 2002, Fernandez et al., 2003), and has therefore been suggested to act as an adaptor recruiting different HAT activities to Myc target promoters during transcriptional activation (Liu et al., 2003). The ATPase domain-containing proteins, TIP48 and TIP49 have also been found to interact with the TAD of Myc and the ATPase activity of Tip49 has been shown to be important for c-Myc oncogenic activity. TIP48 and TIP49 have also shown to interact with BAF53 which is one component of the GCN5-containing so called STAGA complex. In addition, GCN5, TIP48, TIP49, and BAF53 proteins have shown to play essential roles in Myc-dependent cell transformation. And all are part of a variety of both shared and distinct multiprotein complexes involved in regulation of chromatin structure via either ATP-dependent nucleosome remodeling (*e.g.* BAF53-containing SWI/SNF-like complexes) or histone acetylation. (McMahon et al., 1998, Park et al., 2001, Nikiforov et al., 2002, Frank et al., 2003).
In addition to activation, c-Myc is also able to repress the transcription. The HLH-domain of c-Myc can interact with Miz1 (Myc-interacting Zinc finger protein1) (Adhikary et al., 2003, Wu et al., 2003), and recent evidence suggest that this interaction plays an important role in c-Myc-mediated transcriptional repression of promoters of several of cell cycle related genes, such as $p15^{Ink4\alpha}$, $p21^{Cip1}$ and $p27^{Kip1}$. Miz1 was found to upregulate the expression of these genes by binding to initiator (Inr) like elements at their promoters. However Myc can be antagonized by this process forming complexes with Miz1, and thereby inhibits Miz1-mediated transcriptional activation of these genes (Staller et al., 2001, Adhikary et al., 2003, Kime and Wright, 2003, Wanzel et al., 2003, Wu et al., 2003).

Biological activities of Myc

Several studies in the last two decades have shown that Myc is a multifunctional protein that plays a critical role in regulation of numerous cellular processes including proliferation, differentiation, apoptosis and tumorigenesis. Myc is able to control all these biological activities through turning on and off the transcription of Myc target genes. Therefore, the target genes link Myc to its biological activities. In the following section the evidence for Myc's role in both normal and abnormal cellular activities is described see Figure 5. Several hundred of Myc target genes have been identified during last years, genes that are involved in almost all biological activities, such as cell cycle, transformation,

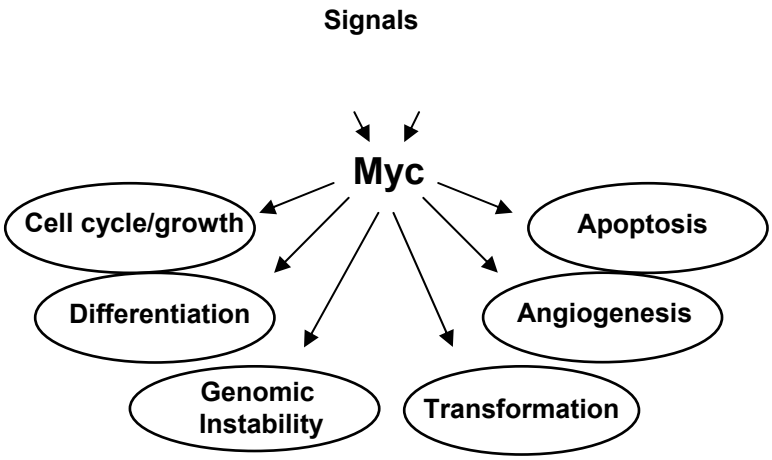


Figure 5: Myc biological activities. In response to extra cellular signals Myc can regulate different target genes in different process

-differentiation, metabolism, apoptosis and DNA-repair (James and Eisenman, 2002, Fernandez et al., 2003).

Myc and cell cycle/ growth

The Myc family of transcription factors has been implicated in cell growth and cell cycle control and Myc:Max DNA binding sites are present in the promoters of several growth and cell cycle regulatory genes. Numerous of studies have shown an essential role of Myc in the transition from G0/G1 to the G1/S phase of the cell cycle, expression of the myc gene is rapidly induced. Upon mitogenic stimulation such as through plated-derived growth factor (PDGF). Activation of Myc expression through mitogenic signaling or through oncogenic events, leads to direct or indirect activation or repression of several genes involved in the cell cycle regulation. Myc activation in quiescent fibroblasts has shown to increase the activation of CycD/CDK4/6 and CycE/CDK2 while inactivation of Myc can results in repression of CDK4/6 kinase activity (Mateyak et al., 1999). The activation of these CDK complexes by Myc can occur through different mechanisms. The phosphatase cdc25A, which activates both CDK2 and CDK4 by removing inhibitory phosphorylations, (see above) is target gene activated by c-Myc (Galaktionov et al., 1996, Amati et al., 1998). Myc also directly activates the CycD1 and CDK4 genes through E-boxes in their promoter regions. The increased expression of CycD1 leads to sequestration of the CDK inhibitor $p27^{Kip1}$into CycD/CDK4/6 complexes, resulting in formation of active CycE/CDK2 through $p27^{Kip1}$ release (Coller et al., 2000, Hermeking et al., 2000, Luscher, 2001). Further, phosphorylation of $p27^{KIP1}$ by CycE/CDK2 targets $p27^{KIP1}$ for degradation through ubiquitin-proteasome pathway by interaction with ubiquitin-ligase complex SCF-Skp2, thereby generate a positive autoregulation loop which further

activate CycE/CDK2. It was recently shown that Cul1, which is an important component of SCF-Skp2, is another downstream target gene for Myc, (O'Hagan et al., 2000).
Inhibition of $p27^{KIP1}$ does not appear to be the only rate limiting cell cycle target of Myc since $p21^{CIP1}$ / $p27^{KIP1}$-deficient fibroblasts are still sensitive to Myc with respect to CycE/CDK2 activation. In contrast, deletion of the pocket proteins p107, p130 and Rb in mice are insensitive to c-Myc, indicating that activation of CycE/CDK2 by Myc, is directly dependent on Rb family members, (Berns et al., 2000, Martins and Berns, 2002). As mentioned above pRb and other pocket proteins are targets for both CycD/CDK4 and CycE/CDK2 complexes, which are direct and indirectly activated by Myc.
This fits with observations suggesting that Myc overexpression induces the activity of the E2F/DP family of the transcription factors since phosphorylation of Rb by CycD/CDK4/6 results in activation of E2F/DP by releasing Rb from the complex (Jansen-Durr et al., 1993, Leone et al., 1997). This allows activation of E2F target genes involving DNA replication as described above. In addition, the E2F family genes (including E2F1, 2 and 3) have also all been shown to be Myc target genes (Leone et al., 1997). c-Myc may also have a role in other phase of the cell cycle than G1 as it has been shown that c-Myc null rat fibroblasts have prolonged both G1 and G2 phases of the cell cycle, (Mateyak et al., 1997). In drosophila, loss of the c-myc homolog dMyc leads to retarded growth and reduced cell size, and overexpression of dMyc resulted in larger cells without a significant changes in cell cycle although the G1-phase become shorter (Johnston and Gallant, 2002). This suggests that c-Myc has a role in the "growth" aspect of cell proliferation, which fits with observations that numerous Myc target genes are involved in macromolecular synthesis and metabolism.

Myc inhibits cell differentiation

In addition to its stimulation of cell proliferation Myc appears to actively repress differentiation and cell cycle arrest programs. Many studies have shown a significant role of the Myc:Mad:Max network in regulating the switch between cell growth and differentiation, (Foley and Eisenman, 1999, Grandori et al., 2000). mad and myc genes are inversely expressed during differentiation, when many mad genes are upregulated in response to differentiation signals whereas myc family genes are downregulated. Indeed down regulation of Myc is one of the earliest events in response to negative growth regulatory signals and is important for cells to exit cell cycle and initiate differentiation processes.
(Chin et al., 1996, Luscher and Larsson, 1999). The differentiation process is usually accompanied by cell arrest, and one possibility is that Mad promotes terminal differentiation through transcriptional repression of genes favoring cell cycle progression while Myc inhibits differentiation by maintaining the expression of the same or similar sites of genes. Foe instance, during myeloid differentiation of HL-60 cells, the downregulated expression of cycD2 and hTERT Myc target genes was proceeded was proceed by a switch of promoter occupancy from Myc to Mad as demonstrated by CHIP analysis (Bouchard et al., 2001, Xu et al., 2001). It is also probable that suppression of specific genes that critically regulate growth arrest and differentiation by Myc, such as $p21^{Cip1}$, $p27^{Kip1}$ (Rama et al., 2003) and Gadd45, (Amundson et al., 1998), and Gas1, (Lee et al., 1997), ensure that cells

are unable to exit the cell cycle to enter a differentiation pathway (Claassen and Hann, 1999). In support of this hypothesis, enforced of the transcription factor C/EBP-α which activates both the $p21^{Cip1}$ and Gadd45 genes, can overcome the inhibition of differentiation by Myc (Freytag, 1988).

