Regulation of Myc Oncoprotein Function by E3 Ubiquitin Ligases

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Abstract

The Myc oncoprotein/transcription factor plays an important role in controlling cell proliferation, and is deregulated in many human cancers. Myc is a short-lived protein that is turned over via the ubiquitin-proteasome pathway. The major aim of this thesis was to identify E3 ubiquitin ligases that regulate Myc turnover and/or function. Further we aimed to understand the mechanisms behind IFN-γ-induced inactivation of Myc.

Through this work we identified the first E3 ligase, SCF^{Skp2}, interacting with Myc and promoting its ubiquitylation and proteasomal turnover. Surprisingly, Skp2 promotes Myc-induced S-phase entry and is required for transcriptional activation by Myc. We show that Myc recruits Skp2 and the proteasome to target promoters and that the proteasome subunit Sug1 is required for Myc transactivation, suggesting that both Skp2 and Sug1 act as coactivators for Myc-induced transcription.

We further found an interaction between Myc and the tumour suppressor/E3 ligase von Hippel Lindau (VHL) that targets hypoxia-inducible factor (HIF) for degradation. Unlike HIF, Myc binds VHL also during hypoxia through a distinct binding site. VHL is shown to play a role in ubiquitylation of Myc, but surprisingly not in degradation, thus displaying a new non-proteasomal VHL function. Further, VHL interacts with a subset of Myc target promoters and is possibly involved in regulation of certain Myc target genes.

We also present results showing that Cyclin E/Cdk2 regulates the stability of Myc by phosphorylating Ser-62 and functions as a Myc cofactor, increasing Myc transactivation and stability. We further show that this phosphorylation is inhibited by the growth inhibitory cytokine IFN-γ via upregulation of the Cdk inhibitor p27, resulting in increased Myc turnover via the ubiquitin/proteasome pathway.

Taken together, this thesis presents three examples of regulation of Myc ubiquitylation with different consequences for Myc function and stability. These findings emphasise the importance of Myc regulation by E3 ligases at different biological levels and could potentially be of importance for the development of novel cancer therapeutics.

Keywords: Myc, Skp2, Sug1, VHL, transcription, ubiquitin-proteasome pathway, cell cycle, cyclin E/Cdk2, $p27^{Kip1}$, interferon-γ

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To my family

Contents

List of Publications

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

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

II von der Lehr N, Johansson S, Larsson LG. (2003). Implication of the ubiquitin/proteasome system in Myc-regulated transcription. *Cell Cycle* 2(5):403-7.

III Fahlén S, Nilsson H, Reza Sharifi H, von der Lehr N, Hydbring P, Su Y, Sangfelt O, Poellinger L and Larsson LG. Regulation of c-Myc function by the von Hippel-Lindau E3 ubiquitin ligase/Tumour Suppressor Protein (manuscript).

IV Bahram F, Yingtao S, Hydbring P, von der Lehr N, Lilischkis R, Fahlén S, Hein N, Henriksson M, Wu S, Vervoorts J, Lüscher B, Larsson LG. CyclinE/Cdk2 functions as a Myc cofactor and regulates Myc stability by phosphorylating serine 62 (manuscript).

Abbreviations

4-OHT	4-hydroxy-tamoxifen
Ab	antibody
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APC/C	anaphase promoting complex/cyclosome
APIS	AAA ATPase independent of 20S
ATP	adenosine triphosphate
bHLHZip	basic helix-loop-helix leucine zipper
BiFC	bimolecular fluorescence complementation
CAD	carbamoyl phosphate synthase-aspartate transcarbamylase-dihydroorotase
CBC	Cul2-elongin B-elongin C
CBP	CREB-binding protein
CDK	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CHIP	Carboxyl-terminus of Hsc70 interacting protein
CKI	cyclin dependent kinase inhibitor
CNS	central nervous system
CoIP	coimmunoprecipitation
CTD	C-terminal domain
DUB	deubiquitylase
E6-AP	E6-associated protein
ER	estrogen receptor
ERK	extracellular receptor kinase
GLUT1	glucose transporter 1
GNAT	Gcn-related N acetyltransferase
GSK3	glycogen synthase kinase3
GTF	general transcription factor

HAT	histone acetyl transferase
HDAC	histone deacetylase
HECT	homologous to E6-AP C-terminus
HIF	hypoxia inducible factor
HPV	human papiloma virus
HRE	hypoxia responsive element
hTERT	human telomerase reverse transcriptase
IFN	interferon
Ig	immunoglobulin
IL-2	interleukin 2
Ink4	inhibitors of Cdk4
IP	immunoprecipitation
Jak	Janus kinase
LDHA	lactate dehydrogenase A
LRR	leucine rich repeates
Luc	luciferase
Max	Myc associated factor x
MB	Myc box
MEF	mouse embryonic fibroblast
Mga	Max giant associated protein
Myc	myelocytomatosis
MycER	Myc-estrogen receptor fusion
MYST	MOZ, Ybf2/Sas3, Sas2 and Tip60 family
N-CoR	nuclear hormone receptor corepressor
NTD	N-terminal domain
ODC	ornithine decarboxylase
PCAF	p300/CBP-binding protein associated factor
PDK1	pyruvate dehydrogenase kinase 1
PHA	phytohemaglutinin
PHD	prolylhydroxylase
PHD finger	plant homeo domain finger
PI3K	phosphatidylinositol 3-kinase
PIC	preinitiation complex
PolII	RNA polymerase II
PolIII	RNA polymerase III
PTEFb	positive transcriptional elongation factor b
Rb	Retionblastoma protein
RCC	renal cell carcinoma
RING	really interesting new gene
SAGA	SPT/ADA/Gcn5/acetyltransferase

SCF	Skp1-Cullin-F-box protein
SID	Sin3 interaction domain
Skp2	S-phase associated kinase associated protein 2
SMRT	silencing mediator of retinoic and thyroid hormone receptor
SPRF	single-polypeptide RING-finger
Stat	signal transducers and activators of transcription
TAD	transactivation domain
TAFs	TBP associated factors
TBP	TATA-binding protein
TF	transcription factor
TGFβ	transforming growth factor β
TRRAP	transactivation/transformation associated protein
Ub	ubiquitin
VEGF	vascular endothelial growthfactor
VHL	von Hippel Lindau
α-proT	α-prothymosin

1 Background

1.1 Development of cancer

For a normal cell to transform into a cancer cell, genes that regulate cell growth and differentiation must be altered (Croce, 2008). Genetic changes can occur at many levels, from point mutations changing one nucleotide to gain or loss of entire chromosomes. The altered gene expression can also be due to epigenetic changes for instance in DNA methylation. Typically, cancers are caused by a series of mutations where each mutation alters the behavior of the cell to some extent. The majority of human cancers have an enhanced mutation rate, the cells are genetically unstable. This instability can be due to defects in either DNA replication repair or the cells ability to repair local DNA damage. Two broad classes of genes are targets for cancerous mutations: proto-oncogenes and tumour suppressor genes. Mutations in proto-oncogenes that cause an overactive protein are "gain of function" mutations and their mutant forms are called oncogenes. Mutations are usually dominant and mutation in one allele is sufficient to become an oncogene. Mutations that inactivate a tumour suppressor gene, a "lose of function" mutation, subsequently drive the cell towards cancer. Mutations in tumor suppressors are typically recessive and both alleles usually need to be affected for the "lose of function" phenotype.

Hanahan and Weinberg suggest that in most or perhaps all tumors the neoplastic cells need to aquire six types of alterations that collectively dictate malignant growth (Hanahan & Weinberg, 2000). The alterations are: self-sufficiency in growth signals, insensitivity to growth-inhibtory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. For proliferation, normal cells need mitogenic growth signals, which are transmitted into the cells via

transmembrane receptors. These growth signals can be diffusible growth factors, extracellular matrix components or cell to cell adhesion molecules and are mimicked in some way by oncogenes in cancer (Hanahan & Weinberg, 2000). To overcome the impact of antigrowth signals, which drives the cell into the resting G0-phase or differentiation, a cancer cell usually has disruptions in the Rb-pathway (described in chapter 1.2). One example of an antigrowth signal is TGFβ that acts in a number of ways to prevent phosphorylation of Rb, for a review see (Massague *et al.*, 2000). Apoptosis is a mechanism that can be triggered by an activated oncogene and has to be overcome in a cancerous cell. Loss of proapoptotic regulators is one strategy to become resistant to programmed cell death. Mutations in the p53 tumour suppressor, is seen in more than 50% of human cancers and results in an impaired DNA damage sensor protein that can induce the apoptotic effector cascade (Harris, 1996). Cancer cells also have to overcome the cell-autonomous programme that limits their multiplication by shortenting of telomeres. Most cancer cells abrogate this by upregulation of the telomerase enzyme which adds hexanucleotide repeats onto the ends of telomeric DNA (Bryan & Cech, 1999; Shay & Bacchetti, 1997). The fifth barrier to overcome in cancer is to maintain oxygen and nutrient supply to the tumor, which means there must be a capillary blood vessel within 100μm from all cells (Tonini *et al.*, 2003). The most important molecule that controls blood-vessel morphogenesis is vascular endothelial growthfactor A (VEGF A) that is required for chemotaxis and differentiation of endothelial precursor cells, endothelial proliferation as well as assembly of endothelial cells into vascular structures and angiogenic remodeling (Adams & Alitalo, 2007). In hypoxic cells the hypoxia inducible factor α activates proangiogenic factors (Liotta & Stetler-Stevenson, 1991). The sixth obstacle to overwin is the limited space for tumor growth, which the cancer cells solve with invasion of other tissues and metastasis. Like the formation of the primary tumor, the success of invation and metastasis depends upon all the other five aquired capabilities (Hanahan & Weinberg, 2000).

1.2 General features of the mammalian cell cycle

The cell cycle is an essential mechanism by which all living organisms reproduce. To produce two identical daughter cells, the cell must replicate its DNA into two perfect copies and then segregate the copies into two genetically identical daughter cells.

The cell cycle consists of four distinct phases: G1 phase, S phase, G2 phase (collectively known as interphase) and M phase. DNA duplication occurs in

S-phase, which takes approximately 10-12 hours in a mammal. When the chromosomes have been duplicated, cells can enter the M-phase where chromosome segregation, mitosis, and cytoplasmic division, cytokinesis, take place. Mitosis and cytokinesis takes typically one hour in a human cell. Mitosis can be divided further into five phases: prophase, prometaphase, metaphase, anaphase and telophase. Between the M- and S phases there are two Gap phases G1 and G2. G1 appears after M-phase and allows the cell to grow and the environment to become favourable before the cell commits itself to enter the S-phase. Therefore the length of G1 can vary depending on external conditions and extracellular signals. If extracellular conditions are unfavourable, cells can delay their process through G1 and even enter a resting state named G0. The second gap-phase G2 takes place between S- and M-phase. There are several checkpoints during the cell cycle. In late G1, there is a restrictionpoint checking that the cell is ready for DNA replication. After passing this point, cells are committed to DNA replication, even if extracellular signals that stimulate growth and cell division are withdrawn. Another restriction point is in the end of G2 when the cells are about to enter M-phase in order to check that all DNA is replicated and that the environment is favourable. In late M-phase, there is an additional checkpoint to check that all chromosomes are attached to the mitotic spindle so the cells can proceed to anaphase and cytokinesis.

In order to maintain correct cell division, the eukaryotic cell cycle is tightly regulated. Temporal activation of different cyclin dependent kinase (Cdk)/cyclin complexes regulates the phases of the cell cycle and drives it forward. Whilst the level of Cdks remains relatively stable during the cell cycle, the level of cyclins generally oscillates. Cyclins are synthesized, bind and activate the Cdk and are then destroyed (Arellano & Moreno, 1997). Cdks are serine/threonine kinases and associate with the cyclin box, a 100 amino acids conserved sequence in all cyclins. The general Cdk activity is very low in early G1, progresses in mid-late G1, S and peak in mitosis for a subsequent rapid decay (Morgan, 1997). There are nine Cdks (Cdk1-9) and at least 16 cyclins, which are linked to different phases of the cell cycle (Johnson & Walker, 1999). In G1, there are three D-type cyclins (D1, D2 and D3) and two E-type cyclins (E1 and E2). The cyclin Ds interact with Cdk4 and Cdk6 whereas cyclin E bind Cdk2, but can also interact and activate Cdk1 (Aleem *et al.*, 2005; Morgan, 1997). Various combinations of D-type cyclins are expressed in different cell types, whereas cyclin E–Cdk2 complexes are expressed ubiquitously. Both the D- and E-type cyclins, and their associated kinases, had been thought to be necessary and "rate-limiting" for entry into and progression through the G1 phase of the cell cycle (Sherr

& Roberts, 2004). The cyclin D-dependent kinases are activated by mitogenic signaling and phosphorylate Rb family members (Rb, p107, and p130), thus releasing E2F that activates a battery of genes whose products are required for DNA metabolism and replication (Trimarchi & Lees, 2002). Additionally, cyclin D–Cdk complexes promote G1 progression by sequestering proteins of the Cip/Kip family, including $p27^{Kip1}$ and $p21^{Cip1}$, both inhibitors of Cdk2 (Polyak et al., 1994; Reynisdottir et al., 1995).

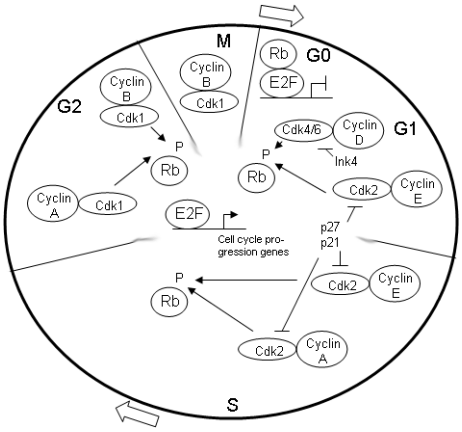

Figure 1. A simplified illustration of the cyclin/Cdks and their inhibitors within the mammalian cell cycle.