Myc and apoptosis

Apoptosis is an important safeguard that protects the organism from cancer by eliminating tumor cells. Studies during the last decade have shown that many oncoproteins that stimulates cell proliferation such as c-Myc and E1A can also induce apoptosis. Early studies have shown that deregulation of c-Myc expression induced apoptosis upon serum deprivation (Evan et al., 1992) or by interleukin-3 deprivation (Askew et al., 1991). Myc has also been shown to sensitize cells to pro-apoptotic stimuli through receptors such as CD95, (Hueber et al., 1997), TNF, (Klefstrom et al., 1994), and TRAIL, (Lutz et al., 2002). Myc expression has also shown to induce the apoptosis through directly activation of p53 by stimulating expression of ARF, which act as potent tumor suppressors by targeting pRb and p53 function through stabilization of p53 by MDM2 inhibition (Zindy et al., 1998). The mechanism by which Myc induces p53-independent apoptos has not been fully clarified. Myc target genes such as ODC, lactat dehydrogenase A (LDH-A) and cdc25A (Hoffman and Liebermann, 1998, Haggerty et al., 2003, Nilsson et al., 2004) has been suggested to play a role in apoptosis. A role in Myc in activation of the pro-apoptotic factor Bax has been shown by analysis of c-Myc null Rat-1 fibroblast cells, were Bax is not activated and cytochrome C is not released by apoptotic stimuli, (Soucie et al., 2001). In addition, Myc has also shown to repress the anti-apoptotic genes such as Bcl2 and Bcl-x, which reside in the outer mitochondria membrane and suppress apoptosis by blocking the membrane permeabilization (MOMP) which is necessary for cytochrome C release (Martinou and Green, 2001). Stimulation of apoptosis after serum starvation or by deregulated Myc expression has also been suggested to occur through induced accumulation of reactive oxygen species (ROS) through inhibition of NF-κB activity by E2F binding, (Tanaka et al., 2002, Vafa et al., 2002).
The phosphorylation sites in TAD of Myc have also been suggested to contribute to Myc-induced apoptosis. Mutation of Ser-71 has reported to result in reduced levels of cytochrome C release from mitochondria, which is one of the characterizes of apoptosis stimulated by Myc, further the antiapoptotic survival factor insulin-like growth factor 1 was reported to suppress phosphorylation of Thr-58, suggesting that the TAD is a direct target of survival signals (Chang et al., 2000). The exact function of these phosphorylation sites in apoptosis however remains unclear.

Myc participation in immortalization, genetic instability and angiogenesis

Myc has also been implicated in the process of cell immortalization. The process of immortality and apoptosis/survival seem to be interconnected, based on results showing that overexpression Myc can promote immortality provided that the cells are rescued from cell death, through loss of p53 or Arf (Eischen et al., 1999). In human cells immortality requires upregulation and constitutive activation of telomerase (hTERT), has been shown to be a Myc target gene in variety of human cell types (Wu et al., 1999, Xu et al., 2001). Like for many, but apparently not all

Myc target genes, Myc induction of TERT is dependent on Myc interaction with TRRAP (Nikiforov et al., 2002).
As mentioned above, cancer cells are characterized by chromosomal aberration, like gene amplifications and translocations, a phenomenon that is referred to as genomic instability. Abnormal Myc expression has been suggested to promote the induction of genomic instability (Felsher and Bishop, 1999, Vafa et al., 2002) by allowing cells to proliferate continuously despite DNA damage or mutations. Replication of damaged DNA correlates with loss of p53 activity, which regulates cell death in response to DNA damage (Yin et al., 1999, Pelengaris et al., 2002). In order for tumors to survive and grow, cancer cells must be able to stimulate the outgrowth of blood vessels around the tumor to provide the tumor cells with oxygen and nutrients. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are two factors involved in stimulation of angiogenesis (Yancopoulos et al., 2000). Myc has been shown to positively regulate angiogenesis through induction of VEGF (Brandvold et al., 2000, Breit et al., 2000, Pelengaris et al., 2002). Myc can also repress the expression of the angiogenesis inhibitor thrombospondin-1, further suggesting that Myc act as a positive and negative regulator of genes involved in angiogenesis (Janz et al., 2000, Ngo et al., 2000).

Posttranslational regulation of Myc

The expression of the c-myc gene is tightly controlled by growth and differentiation signals, but relatively little is known about and posttranslational regulation of Myc.
Myc is a phospho-protein and can be phosphorylated at numerous serine and threonine sites, including Thr-58 and Ser-62 in MB I, Ser-71 and Ser-82 between MBI and MBII and Ser164 upstream MBII in the TAD. The central acid region of c-Myc also contains a cluster of five in vivo phosphorylation sites within residues 240-262. Another group of in vivo phosphorylation sites residue immediately upstream of the DNA binding basic region at Ser-293 and Thr-343, Ser-344, Ser347 and Ser-348 in BR. Ser-373, Thr-358 and Thr-400 in HLH domain (Luscher et al., 1989, Henriksson et al., 1993, Lutterbach and Hann, 1994, Lutterbach and Hann, 1997, Huang et al., 2004). However the role of most of these modifications remains unclear. Mutation at Thr-58 or other mutations in the vicinity which abolish phosphorylation at this site has found in more than 50% of BL. Glycogen synthase kinase 3 (GSK-3) has been reported to phosphorylate Thr-58 in vitro and in vivo and is dependent on prior phosphorylation of Ser-62 residue (Henriksson et al., 1993, Lutterbach and Hann, 1994, Pulverer et al., 1994, Gregory et al., 2003). In addition, mutation at Thr-58 has shown to enhance c-Myc activity in cellular transformation assay while mutation at Ser-62 severely inhibited transformation (Henriksson et al., 1993, Pulverer et al., 1994, Chang et al., 2000). Other studies have suggested that mitogen-activated protein kinase (MAPK) and unidentified CDK could be involved in the phosphorylation of Ser-62(Lutterbach and Hann, 1999). Noguchi et al reported that c-Jun N-terminal kinase (JNK) could phosphorylate Ser-62 and Ser-71, (Noguchi et al., 1999). Other studies have suggested that Ser261 and Ser263 in the central acid region phosphorylates by CKII (Luscher et al., 1989). Recently, it was reported that phosphorylation of Myc at Thr-358, Ser-373 and Thr-400 by Pak2 in response to

stress related signals reduces Myc DNA binding, and phosphorylation of Ser-373 and Thr-400 blocks Myc: Max dimerization. (Huang et al., 2004). Myc phosphorylation has also been suggested to play a role in apoptosis (Noguchi et al., 1999, Chang et al., 2000, Noguchi et al., 2001). (Noguchi et al) showed that activation of c-Jun N-terminal kinase (JNK) by apoptotic stimuli led to phosphorylation Ser-62 and Ser-71 of Myc while mutations of these residues made Myc unable to stimulate apoptosis (Noguchi et al., 1999). The authors suggested that phosphorylation of Ser-71 was somehow involved in cytochrome C release which stimulates apoptosis through caspase activation (Chang et al., 2000).

Myc has a very short-life of 25-30 minutes. We and other have shown that destruction of Myc is mediated by ubiquitin-proteasome pathway, and the mutation of Thr-58 and other hotspot mutations in BL lead to Myc stabilization. This suggest that phosphorylation of Thr-58 is a signal that directs Myc for proteasomal degradation further (Sears et al) have suggested that phosphorylation at Ser 62 is involved in Myc turnover by stabilizing c-Myc, in response to Ras signaling. In addition to phosphorylation, Myc has also shown to be alternatively modified at Thr-58 by O-GlcNAC glycosylation, suggesting additional modification of Myc. Glycosylation of Thr-58 has been suggested to be involved in stability and subcellular localization of Myc (Kamemura et al., 2002). In addition to above mentioned modifications the HAT CBP was reported to acetylate c-Myc in vitro and coexpression of Myc with CBP stimulated acetylation of Myc in vivo. This modification was shown to decrease ubiquitylation and stabilization of Myc (Vervoorts et al., 2003). Taken together it seems that multiple mechanisms exist to regulate Myc protein activity and stability.

Aims of the present investigation

The general objective of this thesis was to gain further insight into post-translational regulation of Myc and thereby make a contribution to the development of better treatment strategies for tumor with deregulated Myc. The thesis can be divided into two parts. The aims of the first part including papers I and II, was to increase the knowledge of Myc degradation pathways and to identify components specifically participating in Myc turnover. The aim of the second part containing paper III and IV was to clarify the mechanisms by which IFN-γ regulates Myc function posttranslationally.

The specific aims of this work were:

1-To clarify whether Myc "hotspot" mutation in BL play a role in Myc turnover.

2- To identify specific SCF-E3 ubiquitin ligase complexes involved in the ubiquitylation of Myc and to clarify their role in Myc function.

3- To elucidate whether the previously describe IFN-γ-induced anti-Myc activity directly affects on Myc function (with response to Myc-induces transcription, DNA binding etc) as a transcription factors.

4- To clarify whether IFN-γ induces ubiquitin/proteasomal degradation of Myc.

Results and discussion

c-Myc hot spot mutations in lymphomas result in inefficient ubiquitylation and decreased proteasome-mediated turnover (paper I)

The objective of this work was to investigate the consequence of the frequently found hotspot mutations in c-Myc in Burkitt's lymphoma. In addition to the obligatory translocation between c-Myc and either of the Ig-loci in Burkitt's lymphoma, mutations in the coding region of Myc in particular of Thr-58 and Pro-57 was frequently found in both in primary tumors and in cell lines derived from lymphomas Burkitt's (Bhatia et al., 1993, Yano et al., 1993, Axelson et al., 1995). Furthermore the Thr-58 be mutation occurs in all v-Myc proteins, and has been shown to increase the transforming potential of Myc. Thr-58 is an in vivo phosphorylation site and has been reported to be phosphorylated in vitro and in vivo by Glycogen synthase kinase 3 (GSK-3), while proline at position 57 has been shown to be a prerequisite for Thr-58 phosphorylation by GSK3 (Bousset et al., 1993, Pulverer et al., 1994, Lutterbach, 1994 #47, Gregory, 2003 #72).