The activity of cyclin E–Cdk2 is maximal at the G1- to S-phase transition and their downstream targets include a subset of the G1 inhibitors that are also targeted by the D-type cyclins: Rb and $p27^{Kip1}$. Cyclin E–Cdk2 does not sequester $p27^{Kip1}$, but rather phosphorylates it on a single threonine residue, T187, (Sheaff et al., 1997; Vlach et al., 1997) providing a recognition motif for SCF^{Skp2}, an E3 ligase that targets phosphorylated $p27^{Kip1}$ for ubiquitination and subsequent degradation (Elledge & Harper, 1998). SCF^{Skp2} will be discussed further in paper I and II. In early S-phase, there is an abrupt decay of cyclin E–Cdk2 activity that results from cyclin E degradation. A prior phosphorylation by both glycogen synthase kinase 3 beta (GSK-3β) and Cdk2 itself is required to target cyclin E for ubiquitination by the SCF^{Fbw7} E3 ligase, leading to proteasomal degradation (Clurman *et al.*, 1996; Welcker *et al.*, 2003; Won & Reed, 1996). In late G1, another cyclin that pairs with

Cdk2, namely cyclin A, makes entrance. Cyclin A-Cdk2 activity steadily increases as cells begin to replicate their DNA, and do not decline until cyclin A is degraded in early mitosis. Cyclin A–Cdk2 is thought to phosphorylate substrates that start DNA replication from preassembled replication initiation complexes (Krude et al., 1997) and in the same manner as cyclin E, cyclin A2 is induced by E2F. During G2 and M-phase cyclin A and and Cyclin B together with Cdk1 control the cell cycle process (Arellano & Moreno, 1997). However, during the last few years knockout studies in mice have shed new light over how cdks regulate the cell cycle. Mouse embryos lacking all interphase Cdks (Cdk2, Cdk3, Cdk4 and Cdk6) undergo organogenesis and develop to midgestation. In these embryos, Cdk1 binds to all cyclins, resulting in the phosphorylation of the retinoblastoma protein pRb and the expression of genes that are regulated by E2F transcription factors, suggesting that Cdk1 compensates for loss of any of Cdk2, Cdk3, Cdk4 and Cdk6 (Santamaria *et al.*, 2007).

There are two major families of Cdk inhibitors (CKIs): the Ink4 family $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ that inhibit Cdk4/6 as well as the Cip/Kip family: $p27^{Kip1}$, $p21^{Cip1}$and $p57^{Kip2}$ that inhibit a broader spectrum of cyclin-cdk complexes (for a review see (Vermeulen *et al.*, 2003; Vidal & Koff, 2000)). The Cip/Kip family proteins have also shown to be involved in other activities such as regulation of actin filaments, for reviews see (Denicourt & Dowdy, 2004; Sicinski *et al.*, 2007). Additionally, Wee-1 catalyzes an inhibitory tyrosine phosphorylation of cyclin B/Cdk1 and operates at the G2 to M-phase checkpoint. Cdc25 is a phosphatase that removes the inhibitory phosphate added by Wee-1, rendering the complex active (for a review see (Pines, 1999)).

1.3 Brief Overview of Transcriptional Regulation

Eukaryotic transcription is an extremely complex and tightly regulated process. The components involved in regulation of gene transcription are: DNA-binding transcriptional activators and repressors and their cofactors, general transcription factors, RNA polymerase I-III complexes and chromatin-structure regulatory factors. RNA polymerase I transcribe rRNA, RNA polymerase II (PolII) transcribe protein coding genes whereas RNA polymerase III facilitate transcription of 5S RNA/tRNA. Only PolII dependent transcription will be discussed here.

1.3.1 Initiation of transcription

Initiation of transcription requires a great mass of proteins including the general transcription factors TFIIB, TFIID, TFIIE, TFIIF, TFIIH and TFIIA. (For a review see (Reese, 2003)). The first step is recognition of the target promoter by TATA-binding protein (TBP) and the TBP associated factors (TAFs), which all are parts of TFIID. TAFs recognize the promoter sequences Inr (TAF2) and DPE (TAF6). Upon binding DNA, TFIID makes a bending of the DNA-helix, creating a platform for the remaining general transcription factors. TFIIB subsequently binds in order to stabilize the interaction between the promoter and TFIID. TFIIB then recruites the PolII-TFIIF complex and thereafter the two final basal transcription factors: TFIIE and TFIIH bind in into this preinitiation complex (PIC). TFIIH possess helicase activity and unwinds DNA at the promoter in an ATP-dependent manner (for a review see (Coin & Egly, 1998; Dvir *et al.*, 2001)). In order to induce promoter release and transcriptional elongation, phosphorylation of the C-terminal tail of polII occurs to release polII from the PIC. PolII is suggested to be phosphorylated by positive transcriptional elongation factor b (PTEFb) which is a complex consisting of Cdk9 and CyclinT1, T2 or K (for a review see (Conaway et al., 2000)). TFIIH is also suggested to be involved in phosphorylation of the CTD tail of PolII (Cowling & Cole, 2007). Also other factors are involved in promoter release. One example is elongin that consists of elongin A, B and C and function by suppressing pausing of PolII at the promoter (Aso et al., 1995).

1.3.2 Coregulators

Besides the general transcription factors, a gene needs specialized coregulators, coactivators and cosupressors, in order to be transcribed. The coactivators can roughly be divided into three classes (Spiegelman & Heinrich, 2004). The first group consists of proteins that modify histones (discussed below), examples are Gcn5, PCAF, p300 and CBP (CREB-binding protein). The second group comprises proteins that bind to transcription factors, recruite RNA polymerase II and interact with the basal transcription machinery, such as the mediator complex. The mediator is a large protein complex containing up to 30 subunits and is conserved from yeast to human. The mediator brings activators and PolII together, functioning as a bridge by transduction of regulatory signals (Kornberg, 2005; Malik & Roeder, 2005). Noteworthy, the mediator is not only activating transcription, but also plays a role in repression of transcription (Kornberg, 2007). The third group of co-regulators is ATP-dependent DNA-unwinding chromatin remodelling complexes such as members of the

yeast Swi/Snf family, with the mammalian homologs BRG1 or BRM (Featherstone, 2002; Spiegelman & Heinrich, 2004), which will be discussed below.

Corepressors have a densing effect on chromatin structure and they generally function by either recruiting histone deacetyl transferases (HDACs) or by physically interfering with activator complexes or PIC. Examples of co-repressors are SMRT (silencing mediator of retinoic and thyroid hormone receptor) and N-CoR (nuclear hormone receptor co-repressor) (Wong & Privalsky, 1998), for a review see (Privalsky, 2004).

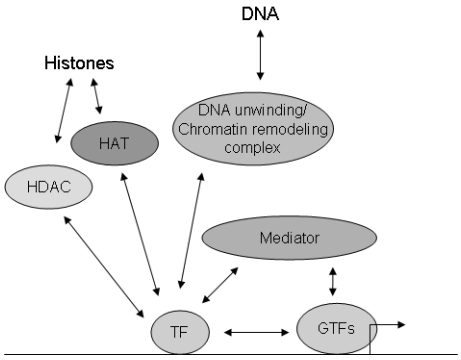

Figure 2. Some of the components involved in transcriptional regulation

1.3.3 Histone modifications and chromatin remodeling

The eukaryotic DNA is neatly wrapped around histone particles and packed into nucleosomes. One histone octamer is build up by two copies of each of the histone subunits H2A, H2B, H3 and H4. DNA is wrapped two superhelical or 1.65 turns around each histone, making 14 contact points between the histone and DNA which results in a very stable protein-DNA complex. Histone H1 is not a part of the histone octamer, but it functions as a linker twisting and folding the nucleosome particles into a very tightly packed chromatin structure. For transcription to occur, the dense heterochromatin must be transformed to a more lose euchromatin to enable the transcriptional machinery to bind DNA. Two mechanisms have evolved to modify chromatin structure: modifications of histones and nucleosome remodeling (Spiegelman & Heinrich, 2004).

Histones are known to be targeted by several post-translational modifications including phosphorylation, acetylation, ubiquitylation and methylation (Strahl & Allis, 2000). Acetylation of histones has proved to be associated with less tight chromatin and activation of transcription whereas deacetylation of histones is connected to silent genes. Histone acetyl transferases (HATs) acetylate lysine residues in the N-terminal tail of histones. Two superfamilies of HATs have been characterized: the GNAT (Gcn-related N acetyltransferase) family and the MYST (MOZ, Ybf 2/Sas3, Sas2 and Tip60) family and additionally, CBP/p300 that not fits into any of these families (Bazan, 2008; Hodawadekar & Marmorstein, 2007). Among the Gcn-related acetyltransferases some examples are Gcn5 and PCAF (p300/CBP-binding protein-associated factor). Gcn5 prefers acetylation of H3, but also acetylate H4, and is a component of the SAGA complex (SPT/ADA/GCN5/Acetyltansferase) which consists of about 20 proteins, most of them conserved from mammals to yeast. CBP/p300 acetylates all four core histones and PCAF interacts with H3 and H4, but not H2A and H2B. Both PCAF and CBP/p300 acetylate other proteins than histones, such as p53. One example from the MYST superfamily is Tip60 that particularly acetylates histone H2A/H4, for a review see (Sapountzi et al., 2006).

Histone deacetylases (HDACs) remove acetyl residues from the N-terminal tail of histones and thereby create a more dense chromatin structure that leads to repression of transcription. Mammalian HDACs are classified into HDAC I-III based upon sequence similarity to the yeast HDACs (Rpd3, Had1 and Sir2). HDAC class I (HDAC1, 2, 3 and 8) and class II (HDAC4, 5, 6, 7, 9 and 10) are the best characterized HDACs. HDACs are recruited to transcriptional units by co-repressors such as Sin3 and NCoR/SMRT (Alland et al., 1997).

Another way of regulating the accessibility of DNA is by ATP-dependent chromatin remodelling. ATP-dependent complexes such as Swi/Snf, RSC, Mi-2/NuRD alter chromatin structure by unwinding DNA and changing the location or conformation of the nucleosome without covalent modifications. The Swi/Snf complex family was first found in yeast and its most known member is Swi2/Snf2 with the mammalian homolog hBRM/hSNF2 and hBRG1/hSNF2b. Swi/Snf complexes can function as either activators or repressors, for a review see (Martens & Winston, 2003). Some transcription factors have been reported to interact with Swi/Snf complexes, including the Myc oncoprotein and the C/EBP transcriptional activator (Naar et al., 2001).

1.4 Ubiquitin regulates biological processes

Before the ubiquitin-proteasome system was discovered, the lysosome was believed to degrade both exogenous and endogenous proteins. However, this rather unspecific lysosomal degradation, in the case of intracellular proteins, proved to have a much more sophisticated "cousin", the ubiquitin proteasome system, which degrades endogenous proteins in a specific manner (Ciechanover et al., 1980; Hershko et al., 1980). The discovery of the ubiquitin-proteasome system resulted also in the realization that regulated protein degradation is involved in a wide range of cellular processes such as the cell cycle and cell division, apoptosis, transcription, antigen presentation, signal transduction, receptor mediated endocytosis, protein quality control and metabolic pathways (Ciechanover, 2005). Altogether, it is estimated that several percent of the human genome is devoted to the ubiquitin pathway taking into account both proteolytic and non-proteolytic functions (Semple, 2003).

1.4.1 Ubiquitylation-a multistep process with different fates

Ubiquitin is a 76-amino acid polypeptide (Goldstein, 1974; Goldstein, 1975; Schlesinger et al., 1975) that is highly conserved and expressed in all eukaryotes. Attaching a chain of ubiquitin molecules on a target substrate is a process termed ubiquitylation. Ubiquitylation is a multistep reaction involving at least three types of enzymes E1, E2 and E3 (Hershko et al., 1983). At first, E1, ubiquitin activating protein, forms a thiol-ester bond with the C-terminal glycine on the ubiquitin protein, a process that requires ATP. Secondly, E2, ubiquitin conjugating enzyme, accepts ubiquitin from the E1 by a transesterification reaction. E3 then binds ubiquitin charged E2 as well as the target protein and facilitates formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and the ε-amino group of a lysine residue on the target substrate. When one ubiquitin molecule has been attached to the target protein, further ubiquitins can be attached to a lysine residue on the surface of the first ubiquitin molecule, in order to build a poly-ubiquitin chain, or the target protein can remain mono-ubiquitylated. (For reviews see (Fang & Weissman, 2004; Pickart & Eddins, 2004; Weissman, 2001)). Ubiquitin has seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) and all seven residues have been found to participate in ubiquitin-ubiquitin interactions, which gives a potential for enormous complexity (Peng et al., 2003). However, ubiquitins are generally linked through K48, K63, or in some cases K29 (Pickart, 2001; Weissman, 2001). The K48 linked polyubiquitin chains are recognized by the 26S proteasome for subsequent degradation, which will be described in more

detail below. K63 linked ubiquitin chains label target proteins for a range of non-proteasomal faiths. K63 linkage has been implicated in DNA-repair (Spence et al., 1995), ribosome modification (Spence et al., 2000), activation of IκB kinase (Deng et al., 2000), endocytosis (for a review see (Bonifacino & Weissman, 1998; Rotin *et al.*, 2000)) and transcriptional activation (Adhikary et al., 2005). K29-linked ubiquitin chains have been suggested to participate in lysosomal degradation of proteins (Chastagner et al., 2006) and K29/K33-linked mixed ubiquitin chains have recently been implicated in regulation of AMP-activated protein kinase (AMPK)-related kinases (Al-Hakim et al., 2008).

The 26S Proteasome

The 26S proteasome recognizes polyubiquitylated proteins and degrades them to small peptides. A minimum of four ubiquitin molecules linked to each other are required for recognition by the proteasome (Thrower et al., 2000). The 26S proteasome is composed of two multisubunit complexes; a core protease, named the 20S proteasome and a regulatory element, termed the 19S regulatory particle. The 20S proteasome has a barrel-formed structure with four ring-formations consisting of two copies of 14 different proteins (α1-7 and β1-7) arranged as α1-7, β1-7, β1-7, α1-7. The proteolytic lumen of the proteasome is build up of the β1, β2 and β7 subunits, which forms a catalytic active site in the middle of the cylinder. The regulatory particle is a 20-subunit complex which binds to one or both sides of the 20S cylinder, positioning itself as a gatekeeper to the catalytic core. The 19S regulatory particle is composed of two multisubunit complexes; the 19S base or APIS, containing six ATPases (Rpt1-6) and three non-ATPase subunits (Rpn1, 2 and 13) and the lid consisting of the remaining Rpn subunits (Rpn3-12). The non-ATPases Rpn1, 2 and 13 bind to the ATPase ring and mediate the interaction to the lid. The function of most subunits of the lid is presently unknown, but some lid-subunits posses deubiquitinating (DUB) activity (see below) and one subunit (Rpn10/S5a) has an ubiquitin interaction motif, which binds polyubiquitin chains (Elsasser et al., 2004; Verma et al., 2004). Also Rpn13 in the 19S base subunit has shown to interact with ubiquitin molecules (Husnjak *et al.*, 2008). The ATPases in the base subunit unfold the deubiquitylated target protein and then promote the opening of the pore and access to the catalytic core (Benaroudj et al., 2003). The overall 26S proteasomal protein degradation is energy consuming and hydrolyzes ATP with an unknown mechanism. It has been suggested that the energy-dependent steps are linked

to substrate unfolding, translocation and deubiquitylation (Baumeister *et al.*, 1998; Pickart & Cohen, 2004).

Ubiquitylation is not an irreversible process. Deubiquitinating enzymes (DUBs) remove ubiquitin molecules from mono- or polyubiquitylated proteins. As mentioned above, one function of DUBs are to be parts of the proteasomal lid subunit, where they cleave off and recycle ubiquitin molecules from the proteins that are about to get degraded by the proteasome (Verma *et al.*, 2002).

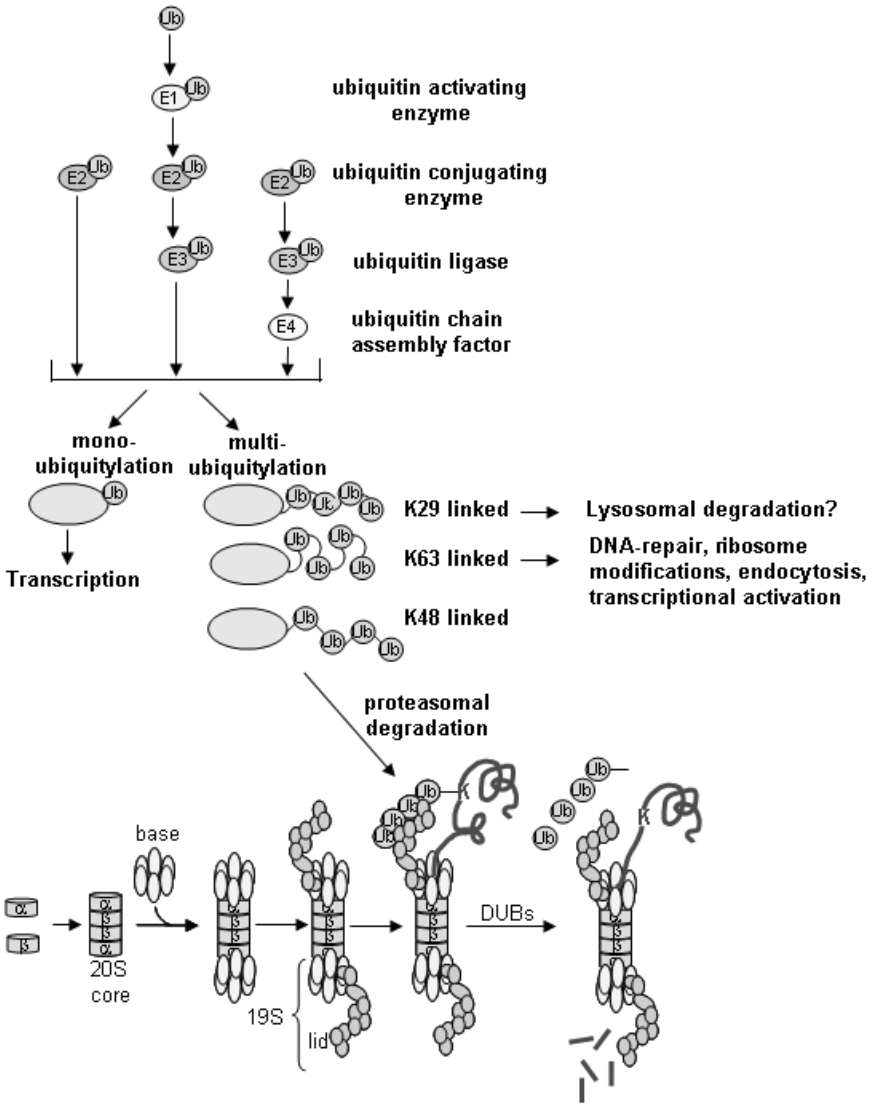

Figure 3. Components involved in the ubiquitin-proteasome system. (Printed with permission from N. von der Lehr.)

1.4.2 E3 ligases

There are two E1 activating enzymes, dozens of E2 conjugating enzymes and hundreds E3 ubiquitin ligases characterized until date (Li & Ye, 2008). Under certain circumstances also an additional enzyme, an E4 ubiquitin elongating factor, are required for extension of the ubiquitin chain (Hoppe, 2005; Koegl et al., 1999).

E3 ubiquitin ligases are classified into three major classes; HECT (Homologous to E6-AP Carboxyl Terminus) (Huibregtse et al., 1995; Scheffner et al., 1993), RING (Really Interesting New Gene) finger (Lovering et al., 1993) and U-box E3 (Aravind & Koonin, 2000; Hatakeyama *et al.*, 2001; Koegl *et al.*, 1999).

HECT

The first E3 ligase to be identified was the E6-AP (E6-Asociated Protein) that mediates ubiquitylation of p53. E6 proteins of the oncogenic human papilomavirus (HPV) were discovered to bind p53 and stimulate its degradation which is catalysed by E6-AP (Scheffner et al., 1993; Scheffner et al., 1990). It was then found that a number of proteins share homology with the carboxyl-terminal half of E6-AP and thus these where given the name HECT (Homologous to E6-AP Carboxyl Terminus) domain proteins (Huibregtse et al., 1995). The HECT domain is approximately 350 amino acids long and highly conserved. A cystein that is positioned about 35 amino acids upstream of the C-terminus accepts ubiquitin from bound E2s for subsequent transfer to the substrate. The amino-terminal part of the HECT E3 ligase recognizes the target substrate and regulate subcellular localization (Weissman, 2001).

RING

The RING finger E3 contains an octet of histidine and cystein residues building up a zinc binding domain and is structurally classified as either RING-H2 which contains two histidines or RING-HC, which only possess one histidine. The majority, but probably not all, of the RING finger domain proteins function as E3 ligases (Lorick et al., 1999). The RING finger E3 ligases can be divided into several subgroups on the basis of structural or molecular similarities. The subclasses are; Cullin-RING, APC/C (anaphase promoting complex/cyclosome) and single-polypeptide RING-finger (SPRF) E3s. The Cullin-RING E3 is the largest subclass and can be further divided into seven subgroups containing the Cullins: 1, 2, 3, 4a, 4b, 5 and 7 respectively, that together with an E2-binding RING finger protein (Rbx1 or Rbx2) form the E3 core. Below I will describe the most well-known Cullin-RING E3s: SCF (Skp1-Cullin-F-box protein) and CBC (Cul2-elongin B-elongin C) in more detail.

The SCF complex consists of Rbx1, Skp1, Cul1 and an F-box protein, which is the target recognition component of the E3 ligase. The F-box protein has an F-box domain that mediates interaction with Skp1 and a substrate interaction domain such as WD40 repeats or leucin rich repeats

(LRR), which interacts with the target protein. There are dozens of F-box proteins identified, but one of the most studied is the LRR F-box protein S-phase associated kinase associated protein 2 (Skp2). The expression of Skp2 varies around the cell cycle and is first detected at the G1/S transition, then it accumulates during S/G2 transition and finally drops as the cells proceed through M-phase (Lisztwan et al., 1998). Skp2 is suggested to recognize many substrates involved in cell cycle progression including; p21 (Bornstein et al., 2003; Yu et al., 1998b), p27 (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999), p57 (Kamura et al., 2003), Cyclin E (Nakayama *et al.*, 2000; Yeh *et al.*, 2001), E2F-1 (Marti et al., 1999) and CyclinD (Yu et al., 1998b). p27 is the most established target of Skp2 and in $Skp2^{-/-}p27^{-/-}$ double knock out mice, most of the cellular abnormalities apparent in $Skp2^{-/-}$ mice were not evident, suggesting p27 to be the primary target of Skp2 (Nakayama *et al.*, 2004). There are several evidences implicating that Skp2 is an oncogene (Yamasaki & Pagano, 2004). Skp2 expression correlates with tumour malignancy and agressivenessnad is associated with poor prognosis in breast carcinomas, colorectal carcinomas, oral squamous cell carcinomas, gastric carcinomas, prostate cancers, lymphomas and human astrocytic gliomas (Bloom & Pagano, 2003; Guardavaccaro & Pagano, 2004).

The CBC complex comprises Rbx1, Cul2, elongin B, elongin C and VHL. The CBC-complex show structural and sequential similarities to the SCF complex. Cul1 and Cul2 as well as Skp1 and elongin C share amino acid sequence homology. Moreover, the pattern of hydrophobic amino acids of the Skp1-binding F-box sequence motif matches that of the VHL alpha-domain. The similarities include both residues involved in elongin C binding and residues involved the alpha-domain stabilization. However, there is little sequential similarity and no cross-reactivity occurs, which suggests a loose structural similarity between the F-box sequence motif and the VHL alpha-domain (Stebbins et al., 1999). VHL recognizes the hypoxia inducible factor α (HIFα) transcription factors; HIF1α, HIF2α and HIF3α for oxygen-dependent degradation (Maxwell *et al.*, 1999; Maynard *et al.*, 2003). Oxygen is needed for hydroxylation of HIFα by prolyl-hydroxylases which is a pre-requisite for recognition by VHL (Bruick & McKnight, 2001; Epstein *et al.*, 2001; Semenza, 2001). In hypoxic conditions, HIFα accumulates and heterodimerize with HIFβ subunits in order to bind DNA and activate transcription of hypoxia induced genes. The features of VHL and oxygen sensing will be described in more detail in chapter 1.6.

The APC/C was identified as an E3 that mediates degradation of cyclin B and thereby promotes M-phase exit. The APC/C complex consists of 11

subunits, including APC2 and APC11, which are similar to Cul1 and Rbx1, respectively (Yu et al., 1998a).

The SPRF E3s consist of a single polypeptide containing a RING-finger domain. The wide range of SPRF E3 include Cbl, that degrades epidermal growth factor and platelet-derived growth factor (Joazeiro et al., 1999), Mdm2, that regulate p53 levels in the cell (Honda & Yasuda, 1999), IAPs (inhibitor of apoptosis) (Yang et al., 2000) and Parkin (Imai et al., 2001; Shimura et al., 2001; Weissman, 2001; Yang et al., 2000).

U-box and others

The U-box domain is distantly related to the RING finger domain in sequence, but without conserved zinc coordinating residues (Fang & Weissman, 2004). UFD2 was the first U-box to be implicated in ubiquitylation. UFD2 was first of all believed to be an E4 ubiquitin chain elongation factor cooperating with the E3 UFD4 (Koegl et al., 1999). However, now UFD2 is known to be a U-box E3 that can mediate ubiquitylation independent of other E3s (Aravind & Koonin, 2000; Hatakeyama *et al.*, 2001). Several U-box E3s have now been discovered, among them the CHIP (Carboxyl-terminus of Hsc70 Interacting Protein) E3 ligase, that interacts with the molecular chaperone Hsp90 in order to degrade unfolded proteins (Connell et al., 2001; Jiang et al., 2001; Meacham et al., 2001).

The PHD (Plant Homeo-Domain) or LAP (Leukemia-Associated Protein) is a RING finger variant, but it possesses a cystein instead of one of the histidines and an invariant tryptophane in the zinc binding motif (Capili et al., 2001). PHD finger dependent E3 activities has been shown for mammalian proteins such as MEKK1 and several herpes virus encoded PHD finger proteins have been implicated in ubiquitylation of major histocompatibility complex (MHC) class I (Boname & Stevenson, 2001; Coscoy *et al.*, 2001; Lu *et al.*, 2002). The PHD finger's involvement in ubiquitylation is not clear and there have been conflicting reports (Scheel & Hofmann, 2003).

1.4.3 Ubiquitin in transcriptional activation

Ubiquitylation has been implicated not only in degradation, but several non-proteolytic processes such as receptor internalization (Terrell et al., 1998), ribosome function (Spence et al., 2000), nucleotide excision repair (Russell et al., 1999) as well as transcriptional activation, which will be discussed here. One way that the ubiquitin-proteasome pathway can regulate transcription is to degrade transcriptional activators that have fulfilled their

mission. The promoter is thereby cleared for fresh proteins to bind and a new round of transcription can take place. Activator turnover has indeed been coupled to transcriptional activation in some systems (Muratani & Tansey, 2003). One of the first examples of this is shown by Natesan and colleagues when they showed an inverse correlation between protein levels and potency as activators (Molinari et al., 1999). Further, the transcriptional activation domain (TAD) of several transcriptional activators overlaps the degradation domain, the degron, (Kodadek *et al.*, 2006; Muratani & Tansey, 2003). This overlap was first shown for the transcription factor c-Myc (Salghetti et al., 1999) and subsequently for other short-lived transcription factors with an acidic domain (Salghetti et al., 2000). Moreover, Tansey and co-workers showed that the ubiquitin ligase Met30 is essential for both the destruction and activity of a VP16 transcription factor implying that activator ubiquitylation is coupled to transcription and might be needed for active transcription (Salghetti et al., 2001). The growing number of E3 ligases that also are transcriptional coactivators adds to the idea that transcriptional activation and ubiquitylation are connected (Muratani & Tansey, 2003).

Active promoters have also been shown to bind the ATPase base of the proteasome. This was first shown for Sug1/Rpt6 and subsequently Sug2/Rpt4, which were first of all identified as activators of transcription and later as parts of the 26S proteasome (Rubin et al., 1996; Russell et al., 1996; Swaffield et al., 1992). Further, Johnston and colleagues showed that ATPases of the 19S subcomplex associates with genes that are being transcribed (Ferdous et al., 2001; Gonzalez et al., 2002). The indications that the human estrogen receptor α (ER-α) is turned over at promoters in a periodical manner gives us further insights into this matter (Reid et al., 2003). Chromatin immunoprecipitation (ChIP) data reveal that ER-α cycles on and off the promoter at a periodicity of approximately 45 minutes together with various transcriptional regulators including general transcription factors, polymerase components, coactivators and ATPases that cycled with a similar periodicity (Reid *et al.*, 2003).

Several models have been suggested to clarify the link connecting transcriptional activation and ubiquitin-dependent degradation. I will give an overview here. The "timer" model (Conaway et al., 2002; Gonzalez et al., 2002), assumes that monoubiquitylation of the activator is a prerequisite for high level gene expression and that extension of this ubiquitin into a K48 linked ubiquitin chain would occur subsequently. During the time frame between these activities, the activated activator activates transcription followed subsequent degradation. In the "black widow", "kamikaze",

"suicide" or "licensing" model (Muratani & Tansey, 2003; Thomas & Tyers, 2000) it is also proposed that ubiquitylation is required for activation of transcription and that the activator has a window of time before it gets degraded. In this model however, it is suggested that not only the ubiquitylation but also the destruction of the activator is a necessary step in order to activate transcription. However, Kodadek and colleagues (Kodadek et al., 2006) suggest a "Second-generation timer model". In this model monoubiquitylation and polyubiquitylation are considered as two separate events which might occur at different rates or even by different E3 ligases. Also the DUBs are suggested as a possible mechanism for inhibition of ubiquitin chains or even reversing the mono-ubiquitylation event. In a very recent report, Kodadek and colleagues extend their model by showing that Sug1/Rpt6 and Sug2/Rpt4 in the 19S base interact with the Gal4 activation domain and exercise unfolding activity on the activator. A prior monoubiquitylation of the activator protect it from unfolding activity and inactivation by the ATPases. If the activator is not monoubiquitylated, the ATPases will unfold the activator, disrupt the activator-promoter complex and inhibit transcription. They expand their model by speculating that the recruited 19S base ATPases transfer to the transcription complex and stimulate promoter release and elongation (Archer et al., 2008).