Decreased turnover of c-Myc mutated at Thr58 or Pro57 in Burkitt's lymphoma cells

The turnover of many transcription factors and other regulatory proteins is often controlled by phosphorylation or other modifications. We therefore hypothesized that the Thr-58 and Pro-57 hotspot mutations might play a role in Myc turnover. We first investigated the turnover of c-Myc in the Burkitt's lymphoma (BL) cell lines Daudi, Jijoye and BJAB (with wild type [wt] c-myc), Raji, and CA46 (with c-myc mutations, including Thr58 and Pro57), Mutu and Rael, both with a single mutation of Thr58 and finally ST486 with mutation in Pro57. Cycloheximide chase analysis showed that Myc half-life was fourfold longer in Burkitt's lymphoma containing Myc with mutations in Thr-58 or Pro-57 compared to wildtype Myc. Simultaneous measuring of the half-life of endogenous c-and exogenous v-Myc in v-Myc transformed monocytic U937 cells, showed that c-Myc turned over rapidly (around 30 minutes), while v-Myc which carries a Thr-58 mutation turned over at a much slower rate (half-life approximately 120 minutes) in the same cells. Taken together these results demonstrated that Thr-58 and Pro-57 mutations led to Myc stabilization.

The role of Thr-58 in Myc ubiquitin-proteasome mediated degradation

Previous work had implicated the ubiquitin-mediated proteolytic system in the turnover of short-lived transcription factors and cell cycle regulatory proteins (Weissman, 2001). Ubiquitylation of c- and N-Myc in vitro was reported previously (Ciechanover et al., 1991) but this had not been demonstrated in vivo. We therefore investigated the effects of proteasome inhibitors on c-Myc stability

in BL and in v-Myc-transformed U937 cells. We observed that c-Myc was noticeably stabilized and v-Myc was somewhat further stabilized by several of proteasome inhibitors but not by other protease inhibitors. The observation that Myc is degraded by the ubiquitin-proteasome pathway prompted us to investigate whether Thr-58 and Pro-57 might be involved in this process. A pulse chase experiment revealed that the proteasome inhibitor MG132 stabilized wt c-Myc in Daudi significantly but had less effect on the already stabilized Myc in CA46 cells. Together these results suggested that Thr-58 and Pro-57 mutations led to impaired proteasome-mediated turnover.

Proteasome-mediated degradation is dependent on ubiquitylation of the target protein, (Hershko and Ciechanover, 1998). We therefore next examined whether Thr-58 mutation could interfere with Myc ubiquitylation, using in vivo ubiquitylation assays. U2OS cells were cotransfected with wild type or T58A c-Myc together with an expression vector containing His-tagged ubiquitin. Western blot analysis showed that cotransfection of wt-c-Myc together with His-Ub generated a strong smear of ubiquitylated Myc. The T58A Myc mutant was also ubiquitylated but to a reduced extent in comparison with wt Myc. These observations thus indicate that Myc ubiquitylation is regulated by Thr-58 phosphorylation. However, ubiquitylation did not seem to be entirely dependent on Thr58 since the T58A mutant was still ubiquitylated. In conclusion the phosphorylation of Thr-58 seems to have an important role in regulation of c-Myc ubiquitin-proteasome mediated degradation.

Our observations within this work is in part in agreement with the reports by (Salghetti et al., 1999, Gregory and Hann, 2000, Sears and Nevins, 2002), which reports demonstrated that Thr-58 mutation led to c-Myc stabilization. Despite this, Gregory et al argued that c-Myc stabilization in BL might was due to some disorder of the ubiquitin-proteasome machinery in these cells rather than the c-Myc mutation, since wt c-Myc transfected into CA46 BL cells was also stabilized. This conclusion disagrees with our results since we have demonstrated that transfected wt c-Myc turned over rapidly while endogenous Myc with Thr-58 mutation was stabilized in the same Raji BL cells. The reason for this discrepancy is unclear, but might indicate that additional oncogenic events affecting the ubiquitin-proteasome pathway contributes to further Myc stabilization in some BL but not in others. We find it unlikely that this is a general explanation for the stabilization of c-Myc in BL, since independent reports from at least three groups found that mutation in Thr-58 stabilize Myc also in other cells than BL. We have also found that too high overexpression of wt c-Myc often results in stabilization, and consequently the amount of transfected Myc must be carefully titrated.

Our results suggest that phosphorylation of Thr-58 trigger ubiquitin-mediated degradation. We hypothesize that such phosphorylation site could be recognized by E3-ligase involved in Myc degradation. However, we still don't have evidence demonstrating that Thr-58 is directly involved ubiquitylation and degradation of Myc. An alternative hypothesis is that phosphorylation of Thr-58 is involved in subcellular localization of Myc which in turn affects its ubiquitylation and degradation. GSK3 which phosphorylates Myc-Thr-58 (Henriksson et al., 1993, Lutterbach and Hann, 1994, Pulverer et al., 1994, Gregory et al., 2003) has been shown to regulate both subcellular localization and degradation of CycD1 via Thr-286 phosphorylation. CycD1 mutated at Thr-286 remained in the nucleus and was

stabilized (Diehl et al., 1998). It is possibly that GSK3 could regulate c-Myc through a similar mechanism.
In agreement with our observation, Salghetti et al showed that Thr-58 mutation and deletion of the first 128 residues in N-terminal stabilized Myc. Further, (Flinn et al) (Flinn et al., 1998) showed that deletion of MBI increased the half-life of c-Myc in agreement with our observation (paper II) and (data not shown). In contrast, Gregory et al (Gregory and Hann, 2000) showed that c-MycS, which lack the N-terminal 100 amino acids of c-Myc, is rapidly degraded and was highly ubiquitylated. This result is contradictory to results from Flinn, Salghetti and our work because c-MycS have also deleted MBI. Contradictory like this are discussed further below.
In addition to MBI, several groups has shown that deletion of MBII (Flinn et al., 1998, Salghetti et al., 1999, von der Lehr et al., 2003) or deletion of the first 158 aa N-terminal sequences, (Gregory and Hann, 2000) stabilized Myc (see discussion below).
In addition to MBI and MBII. (Gregory et al)(Gregory and Hann, 2000) suggested that the centrally located region, amino acids 226-270, was necessary for rapid c-Myc degradation, but not for ubiquitylation. Another report suggested that the region 127-189 is essential for TNF-induced Myc stabilization (Alarcon-Vargas et al., 2002). Salghetti et al has also suggested that deletion in Miz1 binding region (within HLH-motif) in the C-terminus of c-Myc reduced Myc stability, while overexpression of Miz1 stabilized c-Myc (Salghetti et al., 1999). Herbst et al has recently identified a second element (D-element) which is centrally located in c-Myc. Deletion of the D-element stabilized Myc without affecting its ubiquitylation (Herbst et al., 2004). Taken together all these observations suggested that several different regions of Myc could be independently involved in Myc stabilization/degradation.
The discrepancies of the different studies might be due several factors. One explanation is that the c-Myc mutants used were not identical. Further, deletion of certain regions can also have structural consequences which potentially could affect c-Myc stability.
If several different regions of c-Myc is involved in regulating c-Myc stability, they could be of different importance in different tissue. Different cell lines used in the different studies can therefore lead to different conclusions.

SCF-Skp2 is involved in Myc ubiquitin- proteasome mediated degradation and transcriptional activation (paper II)

The results form paper I showed that Myc oncoprotein like many other transcription factors was degraded through ubiquitin-proteasome pathway. The ubiquitylations process requires three activities; ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and finally the ubiquitin ligase E3, (Hershko and Ciechanover, 1998) as described above. Since we and others had observed that this degradation pathway seemed to be phosphorylation dependent and an increasing amount of regulatory factors were reported to bind to SCF-complexes prior to their degradation we aimed for identifying a specific E3 ligase, possibly of SCF nature, for c-Myc.

c-Myc binds to the F-box-protein Skp2 in vivo and in vitro

To establish whether c-Myc does interact with a SCF-complex we initiated our work by investigating if c-Myc can interact with Cul1, which is one of the common components of SCF complexes. Cotransfection of c-Myc and Cul1 into Cos7 cells followed by immunoprecipitation and western blot analysis demonstrated that c-Myc interact with Cul1 in vivo, indicating that Myc can interact with SCF-complexes, possibly through endogenous bridging F-box proteins. Accordingly, we tested several known F-box proteins belonging to different classes to clarify whether any of them could interact with c-Myc. We found that Skp2 strongly and another member of the LRR-family, Fb15 weakly interacted with c-Myc in vivo after cotransfection while other F-box proteins did not. Further, we found that overexpression of Skp2 strongly promoted c-Myc degradation in contrast to other F-box proteins showed only minor effects. Therefore we concentrated our work on Skp2, and could demonstrate an interaction between Myc and endogenous Skp2 in HeLa cell extracts and also in vitro by GST-pulldown experiments. In order to identify the c-Myc regions that interact with Skp2, we constructed a panel of deletion mutants which used together with Skp2 cotransfection. We found that both MBII and the bHLHZip domain were essential for Skp2 interaction. These observations were also confirmed by in vitro GST-pulldown assays. In addition we also utilized the bimolecular fluorescence complementation assay (BiFC) as described by (Hu et al., 2002a) to study interactions between Myc and Skp2 in living cells. This technique is based on reconstitution of yellow fluorescent protein (YFP) from two non-fluorescent fragments when brought together by the association between two interaction partners fused to the fragments. The N-terminal (YN) and C-terminal (YC) fragments of YFP were fused to Skp2 and c-Myc, respectively, and expression vectors containing these fusion proteins were transfected into Cos7 cells either alone or together. Through this method we found that Skp2 and c-Myc interacted in the nucleus. In conclusion we showed that c-Myc interacts directly with Skp2 in vivo and in vitro through the MBII and the bHLHZip domains and that this interaction occurs in nucleus.
In agreement to our work, Kim et al has identified the same region of Myc that involved in Skp2 binding