1.5 The c-Myc transcription factor

The proto-oncogene *c-myc* was first discovered as a cellular homolog of the oncogene of avian myelocytomatosis virus, *v-myc* (Sheiness & Bishop, 1979; Vennstrom *et al.*, 1982). A chromosomal translocation was found in Burkitt's lymphoma involving *c-myc* and the *Ig* loci, where deregulated c-Myc expression was giving rise to constantly high levels of c-Myc (Dalla-Favera et al., 1982). In addition to Burkitt's lymphoma, that is a cancer of the B lymphocytes, Myc deregulation has been detected in a wide range of other human cancers and is often associated with aggressive tumours. Cancers include: lung, breast, cervical, ovarian, prostate and colon carcinoma, as well as lymphoma, melanoma and leukaemia (Dang et al., 1999; Nesbit et al., 1999). The *myc* family has several members which include *N-myc* and *L-myc*. *N-myc* was discovered as homolog to *v-myc* that is amplified in neuroblastoma cell lines (Kohl et al., 1983) and *L-myc* was identified as a *myc* homolog that is amplified in small cell lung cancer (Nau et al., 1985). Myc is highly conserved during evolution and found in all vertebrates, as well as *Drosophila* (Gallant et al., 1996) and Northern sea star, *Asterias vulgaris* (Walker et al., 1992).

1.5.1 Structure of c-Myc and the Myc/Max/Mad network

The structure of the Myc protein comprises an N-terminal transactivation domain (TAD) and a C-terminal bHLHZip (basic helix-loop-helix leucine zipper) domain (Kato et al., 1990; Murre et al., 1989). Within the TAD there are two conserved regions named Myc homology box I and II (MBI and MBII). MBI and MBII were found to be necessary for Myc to transform rat fibroblasts together with Ras. The Myc boxes proved also to be essential for Myc to induce apoptosis and block differentiation (Evan et al., 1992; Freytag et al., 1990). MBII, but not MBI has proved to be necessary for transactivation and coactivator interaction (Kato et al., 1990; Oster et al., 2003). More recently, two additional Myc homology boxes have been identified: MBIII and MBIV, both necessary for cell transformation. MBIII seems to negatively activate pro-apoptotic activity (Herbst et al., 2005) whereas MBIV is needed for Myc-induced apoptosis. Deletion of MBIV seems to promote G2-arrest (Cowling et al., 2006). Both MBIII deletion and MBIV deletion respectively, have defects in activation and repression of several target genes even though they affect Myc transactivation to a much lower extent than a MBII deletion mutant (Cowling et al., 2006; Herbst et al., 2005).

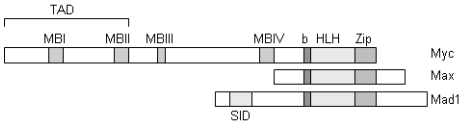

Figure 4. Protein structure of Myc, Max and Mad

Myc binds DNA as a heterodimer together with its obligatory partner-protein Max (Blackwood & Eisenman, 1991). The bHLHZip domains of Myc and Max dimerize and the basic region interacts with the E-box sequence CACGTG and similar sequences in the major groove of DNA. Max on the other hand can bind DNA as a homodimer (Luscher & Larsson, 1999). Furthermore, Max can also heterodimerize with other members of the bHLHzip-family including Mad (Mad1, Mxi1, Mad3 and Mad4, also known as Mxd1-4 (Hurlin & Huang, 2006)), Mnt and Mga. Like Myc these proteins cannot homodimerize and bind DNA on their own, but when forming a heterodimer with Max they recognize and bind E-box sequences.

The Mad proteins antagonize Myc and the Mad/Max complex forms a strong repressor of transcription. The Mad/Max heterodimer repress transcription, at least partly, by recruiting a co-repressor complex Sin3A and Sin3B that interacts with a number of proteins including histone deacetylases HDAC1 and HDAC2 that deacetylates histones in order to produce a more tightly packed chromatin structure, for a review see (Ayer, 1999). Myc is expressed at very low levels in resting cells and its expression is induced when cells begin to grow whereas Max is expressed equally in both resting and proliferating cells and Mad is only expressed in resting cells. Hence, the expression of Myc dependent genes will be repressed by Mad/Max in resting cells, where Mad is expressed at high levels (for a review see (Latchman, 1998)). Mnt, as well as the Mad proteins, possesses a Sin3 interaction domain (SID) and also recruite HDACs in order to repress transcription. However, Mnt is constitutively expressed during the cell cycle (Hurlin et al., 1997) in contrast to the Mad proteins that forms dimers with Max predominantly in resting or differentiated cells (Ayer & Eisenman, 1993). Mga (Max's giant associated protein) is also a transcriptional repressor that binds E-box sequences. Mga's biological function remains unknown, but it has been identified as a part of an E2F6 repression complex (Ogawa et al., 2002).

1.5.2 Myc and transcription

Myc has approximately 25.000 binding-sites in the human genome and potentially regulates a large portion of all human genes. The amount of Myc binding-sites however exceeds the number of Myc molecules in proliferating cells, which indicates that Myc is binding each site only temporarily and work with a "hit and run" mechanism (Adhikary & Eilers, 2005). Myc activates transcription by recruiting cofactors to target gene promoters. These cofactors include histone acetyltransferases (HAT), chromatin modulating proteins, basal transcription factors, DNA methyl-transferases, histone demethylases, E3-ligases, DUBs, AAA ATPases, mediator subunits, kinases and other factors (Adhikary & Eilers, 2005; Eilers & Eisenman, 2008). The first co-factor described was TRRAP (Transactivation/ transformation associated protein) which is a huge protein that binds Myc's MBII in a direct manner (McMahon et al., 1998). TRRAP is a component of the SAGA complex (SPT/ADA/Gcn5/Acetyltansferase) which consists of about 20 proteins, most of them conserved from mammals to yeast (Grant et al., 1998; Saleh et al., 1998; Vassilev et al., 1998). Gcn5 possess histone acetyltransferase (HAT) activity but many of the proteins within the SAGA complex have unknown functions. TRRAP can also

interact with the H2A/H4 histone acetylase TIP60 (Ikura et al., 2000). Histone H4 acetylation has shown to be tightly linked to Myc transactivation (Frank et al., 2001). TIP48 and TIP49 ATPase domain-containing cofactors are also found in complex with TRRAP, but mutational analysis have shown that they can bind Myc independently of TRRAP (Ikura et al., 2000; Wood et al., 2000). A mutation in TIP49 that disrupts the ATPase activity inhibits Myc-induced transformation, probably by enhancing Myc-induced apoptosis, shows that TIP49 is biologically important. TIP48 and TIP49 do not possess HAT activity, but they have been found in complexes with chromatin remodeling activity which displaces nucleosomes (Jonsson et al., 2004). Their structural similarity to the AAA ATPase chaperones (Vale, 2000) suggests that they may have a function in protein folding or assembly of complexes.

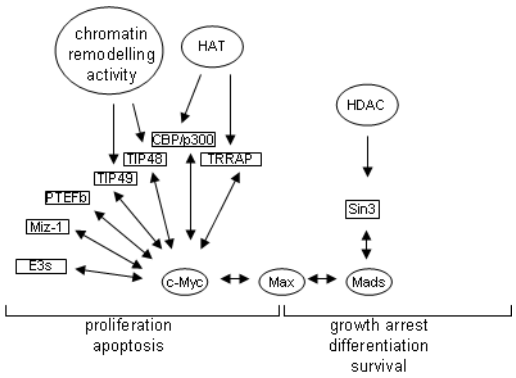

Figure 5. Myc-Max-Mad network and some interacting coactivators. (This figure was remade from an original figure, printed with permission fron N. von der Lehr.)

CBP (CREB-binding protein) and p300 are other HATs that interact with Myc and are recruited to Myc target genes in vivo. There are conflicting reports regarding where in the protein structure CBP and p300 bind Myc. One is implying the transactivation domain (Faiola et al., 2005), but others report that CBP and p300 interact with the C-terminal domain of Myc (Vervoorts et al., 2003). Further, Myc interacts with the mediator complex both *in vitro* and *in vivo* in a TAD dependent but MBII independent manner

(Bouchard *et al.*, 2004). Moreover, CDK9 (cyclin dependent kinase 9) and cyclin T1 subunits of P-TEFb (positive transcription elongation factor b) that phosphorylates RNA polymerase's C-terminal tail prior to promoter release, have shown to interact with Myc's extreme N terminus (Bouchard *et al.*, 2004; Eberhardy & Farnham, 2002; Kanazawa *et al.*, 2003). This is the same region where the mediator is believed to bind Myc (Bouchard et al., 2004). PARP-10 is a protein possessing monoADP-ribose activity and has been co-purified with Myc in Jurkat T cells. Interestingly, it has been shown that PARP-10 overexpression inhibit Myc+Ras transformation of cells, however no effects on Myc transactivation has been observed (Kleine *et al.*, 2008; Yu *et al.*, 2005). Further, Myc has been proposed to promote clearance of RNA pol II from Myc-activated targets and inhibits clearance from Myc-repressed target genes (Barsyte-Lovejoy *et al.*, 2004; Bouchard *et al.*, 2004; Eberhardy & Farnham, 2001; Eberhardy & Farnham, 2002). Moreover, E3-ligases have been implicated in Myc activated transcription. Ubiquitylation by Skp2 (which will be discussed in paper I and II) and HectH9 have proven to increase Myc's transactivational capacity (Adhikary *et al.*, 2005; Kim *et al.*, 2003; von der Lehr *et al.*, 2003).

Myc has also been reported to be involved in transcriptional repression of $p15^{Ink4b}$ and $p21^{Cip1}$. This repression is mediated by Miz-1, which recruits Myc-Max complexes to target promoters where they bind DNA via Miz-1 in order to repress transcription. This Myc-Max recruitment is believed to block Miz-1 from binding p300 and thereby not being able to activate transcription (Peukert et al., 1997; Seoane et al., 2001; Staller et al., 2001; Wu et al., 2003). Additionally, Myc has proven to recruit DNMT3a (DNA methyltransferase 3a) to Myc-Miz complexes suggesting that methylation of DNA is a component of Myc-Miz mediated gene repression (Brenner et al., 2005).

1.5.3 Post-translational modifications and degradation of Myc

The Myc protein has a half-life of 20-30 minutes and is degraded via the ubiquitin-proteasome pathway (Bahram *et al.*, 2000; Flinn *et al.*, 1998; Gross-Mesilaty *et al.*, 1998; Salghetti *et al.*, 1999). Several regions within Myc have proved to be important for its stability including the phosphorylation sites Threonine 58 (T58) and Serine 62 (S62) in MBI. T58 is a mutational hotspot in Burkitt's lymphoma and is frequently mutated in other lymphomas (Bhatia et al., 1993). The absence of T58 results in inefficient ubiquitination and decreased proteasome mediated turnover (Bahram et al., 2000). T58 has been suggested to be phosphorylated by glycogen synthase kinase 3 (GSK3) and S62 by extracellular receptor kinase

(ERK) (Henriksson *et al.*, 1993; Lutterbach & Hann, 1994; Pulverer *et al.*, 1994). The phosphorylation of T58 is dependent on a prior phosphorylation of S62 (Lutterbach & Hann, 1994; Sears *et al.*, 2000). Sears and colleagues have reported that phosphorylation of S62 stabilizes Myc, while T58 phosphorylation destabilizes Myc (Sears et al., 2000). This results in a model with a stable phospho-S62Myc intermediate, which switches to an unstable phospho-S62-phospho-T58Myc when GSK-3 recognizes phospho-S62Myc for further phosphorylation. Sears and co-workers further suggest that the serine/threonine phosphatase PP2A dephosphorylate S62 after a prior cis-trans conformational change performed by Pin1 (Sears, 2004; Yeh et al., 2004). Several E3 ligases have been identified to interact with Myc which will be discussed in the results and discussion chapter.

1.5.4 Biological function of Myc

The Myc transcription factor has been estimated to potentially regulate as many as 15% of the total number of genes in the human genome. Myc affects genes involved in cell cycle regulation, metabolism, protein biosynthesis, cell adhesion, angiogenesis and apoptosis (Dang et al., 2006).

As mentioned earlier, Myc is connected to many human cancers and is often associated with aggressive tumours. Cotransfection of Myc and Ras oncogenes is sufficient to converte rat embryo fibroblasts into tumour cells (Land et al., 1983). Myc is also contributing to tumour formation by upregulating hTert that prevents telomere shortening and thereby promoting imortalisation of cells (Wang et al., 1998; Wu et al., 1999a). Further, Myc has been implicated in genetic instability. Overexpressed Myc induces DNA-damage and abrogates the p53 mediated DNA-damage response, which in turn is enabling cells with damaged genomes to enter the cell cycle (Felsher & Bishop, 1999; Vafa *et al.*, 2002).

Myc affects the cell cycle

The Myc transcription factor is not distributed evenly throughout the cell cycle. Myc levels are increasing from the early G1 phase and peaking at the G1/S transition, for subsequent reduction to a low basic level. Myc is able to overcome the G1/S checkpoint and induce cells to go into S-phase. Myc directly regulates many genes involved in cell cycle regulation. Chromatin immunoprecipitation assays (ChIP) reveal that Myc is present on the promoters of cyclin D1 and D2, cyclin dependent kinase 4 (Cdk4) and cyclin B1 (Bouchard *et al.*, 2001; Fernandez *et al.*, 2003; Hermeking *et al.*, 2000; Menssen & Hermeking, 2002). Additionally, Cdk4 has been implicated in Myc-mediated transformation (Miliani de Marval et al., 2004).