The role of SCF-Skp2 in c-Myc ubiquitylation and degradation

To clarify whether Skp2 has any role in c-Myc degradation we attempted several approaches. We could first demonstrate that Skp2 promoted c-Myc degradation when cotransfected with Myc and Cul1. To investigate whether c-Myc degradation was affected in cells with compromised Skp2 activity, we utilized a dominant negative Skp2 mutant (Skp2ΔF) which lacks the F-Box and therefore is not able to bind Skp1 and thereby can not recruit a SCF functional E3 ligase. Cycloheximide chase assays demonstrated that Skp2ΔF increased c-Myc half life fourfold. To confirm that Skp2ΔF operated as a dominant negative mutant and did not cause general affects on the degradation system, we first stabilized c-Myc by Skp2ΔF and then titrates in increasing amounts of cotransfected wt-Skp2. The results showed that Skp2 restored c-Myc degradation further indicating that Skp2 is participating in c-Myc degradation. We have also confirmed these results by

utilizing the siRNA technique, which is based on interference of small specific synthetic dsRNA oligos with a particular transcribed mRNA. Skp2 siRNA transfection into HeLa cells showed followed cycloheximide chase analysis showed that Skp2 siRNA decreased Myc degradation, further demonstrating that Skp2 is important in c-Myc degradation. In addition to these results we also observed that a c-Myc mutant lacking MBII and the C-terminal and therefore is unable to bind Skp2 was stabilized. Taken together the results suggested that SCF^{Skp2} has an essential role in c-Myc degradation. Since we and others had established that c-Myc is degraded through the ubiquitin-proteasome pathway (paper I) and (Flinn et al., 1998, Salghetti et al., 1999, Bahram et al., 2000, Gregory and Hann, 2000). We next investigated whether Skp2 participates in c-Myc ubiquitylation, by doing an in vivo ubiquitin assay as above. We found that Skp2 cotransfection increased the amount of ubiquitylated c-Myc, while Skp2ΔF overexpression strongly reduced the intensity of the polyubiquitylated detected smear. Further titrating in increased amounts of wt Skp2 into Skp2ΔF cotransfected cells restored polyubiquitylation c-Myc. Taken together these observations suggested that Skp2 participates in c-Myc ubiquitylation.

Skp2/c-Myc interaction is regulated in the cell cycle

To study the biological function of c-Myc/Skp2 interaction we investigated the kinetics of the interaction between endogenous Skp2 and c-Myc during cell cycle activation and progression in peripheral blood lymphocytes (PBL). PBL cells were stimulated with PHA and IL-2 to become activated and to enter into the cell cycle. The expression of c-Myc was undetectable in resting cells, but increased strongly in early G1-phase and then declined starting from the G1/S-phase transition. The expression of Skp2 was also invisible in rested cells and gradually increased during late G1-phase reached a peak in at the G1/S-phase transition in agreement with previous reports (Carrano et al., 1999, Marti et al., 1999, Sutterluty et al., 1999). The interaction between Skp2 and c-Myc was strongest in S-phase correlating with high expression of Skp2 and with increased c-Myc degradation. We conclude that Skp2 interact with Myc during the S-phase in normal lymphocytes.
We utilized Rat1 cells containing inducible MycER construct and cotransfected these with Skp2 or Skp2ΔF and pEGFP. The GFP-positive cells were then sorted by FACS. Myc-induced G1/S-phase transition upon activation of MycER by 4-OHT was studied by cell cycle analysis using FACS. The results showed that cotransfection with wt Skp2 led to increased number of S-phase-cells, while Skp2ΔF expression resulted in inhibition of c-Myc induced S-phase transition. This effect of Skp2 as due to indirect effects through Skp2-mediated degradation of the $p27^{Kip1}$, since similar results were observed in MycER-expressing p27-/- MEF cells. Taken together our findings suggested that Skp2 is a positive regulator of c-Myc-dependent S-phase entry.

Skp2 promotes c-Myc induced transcription

Our observation that Skp2 promotes c-Myc-induced G1/S-phase transition incited us to investigate whether enforced Skp2 expression had any effects on c-Myc-induced transcription of its target genes. We therefore examined the effect of Skp2

on c-Myc-activated transcription from the α-prothymosin (α-ProT) promoter, a wellknown c-Myc target gene, in transiently cotransfection with c-Myc and a α-ProT promoter/luciferase (Luc) reporter gene in HeLa cells. In agreement with previous reports (Desbarats et al., 1996) and paper III, the result showed that the reporter activity of the wt α-ProT promoter/reporter was induced more than threefold by c-Myc compared with an empty expression vector, while a α-ProT mutant lacking the E-boxes in the first intron of the gene was not activated by Myc. Surprisingly, transfection with Skp2 alone also induced the reporter activity in an E-box dependent manner. The α-ProT-E-boxes have been shown to be c-Myc specific (Desbarats et al., 1996), indicating that the effect of Skp2 was mediated by endogenous c-Myc. In contrast, cotransfection with Skp2ΔF did not activate the promoter activity, indicated that Skp2 stimulated transcription is dependent on other component in E3-ligase complex. A Myc mutant with deleted MBII, which can not bind TRRAP and has reduced binding to Skp2, could not activate the reporter. Moreover Skp2ΔF had strong inhibitory effect on c-Myc-induced activation of the transcription, whereas cotransfection with wt Skp2 stimulated the c-Myc induced activation of the promoter. In conclusion these observations suggested that Skp2 is required for c-Myc induced transcription activation and Skp2-mediated activation of c-Myc target gene is c-Myc dependent. This conclusion further was strengthened by experiments in Skp2-/- and c-Myc-/- cells which showed that Skp2 transfection into c-Myc-/- cells and c-Myc transfection into Skp2-/- cells, respectively, both failed to activate the transcription of the α-ProT/luc promoter. We observed similar effects on α-ProT -promoter activity by utilizing c-Myc or Skp2 siRNA, demonstrating that Skp2 siRNA and c-Myc siRNA reduced the promoter activity to a similar degree. We next investigated whether c-Myc-induced activation of endogenous target genes was affected by Skp2 or its mutant. The results showed that Myc activation by 4-OHT in MycER expressing p27-/- MEF cells resulted in threefold increased mRNA expression of ODC and CycD Myc target gene as previously reported (Bouchard et al., 2001, Frank et al., 2001, Haggerty et al., 2003) whereas the Skp2ΔF mutant inhibited their induction by c-Myc. Taken together these results suggested that Skp2 and c-Myc collaborate directly or indirectly to activate the transcription of c-Myc target genes and raises the question whether Skp2 act as coactivator for c-Myc in transcriptional activation. To examine this hypothesis we investigated whether Skp2 is also associated with c-Myc at the CycD2 promoter in vivo utilizing chromatin immunoprecipitation (CHIP) assays. Our results showed as expected that c-Myc and Max were present at the CycD2 promoter. Interestingly, we also found Skp2 and Cul1 associated to the E-box region of CycD2 promoter. Although both Skp2 and c-Myc interacted with the CycD2 promoter in Rat1 myc+/+ cells, neither Skp2 nor c-Myc was observed at the CycD2 promoter in Rat1 myc-/- cells. This result suggested that Skp2 requirement to the CycD2 promoter required c-Myc. We further investigated whether ubiquitylated proteins and proteasome subunits could be detected at the promoter. Indeed, we found that the Sug1, Rpt3, Rpn7 components of the regulatory 19S subunit of the proteasome and α 2 subunit of the 20S proteasome as well as ubiquitylated proteins bound to CycD2 promoter. This observation suggested that Skp2, ubiquitylated proteins and several proteasome subunits is associated to CycD2 promoter region in a c-Myc dependent manner.

In this paper we provide evidence that Skp2 a subunit of an E3 ubiquitin ligase complex is required for ubiquitylation and degradation for c-Myc. We demonstrated that Skp2/Myc interaction was dependent on two conserved regions, MBII and HLH-Zip. Both MBII and bHLHZip are essential for Myc function. The bHLHZip is within DNA binding domain of Myc and MBII is an important sequence for recruitment of the transcription coactivator TRRAP and for most biological activities of Myc protein. Surprisingly, Skp2 did not require MBI for Myc binding, and Thr-58 or Ser-62 did not seem to be involved in Myc Skp2 binding. Furthermore the absence of phosphorylation sites in the Skp2-binding region of Myc suggests that Myc and Skp2 interact in phosphorylation independent manner in contrast to other substrate-SCF interaction. We have also showed that Skp2 interact with Myc in vivo and induces its degradation and ubiquitylation, and this interaction seems to be direct since it was also shown in vitro. We have not however been able to demonstrate Skp2-dependent ubiquitylation and degradation of Myc in vitro, and therefore we can not exclude other E3 ligase mediated Myc ubiquitylation. Our observations also do not exclude other pathways of c-Myc degradation independent of Skp2 in different cell types or in response to different signals.

Our result showed that Myc interact with Skp2 in late G1/S-phase transition during activation of normal lymphocytes. At this time which c-Myc is known to be required for S-phase entry. One possibility is that degradation is required to prevent constant c-Myc activity, which cans damage the cells. Surprisely our results demonstrate that Skp2 did not decrease Myc activity instead promotes c-Myc-induced S-phase entry. Skp2 expression was required for Myc-induced transcription of its target genes. On the other hand c-Myc expression and activity was also required for Skp2-inducd transcription of c-Myc target genes. Our data suggest that Skp2 binds specifically to the Myc binding site (E-boxes) at promoter of the target gene, only when c-Myc is present, suggesting that Skp2 is required to target promoters as a coactivator of c-Myc-induced transcription. Therefore our data support a model of a positive relation between the activity of the transcription factors and their ubiquitylation, which has been suggested previously (Chi et al., 2001, Ferdous et al., 2001, Gonzalez et al., 2002)for review see (Muratani and Tansey, 2003). How can E3 ligase activity stimulate c-Myc-induced transcription? One possibility is that SCFSkp2 induce degradation and ubiquitylation of negative regulators of the transcription at the promoter. This could also be part of an autoregulatory loop, where the Myc activator protein needs to be eliminated at some step in order to complete the transcription process. Another possibility is that c-Myc or other substrates ubiquitylated at the promoter play a nonproteolytic function in, for example, protein-protein interactions of importance for transcription, as has been suggested for Met30-directed ubiquitylation of Met4 transcription factor (Kaiser et al., 2000). Further investigation is needed to determine whether degradation of c-Myc is a necessary step for activation of transcription. This general model has been proposed in the 'licensing' hypothesis, linking transcription factor activity to their destruction in order to maintain stringent control of transcription activation in cells.

Posttranslational regulation of Myc function in response to phorbol ester/interferon-gamma-induced differentiation of v-Myc-transformed U-937 monoblasts (paper III)

The goal of the paper III was to clarify the mechanism behind restoration of TPA-induced differentiation in v-Myc transformed U937 cells by IFN-γ.

The expression of Mad1 is not increased in U937cells costimulated with TPA + IFN-γ

Prior to this study, IFN-γ treatment was shown to abolish the differentiation block in v-Myc transformed U937 cells despite continuous expression of v-Myc (Oberg et al., 1991). To investigate the mechanism behind this IFN-γ activity, we first investigated the synthesis of Mad1, c-Myc and v-Myc, and Max proteins, during IFN-γ and TPA costimulation. The expression of Mad1 increased in response to TPA in parental U937 cells in agreement with previous reports, (Ayer and Eisenman, 1993, Larsson et al., 1994) but only transiently in v-Myc transformed U937 cells. IFN-γ costimulation did however not restore the expression of Mad1 indicating that the continued presence of v-Myc directly or indirectly influenced the expression of Mad1. In addition, IFN-γ stimulation did not increase the mRNA expression of other Mad family proteins including Mxi1, Mad3, Mad4 and Mnt (data not shown). Further the different treatment induced some transient changes in the synthesis ratio of the two Max proteins p21 and p22. The expression of Max was not influenced by IFN-γ costimulation compared to TPA alone. In conclusion, Mad1 does not seem to play a major role in the IFN-γ-induced terminal differentiation of U937 cells.

TPA + IFN-γ inhibit Myc-regulated E-box-dependent reporter-gene activity.

To address whether IFN-γ+TPA hampered the activity of Myc as transcription factor, we studied Myc-induced transcription in U937 cells, using the Myc-regulated α-prothymosine CAT promoter reporter gene construct PrT-CAT and the mutant construct GalmE-PrT-CAT (lacking the Myc-responsive E-boxes in the first intron). In U937-neo-6 cells lacking v-myc, the stimulation with TPA, IFN-γ and IFN-γ+TPA all reduced the PrT-CAT activity but had little effect on GalmE-PrT-CAT. In v-Myc transformed U937 cells, TPA did not reduce the activity of PrT-CAT to the same level as in U937-neo-6 cells, but IFN-γ alone and in particular IFN-γ+TPA efficiently inhibited the activity of the reporter activity suggesting that these treatments inhibit Myc-induced transcription. To further investigate whether IFN-γ+TPA inhibited the transactivating properties of c-Myc, PrT-CAT and GalmE-PrT-CAT were cotransfected with c-Myc expression vector (pCMV-Myc), pCMV-Myc expression stimulated PrT-CAT fourfold, but had no effect on GalmE-PrT-CAT activity. IFN-γ particularly IFN-γ+TPA inhibited pCMV-Myc-induced reporter activity, while TPA stimulation had little effect. We obtained similar results with an additional Myc-induced promoter/ reporter

construct m4mintk-Luc driven by four E-box copies upstream of a minimal-tk promoter. Which were stably integrated into U937-myc-6 cells.. Taken together these experiments suggest that IFN-γ alone and particularly IFN-γ+TPA inhibits transactivation by v-Myc and c-Myc.

The DNA-binding activity of Myc is decreased by IFN-γ+TPA costimulation

It could be several different explanations for the IFN-γ-induced regulated transcription.
To investigate whether the capacity of native Myc, Max and Mad1-complexes to bind specifically to DNA was affected in response to IFN-γ+TPA treatment, a solid-phase DNA binding assay was used. In this assay Myc, Mad1 and Max complexes were immunoprecipitated under low stringency condition from U-937 cell extracts, after which the immunocomplexes were incubated with labeled oligonucleotides containing a Myc/Max binding site (CMD) or mutant variant (CMM). The DNA binding activity of immunoprecipitated Myc decreased after TPA in control U937-GTB and U937-Neo-6 cells but not in U937-myc-6 cells. In addition Mad1 was low in untreated but increased after stimulation of U937-GTB but not in U937-myc-6 cells.
A kinetic experiment showed that the DNA binding activity of c- and v-Myc declined gradually and permanently after IFN-γ +TPA treatment in U937-myc-6 cells, but only temporarily after TPA treatment. The DNA binding activity of Mad1 increased only transiently in v-Myc expressing U937 cells after TPA stimulation and neither IFN-γ alone nor IFN-γ+TPA treatment were able to restore Mad1 DNA binding activity. Max DNA binding activity was not significantly affected in response to IFN-γ+TPA stimulation. In conclusion these results suggest that IFN-γ+TPA directly interfere with the DNA binding activity of Myc.

IFN-γ+TPA costimulation induces Myc: Max disassociation.

Since the DNA binding activity of Myc is dependent on interaction with Max, we next addressed whether the decline of Myc DNA binding activity in response to IFN-γ+TPA stimulation could be due to effects on Myc:Max complex formation or stability. The steady state levels of c-and v-Myc:Max or Mad:Max complexes in U937-myc-6 cells. were studied by coimmunoprecipitation under low stringency condition, followed by western blot analysis and compared to the total amount of these proteins in the cells. The results showed that v-Myc:Max complexes and total v-Myc were relatively stable during the first four hours treatment with TPA and IFN-γ+TPA. Thereafter the amount of v-Myc:Max complexes declined and reached a minimum at 24 hours. The steady state level of v-Myc was only slightly reduced after IFN-γ+TPA stimulation. The amount of v-Myc:Max was also reduced after IFN-γ alone in compared with total v-Myc, and was not caused by decreased to steady state level of Max. In conclusion, these results suggest that an important consequence of the treatment with IFN-γ+TPA and with IFN-γ alone is the reduction in the fraction of total v-Myc and c-Myc in complex with Max. This suggests that the reduction of Myc-DNA binding activity and the inhibition of Myc-induced transcription in response to IFN-γ mentioned above is the result of disassociation of Myc: Max complexes.

Dephosphorylation of Myc in response to IFN-γ+TPA

Destabilization of Myc: Max heterodimers could be the result of IFN-γ+TPA-induced unidentified proteins competing for Myc or Max, or direct modification(s) of Myc or Max by for instance phosphorylation/ dephosph-orylation. To evaluate whether the overall level of phosphorylation of v-Myc and c-Myc changed in response to IFN-γ+TPA treatment in parental and v-Myc transformed U937 cells, aliquots of untreated and induced cultures were in vivo labeled with ^{35}S-methionine and ^{32}P-orthophosphate in parallel, where after the Myc proteins were immunoprecipitated and analyzed. The results showed that the relative intensity of the ^{32}P-labeled v-Myc and c-Myc proteins were significantly decreased compared to the corresponding ^{35}S-labeled protein level in response to IFN-γ+TPA costimulation, suggesting that Myc was dephosphorylated. To further study phosphorylation of c-and v-Myc, the proteins were also analyzed by 2D-electrophoresis. The assays showed that IFN-γ+TPA stimulation induced a shift of v- and c-Myc towards the basic side of the gel. As similar shift was observed after phosphatase treatment, suggesting that c-and v-Myc are dephosphorylated in response to IFN-γ+TPA treatment. However, no effect on Max phosphorylation was observed after IFN-γ+TPA treatment (data not shown). We could also show that phosphatase treatment of Myc: Max complexes resulted in disassociation of Max from the complex, supporting the hypothesis that dephosphorylation of Myc results in destabilization of Myc: Max complexes.
In conclusion our results in this paper suggest that IFN-γ+TPA stimulation restores differentiation and cell cycle arrest of v-Myc expressing U937 through negative regulation of Myc/DNA binding activity correlating with post-translational modification of c-and v-Myc by dephosphorylation.