In Drosophila, cyclin A, cyclin B and Cdk4 have been identified as Myc target genes (Orian et al., 2003). Together with Miz1, Myc represses the Cdk-inhibitors $p21^{Cip1}$ and $p15^{Ink4b}$ (Seoane et al., 2001; Wu et al., 2003). $p21^{Cip1}$ repression by Myc is responsible for the failure of Myc-transformed cells to arrest in G1 after DNA damage (Herold et al., 2002; Seoane et al., 2002). The tumour suppressor p53 upregulates $p21^{Cip1}$ after exposure to DNA damage in normal cells. Additionally, Myc represses the Cdk-inhibitor $p27^{Kip1}$ directly as well as indirectly by inducing transcription of SCF^{Skp2} E3 ligase component Cks1 that is involved in ubiquitylation and degradation of $p27^{Kip1}$ (Keller et al., 2007). In addition, Myc represses genes involved in growth arrest e.g. Gadd45 (Barsyte-Lovejoy et al., 2004; Marhin et al., 1997) and Gas1 (Lee et al., 1997). Taken together, these activities of Myc result in activation of the cyclin E/Cdk2 complex, which in turn phosphorylates the Rb protein, resulting in release of the Rb-mediated repression of E2F, which is transformed into an activator. E2F subsequently activates a large number of genes involved in cell cycle progression.

Cell growth and metabolism

An increased cell cycle and proliferation rate demands increased synthesis of proteins. The rate of protein synthesis is increased almost three-fold in Myc-overexpressing fibroblasts in comparison to Myc knock-out cells (Mateyak et al., 1997). A number of ribosomal proteins have been identified as Myc targets to meet the need of increased protein synthesis, including Nucleolin and BN51 (Greasley et al., 2000). Furthermore, overexpression of Myc induces a Drosophila phenotype with large cell size (de la Cova *et al.*, 2004; Moreno & Basler, 2004), but this could not be confirmed in mice (Trumpp et al., 2001). Myc has also proven to be involved in regulation of RNA polymerase I and RNA polymerase III (PolIII) by several groups (Arabi *et al.*, 2005; Gomez-Roman *et al.*, 2003; Grandori *et al.*, 2005; Grewal *et al.*, 2005). Myc binds to the Pol III specific transcription factor TFIIIB and and directly activates Pol III transcription. ChIP experiments revealed that Myc directly binds Pol III-transcribed tRNA and 5SrRNA genes, which are critical for protein synthesis (Gomez-Roman et al., 2003). As mentioned above, Myc has also been demonstrated to be involved in RNA pol I transcription. Myc has been shown to directly stimulate rDNA transcription in response to mitogenic signals and thereby play a key role in regulating ribosome biogenesis and cell growth (Arabi et al., 2005; Grandori et al., 2005; Grewal et al., 2005). To sum up, these observations suggest that overexpression of Myc induces large cell size by activating ribosome biogenesis.

Myc is involved in several metabolic pathways. Many of the key-enzymes in glycolysis are regulated by Myc which is consistent with the implication of Myc as an enhancer of glucose uptake. Target genes in the glycolysis includes hexokinase II, phosphofructokinase, enolase A glucose transporter I (GLUT1) and lactate dehydrogenase A (LDHA) (Kim *et al.*, 2004; Menssen & Hermeking, 2002; O'Connell *et al.*, 2003; Osthus *et al.*, 2000). LDHA has been suggested to be required for transformation since depletion of LDHA abrogates Myc induced transformation of fibroblasts. Another metabolic pathway where Myc plays a key role is the iron metabolism. The genes that encode the iron-responsive element-binding protein-2 (IRP2) and the transferrin receptor are induced by Myc and the H-ferritin and NRAMP1 are repressed by Myc (Bowen *et al.*, 2002; O'Connell *et al.*, 2003; Wu *et al.*, 1999b). This results in an increase of the iron pool available in the cytosol. Further, Myc regulates nucleotide metabolism. This is accomplished by inducing ornithine decarboxylase (ODC) and carbamoyl phosphate synthase-aspartate transcarbamylase-dihydroorotase (CAD) which are essential for nucleotide synthesis (Bello-Fernandez et al., 1993; Miltenberger et al., 1995).

Myc in apoptosis

Myc was found to be a potent inducer of apoptosis in the beginning of the nineties, which at the time seemed somewhat surprising for an oncogene, but was suggested to be a build-in safety mechanism to defend the cell against inappropriate proliferation (Askew et al., 1991; Evan et al., 1992). Curiously, Myc-induced apoptosis requires the same regions of Myc as transactivation and transformation do (Evan et al., 1992). Shortly after Myc had proven to be involved in programmed cell death, the oncoprotein E2F1 and the viral oncogene E1A also showed involvement in apoptosis indicating a general role for oncoproteins in apoptosis (Kowalik *et al.*, 1995; Mymryk *et al.*, 1994; Qin *et al.*, 1994; Rao *et al.*, 1992; Wu & Levine, 1994). Further, Bcl-2 is an oncoprotein that inhibits apoptosis and proved to cooperate with Myc (Vaux et al., 1988). The Eμ-bcl-2/myc mice, overexpressing Myc and Bcl-2, show hyperproliferation of B cells and develop tumours much faster than Eμ-myc mice, that solely overexpress Myc (Strasser et al., 1990). In cell cultures Bcl-2 showed to abrogate Myc-driven apoptosis in a manner that did not affect Myc's proliferative activities (Bissonnette et al., 1992; Fanidi et al., 1992; Wagner et al., 1993).

Myc has shown to induce apoptosis in both a p53-dependent and a p53-independent manner. The p53-dependent pathway is well established and involves Myc activating ARF that inhibits Mdm2 and thereby stabilizing

p53 which leads to either growth arrest or apoptosis (for a review see (Meyer et al., 2006)). Cytochrome c release from the mitochondrial membrane, which is a requirement for formation of the apoptosome and subsequent caspase activation, has shown to be triggered by Myc in several studies (Juin et al., 1999). The pro-apoptotic protein Bax of the Bcl-2 family, has shown to functionally cooperate with Myc (Juin et al., 2002). Bax also mediates apoptosis triggered by Myc and inhibits Myc-induced lymphomagenesis (Eischen et al., 2001). Since apoptosis can be triggered by an activated oncogene it has to be overcome in a cancerous cell. Loss of proapoptotic regulators is one strategy to become resistant to programmed cell death. Mutations in the p53 tumour suppressor, is seen in more than 50% of human cancers and results in the removal of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996).

1.6 The von Hippel-Lindau (VHL) tumour supressor

The VHL tumour suppressor gene was mapped to the short arm of chromosome 3 (3p25-26) in 1988 (Seizinger et al., 1988) and a few years thereafter the gene was cloned (Latif et al., 1993). The VHL gene is widely expressed in most tissues (Richards et al., 1996) even tough tumours of von Hippel-Lindau disease are predominantly seen in the central nervous system (CNS), kidneys, adrenal glands, retina, pancreas, endolymphatic sac and reproductive organs. von Hippel-Lindau disease is a hereditary cancer syndrome that occurs roughly 1 in 36.000 child births (Maher *et al.*, 1991; Neumann & Wiestler, 1991). VHL disease was described in the literature as early as in 1894 by Treacher Collins, but it got its name from discoveries made a decade later by Eugene von Hippel and Arvid Lindau.

1.6.1 The VHL protein and its interaction-partners

The VHL locus encodes a single mRNA that gives rise to two protein products: pVHL30 and pVHL19, due to an internal initiation of translation (Iliopoulos et al., 1998). pVHL19 starts at amino acid 54 of pVHL30. The first "53 amino acid-polypeptide" consists of eight acidic pentameric amino acid repeats and has an unknown function. Very few tumour-associated mutations are found in this region, indicating that both the VHL19 and VHL_{30} gene products are required to be dysfunctional for tumours to arise (Barry & Krek, 2004). No functional distinction is known between pVHL30 and pVHL19 but they localized to different subcellular compartments. pVHL19 is predominantly found in the nucleus and pVHL30, is shuttling between the cytoplasm and nucleus (Duan et al., 1995). There are also

indications that pVHL30 can associate with the microtubule network (Hergovich et al., 2003). Moreover, VHL has proven to be essential during embryonal development. Homozygous *vhl* $^{-/-}$ embryos die between E10,5 and E12,5 due to lack of vasculogenesis of the placenta (Gnarra et al., 1997).

As described above, VHL possess E3 ligase activity and targets hypoxia inducible factor α (HIFα) for oxygen dependent degradation. HIFα comprises HIF1α, HIF2α and HIF3α, with the function of HIF3 being poorly understood (Gordan & Simon, 2007). HIF1 is widely expressed in many cell types whereas HIF2 is mainly expressed in endothelial cells, kidney, heart, lung, small intestine and liver (Gordan & Simon, 2007; Rankin *et al.*, 2007). The HIFs consists of a constitutively expressed β subunit (ARNT) and an oxygen-dependent α subunit. In aerobic conditions the α-subunit, HIFα, gets hydroxylated by prolylhydroxylases (PHD1-3) and recognized by VHL for ubiquitylation and subsequent proteasomal degradation. During limited oxygen conditions, which is often the case in tumours, the PHDs cannot hydroxylate HIFα and the subsequent recognition by VHL is therefore abolished. HIFα accumulates in the cell and dimerise with HIFβ at hypoxia responsive elements (HRE) on target promoters in order to induce hypoxia inducible genes. p300/CBP is recruited to the promoter and a hypoxia response to adapt the cell to low oxygen conditions is turned on. The target genes differ somewhat between HIF1 and HIF2. HIF1 regulates glycolytic enzymes (Hu et al., 2003) whereas HIF2 preferentially regulates EPO (Rankin et al., 2007), OCT4 (Covello et al., 2006), cyclin D1 and TGFα (Raval et al., 2005). It should be noted that HIF2 regulates cyclin D1 only in RCC (renal cell carcinoma) cells (Bindra et al., 2002). VEGF (vascular endothelial growth factor) and ADRP (adipose differentiation-related protein) are examples of common targets for HIF1 and HIF2.

HIF and Myc

During hypoxic conditions, glycolysis replaces oxidative phosphorylation and the cell cycle is slowed down by induction of cyclin dependent kinase (cdk) inhibitors (Dang et al., 2008). The cdk inhibitors p21 and p27 have been reported to be induced by HIF-1 (Carmeliet et al., 1998; Goda et al., 2003). During the last few years, there have been several reports showing functional interaction between HIF and Myc.

Huang and co-workers suggest that HIF-1α induces cell cycle arrest by functionally counteracting Myc which is achieved by displacing Myc-binding from the $p21^{cip1}$ promoter (Koshiji et al., 2004). They further suggest that this also is the case for the Myc-activated genes: MSH2, MSH6 and

Nbs1, which are involved in DNA-repair, where HIF displaces Myc from the transcription factor Sp1 in the Myc target promoter (Huang, 2008; Koshiji et al., 2005; To et al., 2006).

HIF2 promotes cell-cycle progression in hypoxic cells. This correlates with enhanced c-Myc promoter binding, transcriptional effects on both activated and repressed target genes, and interactions with Sp1, Miz1 and Max (Gordan et al., 2007a). HIF-1α contributes to the regulation of growth factor-stimulated glucose metabolism even in the absence of hypoxia (Lum et al., 2007). Both Myc and HIF-1α induce basically all glycolytic enzymes, but additionally Myc promotes mitochondrial biogenesis in order to produce biosynthetic substrates (for a review see (Gordan et al., 2007b).

Semenza and colleagues suggest that HIF1 activates the Myc repressor Mxi, and that HIF1 promotes proteasome dependent degradation of Myc independent of Mxi (Zhang et al., 2007). The Semenza and Dang labs further show that HIF1 and dysregulated Myc cooperate to promote glycolysis by inducing hexokinase2 and pyruvate dehydrogenase 1 (PDK1) as well as VEGF by using a Burkitt's lymphoma model system with inducible Myc (Kim et al., 2007). They also suggest that HIF1 is needed for Myc-mediated tumourigenesis (Gao et al., 2007).

1.6.2 Clinical manifestations of von Hippel-Lindau disease

Before thorough screening of VHL-patients became routine, mean survival age was below 50 years of age. The main cause of death was renal cell carcinoma and heamangioblastoma (Maher et al., 1990; Neumann et al., 1992). The VHL disease can be classified into two subgroups depending on the presence or absence of pheochromocytoma. Type 1 patients are subjected to a low risk of pheochromocytoma, but can develop all other tumours associated with the disease, whereas type 2 patients are exposed to a high risk of pheochromocytoma. Type 2 families are further subdivided into three groups: 2A, 2B and 2C. Type 2A patients have a low risk of renal cell carcinoma whereas type 2B patients have a high risk. Families with type 2C have pheochromocytoma only (Kaelin, 2002).

As mention above, VHL tumours are mainly found in the central nervous system (CNS), kidneys, adrenal glands, retina, pancreas, endolymphatic sac and reproductive organs. Haemangioblastoma of the CNS is affecting 60-80% of VHL patients and is the most common tumour in von Hippel-Lindau disease (Lonser et al., 2003). Haemangioblastoma are highly vascularised, benign tumours in the CNS that can be a risk due to pressure on surrounding tissue and are a major cause of death. Haemangioblastoma can also affect the retina, where it can cause vision defects or blindness. The

major malignant neoplasm in von Hippel Lindau disease is renal cell carcinoma (kidney cancer arising from the renal tubule), which is seen in 24-45% of VHL patients (Choyke et al., 1995; Neumann et al., 1992) Renal lesions are often multiple (Walther et al., 1995) and commonly give rise to metastasis. Metastatic RCC has a very poor prognosis with a mean survival of less then one year (Barry & Krek, 2004). Pheochromocytoma is a neuroendocrine tumor of the adrenal glands which secretes excessive amounts of catecholamines. It arises in 10-20% of VHL patients and is rarely malignant (Walther et al., 1999). Pancreatic involvement is seen in most patients with VHL disease. Although pancreatic cysts are most common, 8-17% of patients with VHL disease develop tumours in pancreas which can be malignant (Hammel et al., 2000).

1.6.3 Mutations in the VHL gene

As mentioned above, VHL is a hereditary cancer syndrome and inheritance of a mutant VHL allele predisposes to tumour formation. VHL behaves as a classic Knudson two-hit tumour suppressor gene. The two-hits occur when heterozygous VHL carriers lose or inactivate their remaining wild type VHL allele by somatic mutation (second hit) (Knudson & Strong, 1972). As many as 20% of VHL patients have no family history of VHL disease, i.e. the first hit mutation occurs de novo (Glenn G, 1999; Sgambati *et al.*, 2000).

Some genotype-phenotype correlations have been found in von Hippel-Lindau disease. Low risk of pheochromocytoma (type 1 VHL disease) individuals, almost exclusively express a truncated or misfolded VHL protein or harbour a mutation that lead to the complete loss of VHL protein. Type 2 VHL disease individuals on the other hand usually carry a missense mutation (Chen *et al.*, 1995; Neumann & Bender, 1998; Zbar *et al.*, 1996). Taken together, this leads to the speculation that pheochromocytoma development reflects a VHL "gain of function", or that pheochromocytoma development requires partial but not complete loss of VHL protein function (Kaelin, 2002).