Interferon-gamma inhibits CycE/Cdk2-mediated phosphorylation of c-Myc via p27^{Kip1} resulting in increased Myc ubiquitylation and degradation (Paper IV)

We have shown in paper III that IFN-γ +TPA antagonize Myc activity posttranslationally through unclear mechanisms. In this work we continue this study investigating the mechanisms behind IFN-γ +TPA induced inactivation and dephosphorylation of Myc.

IFN-γ +TPA increase the rate of degradation and ubiquitylation of Myc.

To address the possibility that IFN-γ-induced inactivation of Myc occurs through increased degradation, we first analyzed the half-life of c-and v-Myc by pulse chase analysis in IFN-γ +TPA costimulated or TPA–stimulated v-Myc transformed U937 cells. We observed that v-Myc, which is mutated at Thr58,

exhibited a prolonged half life, as mentioned above see paper I (Bahram et al., 2000). IFN-γ + TPA treatment increased both c and v-Myc turnover from 25 to 11 and 120 to 37 minutes respectively, while TPA treatment did not affect Myc turnover. We conclude from these results that IFN-γ and TPA costimulation increases the rate of both c-and v-Myc degradation in U937 cells and similar results were obtained in Colo320 carcinoma cells with c-Myc amplification. It is intriguing that the turnover of v-Myc also increased in response to IFN-γ + TPA treatment despite the Thr-58 mutation. This suggested that IFN-γ signaling utilized a pathway of Myc degradation distinct from the Thr-58 pathway. To investigate whether IFN-γ induced Myc degradation was mediated by the Jak–Stat pathway we treated 2fTGH human fibrosarcoma cells or sublines of these cells lacking Stat1 (U3A) or lacking JAK1 (U4A) with IFN-γ. ^{35}S-methionine pulse chase analysis showed that IFN-γ treatment increased Myc turnover in parental 2FTGH cells but not in STAT1 deficient or Jak1 deficient cells, indicating that both STAT1 and JAK1 are required for IFN-γ induced Myc degradation.

As pointed out above the ubiquitin-proteasome system is involved in Myc turnover, (Ciechanover et al., 1991, Bonvini et al., 1998, Flinn et al., 1998, Salghetti et al., 1999, Bahram et al., 2000, Gregory and Hann, 2000). To investigate whether IFN-γ -induced Myc degradation occurred through this pathway, v-Myc expressing U937 cells were treated with IFN-γ + TPA in presence of the proteasome inhibitor LLnL. IFN-γ-induced degradation of c- and v-Myc was indeed inhibited by LLnL treatment, confirming that IFN-γ-induced degradation occurs via the proteasome. To investigate whether IFN-γ increases Myc ubiquitylation, immunoprecipitated Myc was analyzed by western blot using ubiquitin antibodies. Our results showed that IFN-γ alone and IFN-γ + TPA but not TPA increased c-Myc ubiquitylation in 2fTGH cells. These results suggested that IFN-γ and IFN-γ + TPA treatment increased c-Myc ubiquitylation and proteasome-mediated degradation.

Interferon-gamma dephosphorylates c/v-Myc at Ser-62

In (paper III) we showed that IFN-γ alone and IFN-γ + TPA treatment led to dephosphorylation of v-Myc- and c-Myc in unidentified sites (Bahram et al., 1999). We speculated if these dephosphorylation events could be connected to the induced degradation of Myc. Since Thr-58 and Ser-62 have previously been shown to be involved in Myc turnover as discussed above, it was therefore of interest to determine if the phosphorylation of these sites were affected by IFN-γ treatment. For this purpose specific anti-phospho-T58/S62-Myc antiserum was used in western blot analysis. The results showed that the level of phosphorylated Myc declined after both IFN-γ alone and IFN-γ + TPA treatment in comparison with total Myc. We concluded from these results that IFN-γ stimulation led to dephosphorylation of Ser-62 irrespective of the Thr-58 status since v-Myc has mutated Thr-58. To examine whether Ser-62 was essential for IFN-γ induced degradation, the turnover of c-Myc-wt and T58A and S62A mutants in response to IFN-γ was studied in 2FTGH cells. Cycloheximide chase analysis showed that IFN-γ increased the rate of degradation of wild type and T58A-c-Myc but not of the S62A-c-Myc mutant, suggesting a rate of Ser-62 in IFN-γ -induced Myc

degradation. In conclusion, IFN-γ treatment decreased Myc phosphorylation on Ser-62 and IFN-γ -induced Myc degradation was shown to be Ser-62 dependent and Thr-58 independent.
Our data thus provide evidence that thus antiproliferative signaling leads to decreased Ser-62 phosphorylation of Myc which in turn increases Myc degradation. Certainly we can not exclude other sites which can be affected in response to IFN-γ treatment.

CDK2 phosphorylates Myc at Ser-62 in vivo and in vitro

Our results indicated that dephosphorylation of Ser-62 leads to destabilization of Myc, suggesting that phosphorylation of Ser-62 might stabilize Myc. The kinase that phosphorylates Ser-62 in vivo has not been clarified, but previous work has suggested that both MAPK and CDC2 can phosphorylate Myc at Ser-62 in vitro (Seth et al., 1992, Lutterbach and Hann, 1994). In an attempt to identify the in vivo Ser-62 kinase, we cotransfected U2OS cells with Flag-c-Myc together with CycE/CDK2 or with constitutively active Ras, which activates the MAPK pathway. In addition, cells were treated with the Cdk2 inhibitor roscovitine, (Meijer et al., 1997) or dominant negative Ras (Dn-Ras) which block this pathway. Western blot analysis showed that the phospho-Myc signal was reduced after roscovitine treatment. Whereas CycE/CDK2 increased phosphor-Myc compared to the total Myc level. However, neither activated nor dominant negative Ras seemed to affect Myc phosphorylation. This suggests that CDK2 stimulates Myc phosphorylation in vivo.
To confirm whether Myc is a CDK2 substrate, we performed an in vitro kinase assay using bacterially expressed wt Myc and mutants Myc GST-fusion proteins incubated in the presence of (γ -32P) ATP with purified CycE/A-CDK2 complexes expressed in baculovirus. The results showed that wt Myc and Myc-T58A but not Myc-S62A were phosphorylated efficiently by the CycE/CDK2 complex, and to a lesser extent by CycA/CDK2. In conclusion, our in vitro and in vivo results suggested that CDK2 is a Ser-62 kinase.
To investigate the effect of roscovitine on Myc turnover, v-Myc expressing U937 cells were treated with roscovitine for two hours followed by cycloheximide chase. The results showed that roscovitine increased the rate of degradation of both c-and v-Myc (from 28, 120 to 20, 40 minutes respectively). We conclude that dephosphorylated of Myc is through inhibition of CDK2 in vivo, by the CDK2 inhibitor roscovitine, leads to increased c-and v-Myc turnover, thus mimicking the effect of IFN-γ.

IFN-γ increases Myc degradation and dephosphorylation through CDK2 inactivation via $p21^{Cip1}$ and $p27^{Kip1}$ upregulation

The current dephosphorylation of Myc could either be the result of activation of a phosphatase or inactivation of a Ser-62 kinase in response to IFN-γ treatment.
To investigate if CDK2 activity was affected by IFN- γ stimulation, v-Myc expressing U937 cells treated with IFN-γ, TPA, or both in combination, an in vitro cdk2 kinase activity assay was performed using histone H1 as a substrate and cdk2 immunoprecipitated from the cells. In presence of [γ-32P]-ATP we readily

detected the phosphorylation of histone H1. We observed that IFN-γ alone and IFN- γ+TPA led to strongly decreased phosphorylation of histone H1 suggesting that IFN-γ treatment inactivated CDK2 complexes. A slight reduction was observed also after TPA treatment

Rb is an in vivo substrate for both CycD/CDK4 and CycE/CDK2. Immuno-precipitation of Rb showed that IFN-γ treatment resulted in reduced Rb phosphorylation in both parental and v-Myc expressing cells. These results confirm that IFN-γ treatment inactivates CDK4 and/or CDK2 in U937 cells.

We next investigate whether the IFN-γ-induced CDK2 inactivation correlated to upregulation of CKIs expression as has been suggested by previous report (Harvat et al., 1997, Takami et al., 2002). Our results confirmed that IFN-γ and TPA costimulation induced the expression of $p27^{Kip1}$ and $p21^{Cip1}$ in v-Myc transformed U937 cells. However in contrast to $p27^{Kip1}$, $p21^{Cip1}$ expression was not increased by IFN-γ alone but required costimulation with TPA, further, increased interaction of CDK2 with $p27^{Kip1}$ was found by coimmunoprecipitation analysis in response to TPA, IFN- γ and IFN- γ+TPA stimulation in U937 cells, suggesting that $p27^{Kip1}$ is a main player in the IFN- γ inactivation of CDK2 complexes. However we can not fully exclude other possible mechanisms such as inactivation of CAK which is involved in activation of CDK. CDK2 inactivation can also occurs through other mechanisms such inactivation of an upstream kinase involved in activation of the kinase that phosphorylates Myc.

We next studied the kinetics of CDK2 inactivation after in IFN-γ + TPA treatment in v-Myc transformed U937 cells, by in vitro CycE/CDK2 kinase assay as above. The result showed that CycE/CDK2 activity was down-regulated within 4 hours of IFN-γ + TPA treatment, thus correlating kinetically with upregulation of both $p21^{Cip1}$ and $p27^{Kip1}$.