Known VHL mutations can be viewed in the "VHL mutation database" (http://www.umd.be/VHL/) (Beroud et al., 2000). 823 point-mutations are posted here to date. The most common are R167 (54 cases), R161 (33 cases), V155 (24 cases) and Y98 (23 cases), whose functions are packing of α-helices, elongin C-interaction (R161 and V155) and HIF-interaction, respectively.

1.7 Signalling pathways

Multicellular organisms need a communication system between cells to be able to coordinate their own behaviour with that of other cells for the benefit of the organism as a whole. The mechanism of communication is mainly constituted of extracellular signal molecules produced by cells to attend their neighbouring cells or cells further away in the organism, cell-surface receptor proteins, which bind the signal molecule and transmit the signal into the cell, and intracellular signaling proteins that distribute the signal to appropriate part of the cell. The signaling pathways in mammalian cells can be broadly divided into three pathways: signaling through G-protein linked cell-surface receptors (e.g. adenylyl cyclase (cyclic AMP)), signaling through enzyme-linked cell surface receptors (e.g. Ras, PI 3-kinase, Jak-Stat) and signaling pathways that depend on regulated proteolysis (e.g. Wnt, Notch, Hedgehog, NF-κB) (Alberts *et al.*, 2002).

Of particular relevance for this thesis is the cytokine IFN-γ and its signalling pathway, which will be discussed below.

1.7.1 Interferon-γ

Interferons (IFN), interleukins, growth factors and tumour necrosis factors are members of the large cytokine family involved in cellular communication during cell growth and differentiation, immune response, embryogenesis and many other processes. IFNs respond to double-stranded RNA, a key feature of viral infection and are important in limiting the spread of certain viruses. The most wellknown interferons are α, β and γ. Hematopoietic cells are the major producers of IFN-γ and fibroblasts are a major cellular source of IFN-γ are produced by the infected cell whereas IFN-γ is produced by T-cells and natural killer cells upon antigen presentation (Bach *et al.*, 1997). The interferons are produced very early in infection and are counted as the first line of defence to many viruses. IFN-γ represses proliferation, practices antiviral activities and activates the immunesystem by inducing macrophages, class I and class II MHC, natural killer cells and T-cells (Roitt *et al.*, 1998). IFN-γ is also possessing anti-tumour properties for a review see (Schroder *et al.*, 2004).

The IFN-γ receptor is comprised of two ligand-binding IFN-γ receptor 1 chains associated with two signal-transducing IFN-γ receptor 2 chains and associated signaling machinery. IFN-γ mainly signals through the Jak (Janus kinase) - Stat (signal transducers and activators of transcription) pathway. There are four known Jaks (Jak1-3 and Tyk2) and seven Stats (Stat1, 2, 3, 4, 5a, 5b and 6). For IFN-γ, the associated Jaks are Jak1 and Jak2 which

preferently activate Stat1, but other Stats have also been implicated in IFN-γ signalling. IFN-γ binding causes a conformational change of the IFN-γ receptor, inducing inactive Jak2 kinase to undergo autophosphorylation and activation, which in turn allows Jak1 transphosphorylation by Jak2. Activated Jak1 then phosphorylates each IFN-γ receptor 1 chain to form two adjacent docking sites for Stat1. After binding the IFN-γ receptor, the Stats get phosphorylated and dimerise with each other. The Stat1 dimer enters the nucleus and binds to promoter elements to initiate or suppress transcription of IFN-γ-regulated genes. For a review see (Schroder *et al.*, 2004). Targets genes involved in proliferation includes the G1 repressors Ink4 and the the G1/S repressors Cip/Kip. IFN-γ transcriptionally induces p21 (Kominsky *et al.*, 1998; Xaus *et al.*, 1999) and p27 (Harvat *et al.*, 1997; Mandal *et al.*, 1998; Matsuoka *et al.*, 1999) and downregulate c-Myc (Raveh *et al.*, 1996). IFN-γ affects Myc through several pathways, both Rb dependent and Rb independent as well as by increasing the levels of Mad1 (Dey *et al.*, 1999). In addition, IFN-γ has been reported to destabilize Myc-Max complexes, which correlated with reduced phosphorylation of Myc (Bahram *et al.*, 1999).

2 Aims of this study

The general objective of this study was to gain insight into the function and regulation of the transcription factor and oncoprotein Myc. By gaining more detailed knowledge about the mechanisms which regulate Myc degradation, we wanted to shed light on how Myc is regulated during different cellular conditions. Further we also wanted to investigate the importance of ubiquitylation for the function of Myc in cell cycle regulation and transactivation of target genes. Deeper understanding of Myc regulation and function may potentially result in new strategies and therapeutic applications for treatment of cancer.

2.1 Specific aims

- To identify E3 ligases involved in ubiquitylation and degradation of Myc (paper I, II and III).
- To investigate whether there are E3 ligases that exerts other or additional functions on Myc apart from protesomal degradation (paper I, II and III).
- To elucidate the mechanism by which IFN-γ inhibits Myc function, in particular to evaluate the role of the ubiquitin/proteasome system (paper IV).

3 Results and discussion

3.1 SCF^{Skp2} promotes ubiquitylation and degradation of Myc (paper I and II)

Previous studies have demonstrated that Myc is degraded by the ubiquitin-proteasome pathway and that phosphorylation of Thr-58 plays an important role in this process (Bahram *et al.*, 2000; Flinn *et al.*, 1998; Salghetti *et al.*, 1999; Sears *et al.*, 1999). Our goal was to identify an E3-ligase responsible for ubiquitylation of Myc and screened several E3-ligases, in particular F-box proteins since they often bind phosphorylated substrates. We also checked other proteins of the SCF (Skp1-Cullin-F-box protein) complex for interaction with Myc. We found that the F-box protein Skp2 indeed interacted with Myc both in cells using reciprocal coimmunoprecipitation assays and by bimolecular fluorescence complementation assays, as well as *in vitro* by GST-pulldowns. By cotransfecting the SCF components Cul1, Skp1 and Skp2 together with Myc, we also showed that the Myc-Skp2 interaction takes place in the context of a SCF complex. Skp1 bound Myc only when Skp2 and Cul1 were cotransfected whereas Skp2 interacted with Myc independently of Skp1 and Cul1. We further mapped the interaction domains on Myc to the highly conserved Myc box II (MBII) and the HLHZip domain. Curiously, these regions are also involved in transcriptional regulation and cellular transformation. To address the issue of degradation, we measured Myc protein levels after blocking protein synthesis with cycloheximide (CHX) and found that when cotransfecting Skp2 together with Cul1, Myc half-life was drastically reduced. Also a dominant negative Skp2-mutant, Skp2deltaF which abolishes binding to the SCF-complex, could not degrade Myc. We additionally provided evidence for this by knocking down Skp2 expression with siRNA, which resulted in

more stable Myc. These results imply that Myc is stabilised in cells with reduced SCF^{Skp2} E3-ligase activity. Further, the MBII mutant and the MycdeltaC-terminal+deltaMBII mutant were more stable than Myc wt in CHX-chases, which is compatible with the model that SCF^{Skp2} cannot target Myc for degradation when the Skp2 binding site is deleted. In ubiquitylation assays we showed that cotransfections of Myc and the dominant negative Skp2deltaF drastically reduced the amount of ubiquitylated Myc. We further showed that this effect could be abrogated by additionally cotransfecting Skp2wt. Taken together these results suggest that Skp2 binds to and promotes ubiquitylation of Myc which subsequently results in proteasomal degradation. However, since we have not been able to demonstrate ubiquitylation of Myc by SCF^{Skp2} *in vitro*, the effect we see on Myc ubiquitylation could also be indirect via another E3 ligase.

3.2 The interaction between Myc and Skp2 is cell cycle regulated (paper I)

Since Skp2 is an important protein in cell cycle regulation, mainly by degrading p27, we wanted to test whether the Myc-Skp2 interaction was cell cycle regulated. For this we used peripheral blood lymphocytes (PBL) that were activated by PHA and interleukin-2 (IL-2). Resting PBLs reached S-phase 36 hours post PHA and IL-2 stimulation which was confirmed by FACS analysis and increased cyclin A expression. The Myc level was low in resting cells and increased steadily as the cells progressed through G1-phase with a peak at 12 hours followed by a subsequent decline to a low level when the cells entered S-phase. Myc-Skp2 interaction coincided with decreased level of Myc, increased level of Skp2 and S-phase entry. Myc mRNA and the rate of protein synthesis were constant during the time, indicating that the decreased Myc level was due to degradation. These results suggest that Skp2 participates in Myc turnover at the G1-S phase transition in the cell cycle.

Previous reports have suggested that Myc promotes S-phase entry (Grandori *et al.*, 2000). To see whether Skp2 affects Myc-induced S-phase entry, MycER (Myc fused to the ligand-binding domain of the estrogen receptor) expressing Rat1 cells were induced by 4-hydroxy-tamoxifen (4-OHT) which causes activation of Myc and induces the cells to enter S-phase. Surprisingly, transfection of Skp2 further enhanced the cells to enter S-phase whereas the Skp2deltaF-mutant reduced the ability of S-phase entry. This was also performed in $p27^{-/-}$ cells in order to determine if the effect was indirect via the p27 Cdk inhibitor. Similar results were seen in the

$p27^{-/-}$MEFs expressing MycER, therefore we concluded that the mechanism was p27 independent.

3.3 SCF^{Skp2} stimulates transcriptional activation of Myc targets (paper I)

Since certain E3 ligases in yeast had been reported to also work as activators of transcription (Kuras *et al.*, 2002; Salghetti *et al.*, 2001), we wanted to test whether this was the case also for Skp2 in mammalian cells. We utilised a reporter assay system with the α-prothymosin promoter, which is an established Myc target, fused to the luciferase (Luc) gene. Luc-activity was then measured as a read-out for promoter activity. We found that the cotransfections of Myc and Skp2 enhanced α-proT promoter activity compared to Myc wt alone. Cotransfections with Myc and Skp2deltaF repressed the α-proT activity indicating that transcriptional stimulation by Myc requires intact Skp2 E3 ligase activity. To further strengthen the importance of Skp2, we performed the assay in $Myc^{-/-}$ cells and $Skp2^{-/-}$ cells, respectively. The results indicated that both Myc and Skp2 were required for activation of the α-proT promoter. Further, siRNA oligos targeting Skp2 reduced the activity on the α-proT promoter to the same extent as siRNA against Myc. To make sure that the observed effects on Myc induced transcription by Skp2 siRNA or cotransfections of Skp2deltaF dominant negative mutant were not due to indirect cell cycle effects, we arrested the cells in G1 or S-phase by novastatin, hydroxyurea or aphidicholin. Neither of these cell cycle blocking agents did affect the activity of the α-proT promoter. To determine if these activating effects on transactivation also applied for endogenous targets, we utilized the MycER system and transfected the cells with Skp2 and Skp2deltaF respectively. After activation of MycER we measured mRNA for the Myc-targets cyclin D2 and ODC and found that both cyclin D2 and ODC mRNA were upregulated when Skp2 was transfected and downregulated by Skp2deltaF. In summary, both Myc and Skp2 seemed to be required for activation of the Myc target promoters α-proT, ODC and cyclin D2.

3.4 Skp2 as well as the proteasomal subunit Sug1 are involved in Myc's transactivation (paper I and II)

To strengthen Skp2's role in Myc activated transcription, we investigated whether Skp2 was physically present together with Myc on Myc target promoters. For this we performed chromatin immunoprecipitation (ChIP)

which revealed that Skp2 indeed is present on the cyclin D2 promoter. Additionally, we also found Cul1, ubiquitin as well as the proteasomal subunits Sug1, Rpt3, Rpn7 and α2 on the cyclin D2 promoter. Also, we showed that this was Myc-dependent since none of the proteins Myc, Skp2, Sug1 or ubiquitin, were associated with the cyclinD2 promoter in $Myc^{-/-}$ cells. Further, we showed in coimmunoprecipitations that Myc and the AAA ATPase subunit Sug1 which is a component of the regulatory base of the proteasome, interacted in transfected Cos7 cells. To further address the role of Sug1, we knocked down endogenous Sug1 expression with Sug1 siRNA in cotransfections with the α-proT-Luc construct. The results showed that siRNA knock down of Myc, Skp2 and Sug1, respectively, reduced the activity of the α-proT promoter to a similar extent. This implies that the Sug1 proteasomal subunit as well as Skp2 are necessary for Myc transactivation of the α-proT promoter.

Taken together, this gives us a speculative model, where Skp2 ubiquitylates Myc at promoters, leading to subsequent recruitment of the base of the proteasome and the remaining lid and core proteasome thereafter. Alternatively, the whole proteasome could be recruited at once. Since we have not shown whether SCF^{Skp2} ubiquitylates Myc at target gene promoters, there is a possibility that SCF^{Skp2} ubiquitylates other proteins on the promoter, named protein x in the figure (paper II, fig 3), which are responsible for recruitment of the proteasome. After recruitment, Skp2, Sug1, ubiquitin and/or the proteasome exert coactivator activity, that potentially activates target gene transcription. Ubiquitylated Myc is then degraded and the promoter is cleared for another round of transcription to take place.

The nature of Skp2's coactivator activity is presently unknown. One suggestion is that Skp2 ubiquitylates and degrades negative regulators of transcription and therefore act in a positive way on Myc's transactivation. Another possible way of action is an autoregulatory loop, where Myc needs to be eliminated in order to complete the transcription cycle. Another possibility is that ubiquitylation of Myc or other proteins at the promoter has a non-proteolytic function and the ubiquitin-modification serves to recruit other proteins important for transcription. Since we have shown that the APIS subunit Sug1 interacts with Myc and that siRNA knocking down Skp2 and Sug1 respectively reduced the activity of the α-proT promoter, one plausible guess is that the coactivator activity is mediated by the APIS complex. The APIS ATPases have been implicated in transcriptional activation in yeast through their chaperon-activity. Kodadek and colleagues suggest in a recent model that Sug1/Rpt6 and Sug2/Rpt4 in the APIS

interact with the Gal4 activation domain and exercise unfolding activity on the activator. A prior monoubiquitylation of the activator would protect it from unfolding activity and inactivation by the ATPases. If the activator is not monoubiquitylated, the ATPases will unfold the activator, disrupt the activator-promoter complex and inhibit transcription. The authors further speculate that the recruited 19S base ATPases transfer to the transcription complex and stimulate promoter release and elongation (Archer et al., 2008). If this model is true also for Myc and for mammalian cells, ubiquitylation or rather monoubiquitylation, would protect Myc from being unfolded, thereby maintaining Myc-driven transcription. However, further studies are required to reveal the role of ubiquitylation and AAA ATPase activity in the regulation of transcription.