The inactivation of CDK2 by IFN-γ could further be connected kinetically to the reduced phosphorylation of Ser-62 as determined by western blot using phospho-Myc antisera. The turnover rate of Myc was determined in v-Myc expressing U937 cells treated with IFN-γ + TPA for 4 and 8 hours and followed by a cycloheximide chase experiment and showed that Myc turnover increased already within 4 hrs of by IFN-γ + TPA treatment, thus confirming that Myc degradation correlated kinetically with Myc dephosphorylation, inactivation of CDK2 and upregulation of CKIs in response to IFN-γ

To exclude that the effect of IFN-γ and roscovitine treatment on Myc-Ser62 dephosphorylation and degradation was not due to indirect effects on the cell cycle, we analyzed the cell cycle of distribution v-Myc- transformed U937 cells kinetically after IFN-γ+TPA, or roscovitine treatments. The result demonstrate that neither IFN-γ+TPA nor roscovitine treatment altered the cell cycle distribution at early time point, when the effects of Myc was observed, whereas G1 accumulation was indeed observed at 24 hours after treatment with IFN-γ + TPA, as previously reported (Oberg et al., 1991). In conclusion these events occurred prior to effects of IFN-γ on the cell cycle, excluding that Myc was affected indirectly through the cell cycle arrest. An alternative explanation for the observed Myc dephosphorylation in response to IFN-γ could be activation of a Ser-62 phosphatase. Analysis of PP2A which was recently reported to dephosphorylate Ser-62 (Yeh et al., 2004) showed that its activity was not altered by IFN-

γ treatment, we can however not entirely exclude other phosphatases that might affects by IFN-γ stimulation.

IFN-γ induced Myc degradation is p27^{Kip1} dependent moreover p27 induced degradation is Ser-62 dependent and Skp2 independent manner

These results suggested that p27^{Kip1} might be a key player in mediating the anti-Myc activity of IFN-γ.
To further investigate the role of p27^{Kip1} in Myc dephosphorylation and degradation. HeLa cells were cotransfected with wt-Myc or T58A and S62A mutants together with p27^{Kip1}. We observed that overexpression of p27^{Kip1} reduced the mount of phospho-Myc relative to the total amount of Myc for both wt-Myc and the T58A mutant. Further p27 ^{Kip1} overexpression induced Myc degradation in both Myc-wt and Myc-T58A mutant but not in S62A mutant, suggesting that p27^{Kip1} induced-Myc degradation is Ser-62 dependent.
To determine whether IFN-γ-induced degradation of c-Myc is p27^{Kip1}-dependent, we used wild-type and p27-/- MEF cells. Western blot analysis showed IFN-γ increased Myc degradation in wt but not in p27-/- MEF cells, suggesting that p27^{Kip1} is indeed required for IFN-γ-induced degradation of c-Myc. Taken together our data suggest that IFN-γ-induced Myc degradation is p27^{Kip1} dependent emphasizing the role of p27^{Kip1} in regulation of Myc degradation and inactivation.
Paper II and (Kim et al., 2003) showed that the E3 ubiquitin ligase SKP2 interact with Myc and directs its ubiquitylation and degradation in Thr-58 independent manner (Kim et al., 2003, von der Lehr et al., 2003). It was of interest to determine whether Skp2-induced Myc degradation is Ser-62 dependent. Cotransfection of HeLa cells with wt-Myc or the S62A mutant together with Skp2 and Cul1, showed that Skp2/Cul1 induced degradation of the S62A mutant as efficient as wt-Myc, suggests that Skp2-induced degradation is independent of Ser-62. Addressed the role of Skp2 in p27-induced-Myc degradation, cycloheximide chase experiments showed that c-Myc was stabilized in Skp2ΔF cotransfected cells as shown in paper II. However, p27^{Kip1} cotransfection increased Myc degradation irrespective of the presence of Skp2ΔF suggesting that Skp2 does not participate in p27^{Kip1}-induced Myc degradation. Interestingly this suggests that a distinct E3-ligase might be involved in IFN-γ and p27^{Kip1}-induced Myc degradation. The identification of this E3 ligase requires further investigation.
In conclusion our results suggest that IFN-γ induced signals inhibit Myc function through dephosphorylation of Myc at Ser-62 resulting in increased c-and v-Myc degradation via the ubiquitin/proteasome pathway. This dephosphorylation and destabilization occur through inactivation of CDK2 via upregulation of p27^{Kip1}. This evidence proves that p27^{Kip1} is both required and sufficient for Myc degradation and Ser-62 dephosphorylation.

Discussion paper III and IV

Interferon-gamma has long been known as antiproliferative cytokine. However the mechanism of its antiproliferative action is not fully understood. Previously has shown that IFN-γ with TPA costimulation restores terminal differentiation and G_1 cell-cycle arrest despite continuous expression of v-Myc. These findings suggested that IFN-γ +TPA combination can interfere with the growth and blocking Myc activity. In paper III we initiated our work with investigating the mechanisms behind IFN-γ effect. We envisioned that these signals could counteract Myc by at least three different mechanisms. First, by increased expression or activity of Myc-antagonist members of the *mad*-family. Second, by acting on Myc directly through inhibition of its activity or through affecting its steady state level by a posttranslational mechanism. Third, by acting downstream of Myc; for instance, through independent regulation of Myc target genes.

We found that induced expression of Mad1 occurred only transiently in v-Myc expressing cells (Figs 2, 4, and 6 . In paper III). This indicates a direct or indirect role of Myc in the regulation of the *mad*1. Since, costimulation with IFN- niether increased the expression of other mad genes or mnt mRNA (data not shown), these results argue against our first hypothesis.

In contrast, IFN-γ costimulation with TPA reduced the both DNA binding activity of both v- and c-Myc and their hetrodimerization with Max. This suggest that IFN-γ+TPA inhibit Myc activity at least, in part by direct interference with the disassociation of Myc:Max complexes and decreased DNA binding rather than competition with Mad. Indeed Myc activity is dependent on dimerization with its obligate partner, Max. These findings likely explain the inhibition of Myc-induced transcription from its target promoters, since Myc needs to interact with Max and bind DNA in order to activate transcription. In addition to these observations we found that IFN-γ alone or in combination with TPA dephosphorylated Myc in one or several phosphorylations sites. In fact, Max is constitutively phosphorylated *in vivo* at protein kinase CKII sites, including Ser2 and Ser11. This phosphorylation has been shown to affect the DNA-binding properties of Max homodimers. (Berberich and Cole, 1992, Bousset et al., 1993, Bousset et al., 1994). Myc also has CK II phosphorylation sites in similar positions, but it is unclear if they affect DNA binding. The central acidic region of Myc also contains several sites of phosphorylation which can be phosphorylated by CKII (Luscher et al., 1989).

Several recent studies has shown that Myc phosphorylation can be involved in stabilization of the protein (see paper I) (Noguchi et al., 1999, Bahram et al., 2000, Gregory and Hann, 2000, Sears et al., 2000, Noguchi et al., 2001) or its ability to promote transformation (Bousset et al., 1993, Lutterbach and Hann, 1994). In addition phosphorylation of Myc at unidentified sites has shown to inhibit Myc DNA binding during mitosis, (Luscher and Eisenman, 1992). Interestingly, recently work from (Huang et al., 2004) has demonstrated that phosphorylation of Myc at Thr-358, Ser-373 and Thr-400 by Pak2 in response to stress related signals, reduces Myc DNA binding and phosphorylation of Ser-373 and Thr-400 blocks Myc: Max dimerization. These observation suggest that different signals involved in phosphorylation or dephosphorylation of Myc can affect its DNA binding and dimerization. It would be interesting to investigate whether IFN-γ can affect phosphorylation at the C-terminus sites regulated by stress signals.

Moreover Myc has been shown to have multiple phosphorylation sites in the N-terminal region, where Thr-58, Ser-62 and Ser71 may be most functionally significant. (As discussed in paper I and II). In an attempt to further elucidate the mechanism(s) of IFN-γ induced Myc inactivation, we addressed whether IFN-γ might be involved in Myc degradation. Our present results show that stimulation with IFN- γ alone or together with TPA increased degradation not only of c-Myc but also of v-Myc, which carries a stabilizing mutation at Thr-58 (see paper I). We have also demonstrated that IFN-γ induced degradation was not cell line specific since we have also observed IFN-γ induced degradation in Colo320 colocarcinoma and in human fibrosarcoma cells. Further, we found that IFN-γ-stimulated turnover of Myc was compromised in STAT1 or JAK deficient cells', suggesting that it is STAT1/JAK1 dependent. It is however, still not unclear how the IFN-γ-induced activation of STAT1 leads to inactivation of Myc.

To our knowledge this is the first report demonstrating that Myc turnover can be regulated by antiproliferative signaling. Since we have shown in paper III that Myc was dephosphorylated after IFN-γ treatment, these observations raised the question whether there might by a connection between dephosphorylation and increased degradation by IFN-γ. Two candidates for Myc phosphorylation sites affected by IFN-γ were Thr-58 and Ser-62, which both has been implicated in Myc turnover (see paper I). Investigating the status of these phosphorylation sites we found that IFN-γ stimulation led to dephosphorylation of Ser-62 irrespective of the Thr-58 status, which in turn increases Myc ubiquitylation and degradation in Ser-62 dependent and Thr-58 independent manner. Surprisingly, mutation of S62 to alanine stabilized Myc in untreated cells. The reason for that is unclear, but may be due to decreased Thr-58 phosphorylation as a result of the prevention of Ser-62 phosphorylation as previously suggested (Bousset et al., 1993 44, Lutterbach and Hann, 1994 47, Pulverer et al., 1994 46). Although regulation of Ser-62 seems to play a crucial role, we can not exclude that also other sites might be affected in response to IFN-γ treatment.