3.5 Myc is targeted by additional E3s (paper I and II)

We show that Myc and Skp2 interact in the G1-S transition of the cell cycle, but Myc degradation is also substantial during other phases of the cell cycle for instance in early G1 when Myc and Skp2 do not seem to interact strongly. This together with earlier studies showing that Myc degradation was phosphorylation dependent made us believe that Skp2 probably was not the only E3 ligase involved in degradation of Myc. Further, considering the importance of Myc in such a variety of cellular functions it would be surprising if Myc was not tightly regulated on different levels and in different phases of the cell cycle.

Since our Skp2-papers have been published, an additional E3-ligase has been identified for ubiquitylation and degradation of Myc. This E3-ligase, Fbw7, interacts with phosphorylated MBI within Myc's transactivation domain (Welcker *et al.*, 2004; Yada *et al.*, 2004). Our initial hypothesis that an E3-ligase would interact with phosphorylated Thr-58 proved to be right, we just found the "wrong" E3-ligase.

Also the HectH9 E3 ligase has shown to interact and ubiquitylate Myc. However, this ubiquitylation is of a different nature. HectH9 was reported to ubiquitylate Myc by attaching K63-linked ubiquitin chains leading to transcriptional activation without subsequent degradation (Adhikary et al., 2005).

3.6 The tumour suppressor VHL interacts with Myc (paper III)

As mentioned above, we initiated the Skp2-study with screening E3-ligases for interaction with Myc. However, apart from Skp2 we found an additional

E3-ligase, VHL, which is part of a CBC (Cul2-elongin C-elongin B) complex to interact strongly with Myc. We further confirmed this interaction with reciprocal coimmunoprecipitations in transfected Cos7 and 293-T cells, endogenously in P493-6 cells, in living cells with BiFC and in *in vitro* assays. We also showed that Myc preferentially bound the pVHL19 isoform, which is nuclear, even though Myc could bind both pVHL19 and pVHL30. BiFC assays also confirmed that the interacting Myc-VHL complex has a nuclear localisation. We then mapped the binding region on Myc to the MBII and the bHLHzip domain. Interestingly, these are the same regions as are required for Skp2 interaction and additionally, these regions are also involved in transcriptional regulation and cellular transformation.

We utilised VHL-deletion mutants to map the binding site on VHL and found that binding to Myc seemed to involve two potential binding sites. However, when studying VHL's 3D-structure and adding that to the results from the CoIPs with Myc and VHL deletion mutants we could narrow down the binding region by educated guesses. Our proposed binding region is a dual binding site comprising the amino acids 91-113 as well as 195-204 located in the β-domain of VHL. As mentioned above, these regions are just hypothetical and there could also be other explanations, for instance that there could be an inhibitory domain in the VHL114-154 region, since a VHLdelta114-154 mutant interacted strongly with Myc, hypothesising that something that interferes with Myc–VHL interaction binds there. We also wanted to test VHL point mutants commonly found in VHL disease for interaction with Myc. Neither the Y98N mutant, which has been reported not to bind HIF-1α, nor the C162F mutant, which has been reported to not interact with elongin C and the CBC complex, showed any reduction in binding to Myc. Neither did the tumour mutants S65 nor S111, remarkably though the S65 mutant did show stronger interaction with Myc than VHL wt.

An interesting task for the future will be to establish if any mutational hot spots are responsible for interaction with Myc and whether this correlates with any particular type of von Hippel Lindau disease. For this purpose we have utilised the "VHL mutations database" (http://www.umd.be/VHL/) to connect the most common mutations with the literature regarding genotype-phenotype correlations. We plan to clone the most common mutations from each disease type (von Hippel Lindau disease type 1, 2A, 2B and 2C) and investigate their properties with respect to interaction with Myc.

Table 1. Mutants involved in von Hippel Lindau disease.

Mutant aa[1]	Hits on UMD[2]	Disease type	Reference
E52K	1(1)	1	(Olschwang, 1999)
E55E	1(5)	1	(Olschwang, 1999)
R64P	1(4)	2C	(Hoffman *et al.*, 2001)
S65W	4(13)	1	(Chen *et al.*, 1995)
S65L	4(13)	1	(Olschwang, 1999)
E70L	1(5)	1	(Olschwang, 1999)
76delF	9(20)	1	(Zbar *et al.*, 1996)
76delFinsC	6(20)	1	(Crossey *et al.*, 1994; Maher *et al.*, 1996)
S80Q	0(8)	2C	(Zbar *et al.*, 1996)
S80N	2(8)	1	(Olschwang, 1999)
S80R	1(8)	1	(Olschwang, 1999)
P81S	17(19)	1	(Brauch *et al.*, 1999; Zbar *et al.*, 1996)
R82P	2(6)	2B	(Gnarra *et al.*, 1994; Olschwang, 1999)
V84L	1(1)	2C	(Hoffman *et al.*, 2001)
P86S	5(19)	2B	(Olschwang, 1999)
P86A	1(19)	1	(Zbar *et al.*, 1996)
P86L	8(19)	1	(Zbar *et al.*, 1996)
P86R	1(19)	1	(Corn *et al.*, 2003; Stolle *et al.*, 1998)
W88S	4(12)	1	(Olschwang, 1999)
Y98H	21(23)	2A	(Corn *et al.*, 2003; Hsu *et al.*, 2006)
Y98N	1(23)	2B	(Liu & Nussinov, 2008)
G104G	1(4)	1	(Olschwang, 1999)
T105P	3(3)	1	(Olschwang, 1999)
S111R	2(9)	1	(Corn *et al.*, 2003)
S111N	4(9)	1	(Zbar *et al.*, 1996)
Y112H	3(6)	2A	(Barry & Krek, 2004; Rathmell *et al.*, 2004)
Y112N	1(6)	2B	(Barry & Krek, 2004)
Y112stop	2(6)	1	(Zbar *et al.*, 1996)
G114	0(20)	1	(Zbar *et al.*, 1996)
H115Q	2(9)	1	(Olschwang, 1999)
W117C	6(12)	1	(Olschwang, 1999)
L118P	4(6)	1	(Olschwang, 1999)
delta 114–154		1	(Corn *et al.*, 2003; Hsu *et al.*, 2006)
F119S	1(6)	2C	(Hoffman *et al.*, 2001)
F119L	5(6)	2B	(Barry & Krek, 2004)

Mutant aa[1]	Hits on UMD[2]	Disease type	Reference
V130L	6(8)	1	(Olschwang, 1999)
N131K	1(3)	2A	(Olschwang, 1999)
F136S	4(11)	1	(Olschwang, 1999)
P154P	1(4)	2A	(Olschwang, 1999)
P154L	2(4)	2A/2B	(Corn *et al.*, 2003; Hsu *et al.*, 2006)
V155	1(24)	1?	(Japan, 1995)
Y156D	1(6)	2B	(Olschwang, 1999)
Y156C	1(6)	1	(Olschwang, 1999)
T157I	5(8)	2A	(Olschwang, 1999)
L158P	7(18)	1	(Gnarra *et al.*, 1994)
R161G	3(33)	2B?	(Zbar *et al.*, 1996)
R161stop	18(33)	1/2	(Stolle *et al.*, 1998; Zbar *et al.*, 1996)
R161P	1(33)	1/2	(Zbar *et al.*, 1996)
R161Q	7(33)	2A	(Olschwang, 1999; Stolle *et al.*, 1998; Zbar *et al.*, 1996)
C162R	6(17)	1	(Zbar *et al.*, 1996)
C162Y	3(17)	1	(Zbar *et al.*, 1996)
C162F	2(17)	1	(Hoffman *et al.*, 2001; Stolle *et al.*, 1998)
C162W	4(17)	1/2B	(Olschwang, 1999)
V166F	4(8)	2A	(Olschwang, 1999)
R167W	24(54)	1/2A/2B	(Olschwang, 1999; Stolle *et al.*, 1998; Zbar *et al.*, 1996)
R167Q	28(54)	1/2B/2	(Olschwang, 1999; Rathmell *et al.*, 2004; Stolle *et al.*, 1998; Zbar *et al.*, 1996)
V170G	2(10)	1	(Olschwang, 1999)
L188P	1(10)	1	(Olschwang, 1999)
L188V	5(10)	2C	(Hoffman *et al.*, 2001; Hsu *et al.*, 2006; Stolle *et al.*, 1998)
L188Q	1(10)	1	(Chen *et al.*, 1995)
Q195 ter	2(2)	2B	(Crossey *et al.*, 1994)
R200W	3(3)	Chuvash Polycythemia	(Rathmell *et al.*, 2004)

[1]Mutant aa refers to the mutated amino acid. [2]Hits on UMD refers to the number of reports on "VHL mutations database" (Beroud et al., 2000) http://www.umd.be/VHL/. The numbers are written in the format: # reports for this exact aa exchange (# reports for this aa position).

3.7 The Myc-VHL interaction is HIF independent (paper III)

Since VHL binds HIF in an oxygen dependent manner, we wanted to test whether the Myc-VHL binding was affected by oxygen conditions. In

CoIPs, we showed that Myc and VHL could interact during hypoxia and further, after 20 hours of hypoxia the Myc-VHL binding had somewhat increased whereas Myc levels seemed to be reduced, implying that hypoxia lower the steady state level of Myc, but increase the Myc-VHL binding.

Even though the VHL-Y98N mutant with diminished binding to HIF-1α (Tanimoto *et al.*, 2000) still interacted with Myc we wanted to establish whether Myc and HIF could compete for binding to VHL. By using a polypeptide comprising a part of the oxygen dependent domain (ODD) of HIF-1α in increasing concentrations we could not abolish the Myc-VHL nor the Myc1-215-VHL interaction. However, when high concentrations of the polypeptide was transfected, the total level of Myc and Myc1-215 respectively were reduced, suggesting that released HIF-1α might regulate the steady state levels of Myc protein or mRNA.

3.8 VHL plays a role in ubiquitylation of Myc but seems not to be involved in proteasomal degradation (paper III)

Since VHL is an E3 ligase, we wanted to test if VHL was involved in ubiquitylation of Myc. Ubiquitin-assays showed that VHL did not seem to affect Myc ubiquitylation, but the VHL-Y98N and VHL-C162F mutants strongly repressed ubiquitylated Myc. This suggests that the VHL mutants work in a dominant negative fashion and that VHL directly or indirectly regulates ubiquitylation of Myc.

We next raised the question whether the potential ubiquitylation of Myc was followed by degradation in the same manner as when Myc is ubiquitylated by the E3 ligases Skp2 and Fbw7. In a panel of cell lines with functional and dysfunctional/absent VHL respectively, we observed that Myc steady state levels were higher in those cells with dysfunctional or absent VHL. This made us believe that VHL was involved in degradation of Myc and we tested this hypothesis by ^{35}S pulse-chases and cycloheximide block of translation. Surprisingly, chase data revealed that absence or presence of functional VHL did not seem to affect the turnover rate of Myc in the cell lines used. However, we the increased protein levels of c-Myc in cells lacking functional VHL correlated with increased *c-myc* mRNA expression, indicating that the VHL status of the cell affects Myc expression pre-translationally.

3.9 VHL is associated with some Myc target promoters and is suggested to activate transcription of Myc target genes (paper III)

Since VHL affected ubiquitylation of Myc without obvious effect on Myc turnover, we wanted to test whether VHL had a function in Myc transactivation. We utilized the reporter assay system with an α-prothymosin promoter-Luc construct as described above and found that VHL had little effect on Myc induced transcription. However, when cotransfecting the VHL-Y98N and VHL-C162F (data not shown) mutants with Myc we noticed repression of Luc-activity already at low concentrations of the mutants. This suggests that the VHL-Y98N and VHL-C162F mutants work in a dominant negative fashion in the same manner as in the ubiquitylation assay. This would imply a possible regulatory role for VHL in Myc induced transcription. To strengthen the evidence for VHL's role in transcription, we performed chromatin immunoprecipitation (ChIP) assays, where DNA-protein interaction was measured. We confirmed that VHL was physically present on the CAD and cyclin D2 Myc target promoters, but not on the ODC promoter. This suggests a model where VHL is involved in transcription of some Myc targets, but not with a general mechanism including all Myc targets.

3.10 Hypothetical model of regulation of Myc by VHL (paper III)

Taking all this data together, we suggest a hypothetical and speculative model (figure 6). In this model we propose that VHL binds Myc-Max complexes on target gene promoters. There VHL ubiquitylates Myc which possibly enhances activation of Myc target genes. This ubiquitylation by VHL does not seem to induce turnover of Myc under the conditions we have used. We cannot exclude however, that VHL not have an effect on Myc degradation in a different cellular context, such as hypoxia.

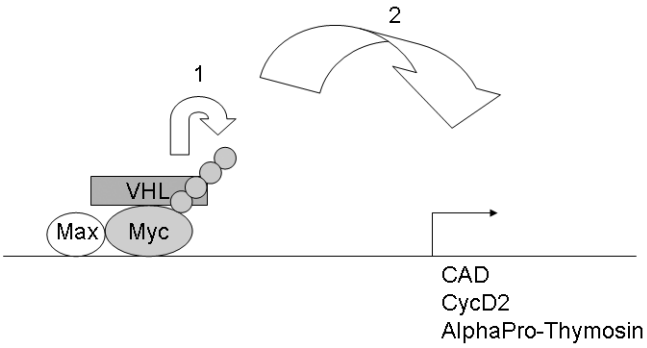

Figure 6. Hypothetical model of VHL-Myc interplay. VHL interacts with Myc on Myc target promoters which leads to ubiquitylation of Myc but without subsequent degradation. VHL, ubiquitylated Myc and/or other factors present, induces activation of Myc targets.

Whether HIF is involved in this regulation is not entirely clear. HIF and Myc do not seem to bind VHL on the same site although possibly in the same region, the β-domain. Neither did HIF seem to compete with Myc for binding which fits with the idea that they not bind exactly the same residues on VHL. Further, the Myc-VHL interaction is not oxygen-dependent, actually Myc and VHL seemed to interact even stronger during hypoxia, which correlated with reduced Myc protein levels. It has been reported that Myc is downregulated during hypoxia (Corn *et al.*, 2005; Zhang *et al.*, 2007). It will therefore be an important task for the future to elucidate whether Myc is downregulated during hypoxia in a VHL dependent manner.