Our results further suggest that CDK2 is a major in vivo and in vitro Ser-62 kinase (Fig 3 paper-IV). Previous reports have suggested that Ras activation can lead to Ser-62 phosphorylation through MAPK which can phosphorylate Myc at Ser-62 in vitro (Seth et al., 1992, Lutterbach and Hann, 1994). However, neither constitutively active nor dominant negative Ras affected Ser-62 phosphorylation in our cell systems arguing against MAPK as a major in vivo Ser-62 kinase. This conclusion is supported by (Lutterbach and Hann, 1999). However, we still can not exclude that MAPK could phosphorylate Ser-62 in other cells or under other conditions.

Several signal-mediated pathways have previously been connected to regulation of Myc stability. Among these is the Ras/MAPK pathway, which has been suggested to increase Myc stability through phosphorylation of Ser-62, (Sears et al., 1999). In addition, the activation of MEKK1/JNK by UV or the necrosis factor-alfa (TNF-α), and has been suggested to stabilized Myc (Park et al., 1998, Yujiri et al., 1998, Alarcon-Vargas et al., 2002, Alarcon-Vargas and Ronai, 2004). The action in this case seems to involve in activation of PI-3K/AKT resulting in inactivation of GSK-3, which in turn leading in preventing of Thr-58 phosphorylation resulting in Myc stabilization, (Sears et al., 2000, Venter et al., 2001)

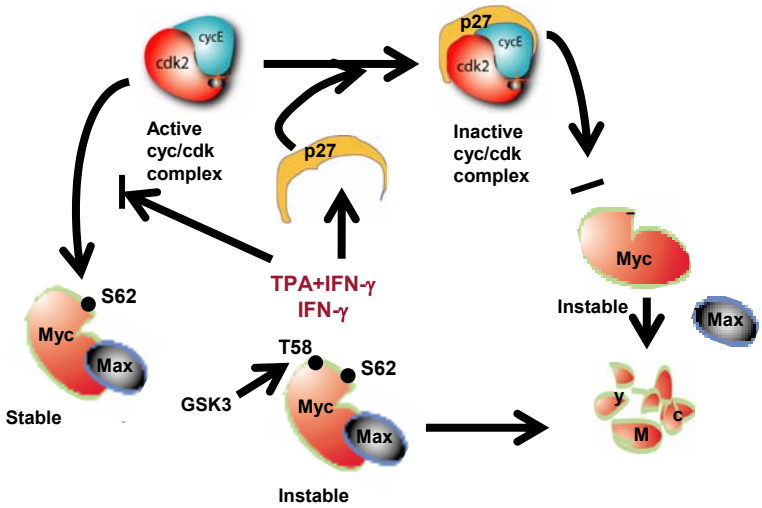
Dephosphorylation of Myc could either be the result of activation of a Ser-62 phosphatase or inactivation of the Ser-62 kinase. The PP2A Ser/Thr phosphatase was recently shown to dephosphorylate Myc at Ser-62 in Thr-58 phosphorylation and Pin1 dependent manner (Yeh et al., 2004). However, we could demonstrate that the activity of PP2A was not altered by IFN-γ treatment. Although we can not entirely exclude the involvement of other Ser/Thr phosphatases, we speculated that IFN-γ stimulation might inhibit CDK2 activity, for instance by upregulation of CDK inhibitors. It has been reported previously that IFN-γ induces $p27^{Kip1}$ and $p21^{Cip1}$ in certain cells (Harvat et al., 1997, Takami et al., 2002). Here we confirmed that IFN-γ and TPA costimulation induces the expression of $p27^{Kip1}$ and $p21^{Cip1}$ in v-Myc transformed U937 cells. However in contrast to $p27^{Kip1}$, $p21^{Cip1}$ expression was not increased by IFN-γ alone but required costimulation with TPA in U937 cells. Our findings suggest that $p27^{Kip1}$ is a main player in the IFN- γ inactivation of CDK2 complexes. However, we can not fully exclude other possible mechanisms such as inactivation of CAK which is an upstream kinase involved in activation of CDK, or alternatively inactivation of the phosphatases that are involved in activation of CDKs such as cdc25 family proteins.

Interestingly, we found that CDK2 inactivation occurs early after IFN-γ+TPA treatment in parallel with the increased expression of $p27^{Kip1}$ and $p21^{Cip1}$. This correlated kinetically with the dephosphorylation of Myc at Ser-62 and the increased c- and v-Myc degradation after IFN-γ treatment. These events occurred prior to effects of IFN-γ on the cell cycle, excluding that Myc was affected indirectly through cell cycle arrest. The role of $p27^{Kip1}$ was further emphasized by the observation that overexpression of $p27^{Kip1}$ decreased Myc phosphorylation at Ser-62 and induced Myc degradation in Ser-62 dependent manner. Furthermore, utilizing p27-/- MEF cells we could show that IFN-γ-induced Myc degradation is $p27^{Kip1}$ dependent. Taken together our data strongly suggest an important role of $p27^{Kip1}$ in regulation of Myc degradation and inactivation.

We and others recently showed that the E3 ubiquitin ligase Skp2 interact with Myc and directs its ubiquitylation and degradation in Thr-58 independent manner (Kim et al., 2003, von der Lehr et al., 2003). Interestingly we found that IFN-γ and $p27^{Kip1}$-induced Myc degradation and inactivation were independent on Skp2. In contrast to IFN-γ, Skp2 cooperates with Myc to induce G1/S-phase transition and to activate target genes Myc transactivation, suggesting that Myc transactivation could be positively or negatively regulated through ubiquitylation by different E3-ligases. This suggests that a distinct E3-ligase might be involved in IFN-γ and $p27^{Kip1}$-induced Myc degradation. The identification of this E3 ligase requires further investigation. We also hypothesizes that Ser-62 phosphorylation might be involved in reducing of E3-ligase binding to Myc resulting in stabilization of Myc. Another option is that Thr-58 and Myc Ser-62 phosphorylations both are involved in intracellular localization of Myc such as nuclear import and export.

In conclusion our results suggest that IFN-γ+TPA stimulation restores differentiation and cell cycle arrest of v-Myc expressing U937 through posttranslational modification of c-and v-Myc by dephosphorylation at Ser-62 resulting in increased v-and c-Myc degradation via the ubiquitin/proteasome pathway. This may not exclude that some of the effects of IFN-γ on Myc observed in paper III may be generated via distinct IFN-γ-induced signals. This dephosphorylation and destabilization occur through inactivation of CDK2 via

activation of $p27^{Kip1}$. Our results suggest that $p27^{Kip1}$ is both required and sufficient for Myc degradation and Ser-62 dephosphorylation. Various reports have shown that Myc regulates $p27^{Kip1}$ activation and degradation in response to proliferative signals. Here we show $p27^{Kip1}$ in turn is able to regulate the activity and stability of Myc in response to anti-proliferative signals. Such negative regulation of c-Myc generally and Myc with mutated Thr-58 by cytokine signals might be a biologically relevant level of control with potential therapeutic relevance for tumors with deregulated Myc-expression.



Φιγυρε 6: Ηψποτηετιχαλ μοδελ οφ ΙΦΝ–γαμμα–ινδυχεδ ινηιβιτιον οφ χ–Μψχ πηοσπ ηορψλατιον τηρουγη Χδκ2/π27Κιπ1 ρεσυλτσ ιν χ–Μψχ υβιθυιτιν/προτεασομαλ δεγ ραδατιον

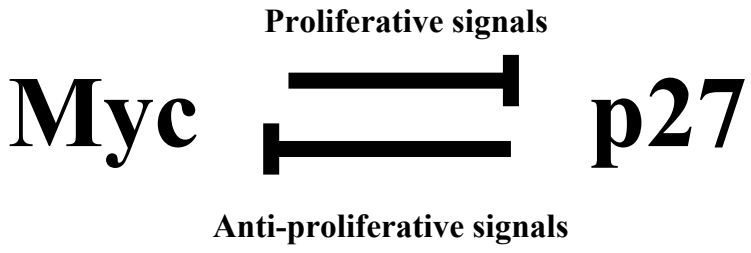


Figure 7: Hypothetical model of Myc and p27
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CONCLUSIONS

1 The Myc oncoprotein is degraded via the ubiquitin-proteasome pathway.

2 Thr-58 mutation results in inefficient ubiquitylation and decreased proteasome-mediated turnover.

3 The SCF^{Skp2} E3 ubiquitin ligase interacts with c-Myc and participates in Myc ubiquitylation and degradation in a Thr-58 independent manner.

4 SCF^{Skp2} is coactivator for Myc induced transcription.

5 IFN-γ costimulation with TPA inhibits Myc-induced transcription and DNA binding through Myc:Max destabilization, correlating with posttranslational modification of Myc by dephosphorylation.

6 IFN-γ decrease Ser-62 phosphorylation and increases Myc ubiquitin-proteasome-mediated degradation in Ser-62 dependent manner.

7 CDK2 is an in vivo and in vitro Ser-62 kinase.

8 IFN-γ increases Myc Ser-62 dephosphorylation and degradation through inactivation of CDK2 via $p27^{Kip1}$.

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