3.11 IFN-γ induces degradation of Myc in a Ser-62-dependent manner (paper IV)

Previous research in the group had revealed that IFN-γ destabilises Myc-Max complexes which correlated with reduced overall Myc phosphorylation (Bahram *et al.*, 1999). We now wanted to further investigate the mechanisms by which IFN-γ inhibits Myc function. We utilised the U-937 differentiation model where Thr-58 mutated v-Myc inhibits TPA-induced differentiation of U-937 monocytes. In this model system, IFN-γ abrogates the v-Myc effects and restores differentiation and G1-arrest (Oberg *et al.*, 1991). We could now show that IFN-γ treatment increases the degradation of both v-Myc and c-Myc in U-937-Myc6 cells as well as in several other

cell lines including Colo320 colon carcinoma cells with amplified c-Myc, 2fTGH fibrosarcoma cells but not in the 2fTGH fibrosarcoma sublines U3A and U3B which lack STAT1 and JAK1, respectively. This indicates a requirement for STAT1 and JAK1 in IFN-γ induced degradation of Myc. Further we showed that IFN-γ enhances the amount of ubiquitylated Myc and that a proteasome inhibitor rescues Myc from degradation when treated with IFN-γ.

Phosphorylation of Thr-58 requires a prior phosphorylation of Ser-62, which has been suggested to be targeted by Erk and unknown Cdks. Phosphorylated Ser-62 has also been suggested to stabilize Myc (Sears *et al.*, 2000). We showed that IFN-γ reduced v-Myc Ser-62 phosphorylation and increased degradation of the Myc-T58A mutant but not of the Myc-S62A mutant. Taken together this implies that IFN-γ decreases phosphorylation of Myc Ser-62 and that IFN-γ induced Myc degradation is Ser-62 dependent.

3.12 Cdk2 phosporylates Myc at Ser-62 (paper IV)

We next asked whether the reduced Myc Ser-62 phosphorylation was due to inhibition of a kinase or activation of a phosphatase or both. To address this question we transfected U2OS cells with the Myc-T58A mutant in order to make sure that Ser-62 would be the only targeted phosphorylation site and then treated these cells with a panel of kinase inhibitors. We found that the Cdk1/Cdk2 inhibitor roscovitine, was the most potent agent in reducing Ser-62 phosphorylation. Since kenpaullone which prominently inhibits Cdk1 had less effect we concluded that Cdk2 is the kinase that phosphorylates Myc Ser-62. We further proved this *in vitro* by using GST-Myc and recombinant cyclin/Cdk2 complexes and showed that Myc was a substrate for cyclinE/Cdk2 and to a lesser extent for cyclinA/Cdk2. Also, we arrested U-937 cells in early S-phase and found that Myc Ser-62 was phosphorylated in these cells. This effect could be abrogated by roscovitine, suggesting that Cdk2 is the major Myc Ser-62 kinase in early S-phase. Moreover, roscovitine increased the turnover rate of both c-Myc and v-Myc in U937-Myc6 cells, further strengthening the role for Cdk2 in Myc degradation.

3.13 IFN-γ represses Cdk2 by upregulation of p27 (paper IV)

To confirm that IFN-γ represses Cdk2 activity, we performed *in vitro* kinase assays which showed that Cdk2 activity was strongly repressed within four hours of IFN-γ exposure. In addition this correlated with reduced

phosphorylation of Rb which is also a Cdk2 target. To find the missing link between IFN-γ and Cdk2 activity, we tested expression of the Cdk2 inhibitors p21 and p27, which have been reported to be upregulated by IFN-γ (Harvat *et al.*, 1997; Kominsky *et al.*, 1998; Mandal *et al.*, 1998; Matsuoka *et al.*, 1999; Xaus *et al.*, 1999). Indeed, p27 protein levels were upregulated by IFN-γ treatment which correlated with increased p27-Cdk2 complex formation, reduced Myc Ser-62 phosphorylation and increased Myc degradation. p21 expression also increased, but only transiently. We excluded the possibility that the effects of IFN-γ were due to indirect effects of the cell cycle by measuring the cell cycle distribution after IFN-γ+TPA or roscovitine treatments. No G1 accumulation at the essential time points was observed. Further, to rule out the possibility of upregulation of phosphatase action by IFN-γ, we measured PP2A activity after IFN-γ treatment and found no changes in PP2A activity.

To further analyze the role of p27, we coexpressed p27 and Myc and found that p27 reduced the levels of Myc wt as well as the Myc-T58A mutant, but not the Myc-T58A/S62A double mutant. Additionally, IFN-γ treatment did not affect Myc steady state level in p27-/-MEF cells.

3.14 A novel E3 ligase is involved in p27 induced Myc degradation (paper IV)

Since the SCF^{Fbw7} E3 ligase previously had been shown to ubiquitylate and degrade Myc via MBI in a phospho-Thr-58-dependent manner (Welcker *et al.*, 2004; Yada *et al.*, 2004), we wanted to test whether Fbw7 was involved in p27 induced Myc turnover. However, p27 expression induced Myc degradation also in *Fbw7/cdc4* $^{-/-}$ HCT116 cells, which is in agreement with the ability of p27 to induce degradation of Myc-T58A. The SCF^{Skp2} E3 ligase did not seem to participate in p27 induced Myc turnover either, which was expected, since Skp2 does not operate via MBI (see paper I). Hence, a novel E3 ligase is involved in p27 induced Myc degradation.

3.15 Cyclin E/Cdk2 and p27 are present on the cyclin D2 promoter and are involved in Myc transactivation (paper IV)

To further clarify the cyclinE/Cdk2-p27-Myc interactions, we performed coimmunoprecipitations in IFN-γ treated U-937 cells. We showed that Myc coimmunoprecipitated with both p27 and cyclin E antibodies, although the interactions were weaker than the Myc-Max binding indicating a transient interaction. In addition, the interactions of these proteins were detected on

chromatin by quantitative chromatin immunoprecipitations (Q-ChIP) on the cyclin D2 promoter. Cyclin E and p27 were further re-ChIPed, protein/DNA complexes immunoprecipitated with Myc antibody were released and IPed over again with Cdk2 and p27 antibodies. The results confirmed a direct interaction. IFN-γ treatment decreased the amount of total and phosphorylated Myc as well as Cdk2, but increased the amount of p27 on the cyclinD2 promoter. Together these data implicate a model where IFN-γ-induced p27 is transiently recruited and inhibits Myc-bound cyclin E/Cdk2 which results in reduced Myc phosphorylation and degradation of Myc via the ubiquitin/proteasome pathway.

Since Myc, cyclin E/Cdk2 and p27 are detected on the cyclin D2 promoter, we wanted to evaluate whether cyclin E/Cdk2 is involved in Myc transactivation. In reporter-assays where the Myc-driven m4mintk-Luc construct was cotransfected with Myc and cyclin E/Cdk2, we showed that cyclin E/Cdk2 substantially enhanced Myc-driven transcription and that this was abrogated in IFN-γ treated cells. This was also confirmed by the Myc-target α-prothymosin-Luc cotransfections with Myc and cyclin E/Cdk2. Additionally, this correlated with mRNA levels for the Myc-activated targets hTERT and ODC as well as the Myc-repressed target p21 measured by RT-qPCR. IFN-γ treatment decreased the hTERT and ODC mRNA levels as well as substantially induced p21 expression.

There are several possible ways how cyclin E/Cdk2 could activate Myc dependent transcription. Firstly, by acting as a coactivator implicating that phosphorylating Myc Ser-62 or other substrates are sufficient for activating transcription. It has been reported that the coactivator CBP which is recruited by Myc is activated by cyclin E/Cdk2. Secondly, the Cdk2 kinase activity may phosphorylate components of the Pol II complex or finally cyclin E/Cdk2 might serve as an adaptor for recruitment of other components that activate transcription.

3.16 Proposed model for the IFN-γ induced p27/cyclin E/Cdk2/Myc interplay (paper IV)

To sum up, our data implicates a model for the IFN-γ induced p27-cyclin E/Cdk2-Myc interplay. At first, cyclin E/Cdk2 is recruited to Myc-Max complexes located at E-box sequences in target promoters. Secondly, cyclin E/Cdk2 phosphorylates Myc at Ser-62 which results in a more stable Myc-Max complex and increased transcription of Myc target genes. In absence of IFN-γ, GSK3 recognises phosphorylated Myc Ser-62 which is a prerequisite for the subsequent GSK3-mediated Thr-58 phosphorylation. Further, for

efficient ubiquitylation by Fbw7, the Ser-62 phosphorylation needs to be removed which is mediated by PP2A assisted by Pin1 (Yeh *et al.*, 2004). In the presence of IFN-γ however, p27 is induced and recruited to the Myc/cyclin E/Cdk2 complex where it represses the kinase activity of Cdk2. Cdk2 cannot phosphorylate Myc Ser-62 and unphosphorylated Myc is transcriptionally less active. Also, unphosphorylated Myc is less stable than Ser-62 phosphorylated Myc which results in ubiquitylation and degradation by a presently unknown E3 ligase.

4 Conclusions

- SCF^{Skp2} interacts with Myc and participates in Myc ubiquitylation and degradation by the proteasome.
- Skp2 is a cofactor for Myc function in cell cycle regulation and transcriptional regulation.
- Sug1 interacts with Myc, is physically associated with the cyclin D2 promoter and is involved in Myc induced transcription.
- VHL interacts with Myc and plays a role in Myc ubiquitylation and regulation of transcription by Myc.
- Cyclin E/Cdk2 regulates the stability of Myc by phosphorylating Ser-62 and functions as a Myc cofactor.
- IFN-γ induces p27 expression, which in turn inhibits cyclin E/Cdk2 phosphorylation of Myc Ser-62, resulting in increased Myc turnover via the ubiquitin/proteasome pathway.

5 References

Adams, R.H. & Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 8(6), 464-78.

Adhikary, S. & Eilers, M. (2005). Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol* 6(8), 635-45.

Adhikary, S., Marinoni, F., Hock, A., Hulleman, E., Popov, N., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., Kogel, U., Scheffner, M., Helin, K. & Eilers, M. (2005). The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. *Cell* 123(3), 409-21.

Al-Hakim, A.K., Zagorska, A., Chapman, L., Deak, M., Peggie, M. & Alessi, D.R. (2008). Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains. *Biochem J* 411(2), 249-60.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. (2002). *Molecular Biology of the Cell*. 4th edition. Garland Science.

Aleem, E., Kiyokawa, H. & Kaldis, P. (2005). Cdc2-cyclin E complexes regulate the G1/S phase transition. *Nat Cell Biol* 7(8), 831-6.

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N. & DePinho, R.A. (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* 387(6628), 49-55.

Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fatyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I., Larsson, L.G. & Wright, A.P. (2005). c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat Cell Biol* 7(3), 303-10.

Aravind, L. & Koonin, E.V. (2000). The U box is a modified RING finger - a common domain in ubiquitination. *Curr Biol* 10(4), R132-4.

Archer, C.T., Burdine, L., Liu, B., Ferdous, A., Johnston, S.A. & Kodadek, T. (2008). Physical and functional interactions of monoubiquitylated transactivators with the proteasome. *J Biol Chem* 283(31), 21789-98.

Arellano, M. & Moreno, S. (1997). Regulation of CDK/cyclin complexes during the cell cycle. *Int J Biochem Cell Biol* 29(4), 559-73.

Askew, D.S., Ashmun, R.A., Simmons, B.C. & Cleveland, J.L. (1991). Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* 6(10), 1915-22.

Aso, T., Lane, W.S., Conaway, J.W. & Conaway, R.C. (1995). Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II. *Science* 269(5229), 1439-43.

Ayer, D.E. (1999). Histone deacetylases: transcriptional repression with SINers and NuRDs. *Trends Cell Biol* 9(5), 193-8.

Ayer, D.E. & Eisenman, R.N. (1993). A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev* 7(11), 2110-9.

Bach, E.A., Aguet, M. & Schreiber, R.D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 15, 563-91.

Bahram, F., von der Lehr, N., Cetinkaya, C. & Larsson, L.G. (2000). c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood* 95(6), 2104-10.

Bahram, F., Wu, S., Oberg, F., Luscher, B. & Larsson, L.G. (1999). Posttranslational regulation of Myc function in response to phorbol ester/interferon-gamma-induced differentiation of v-Myc-transformed U-937 monoblasts. *Blood* 93(11), 3900-12.

Barry, R.E. & Krek, W. (2004). The von Hippel-Lindau tumour suppressor: a multi-faceted inhibitor of tumourigenesis. *Trends Mol Med* 10(9), 466-72.

Barsyte-Lovejoy, D., Mao, D.Y. & Penn, L.Z. (2004). c-Myc represses the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism. *Oncogene* 23(19), 3481-6.

Baumeister, W., Walz, J., Zuhl, F. & Seemuller, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. *Cell* 92(3), 367-80.

Bazan, J.F. (2008). An old HAT in human p300/CBP and yeast Rtt109. *Cell Cycle* 7(12), 1884-6.

Bello-Fernandez, C., Packham, G. & Cleveland, J.L. (1993). The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci U S A* 90(16), 7804-8.

Benaroudj, N., Zwickl, P., Seemuller, E., Baumeister, W. & Goldberg, A.L. (2003). ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. *Mol Cell* 11(1), 69-78.

Beroud, C., Collod-Beroud, G., Boileau, C., Soussi, T. & Junien, C. (2000). UMD (Universal mutation database): a generic software to build and analyze locus-specific databases. *Hum Mutat* 15(1), 86-94.

Bhatia, K., Huppi, K., Spangler, G., Siwarski, D., Iyer, R. & Magrath, I. (1993). Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. *Nat Genet* 5(1), 56-61.

Bindra, R.S., Vasselli, J.R., Stearman, R., Linehan, W.M. & Klausner, R.D. (2002). VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. *Cancer Res* 62(11), 3014-9.

Bissonnette, R.P., Echeverri, F., Mahboubi, A. & Green, D.R. (1992). Apoptotic cell death induced by c-myc is inhibited by bcl-2. *Nature* 359(6395), 552-4.

Blackwood, E.M. & Eisenman, R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* 251(4998), 1211-7.

Bloom, J. & Pagano, M. (2003). Deregulated degradation of the cdk inhibitor p27 and malignant transformation. *Semin Cancer Biol* 13(1), 41-7.

Boname, J.M. & Stevenson, P.G. (2001). MHC class I ubiquitination by a viral PHD/LAP finger protein. *Immunity* 15(4), 627-36.

Bonifacino, J.S. & Weissman, A.M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol* 14, 19-57.

Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M. & Hershko, A. (2003). Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J Biol Chem* 278(28), 25752-7.

Bouchard, C., Dittrich, O., Kiermaier, A., Dohmann, K., Menkel, A., Eilers, M. & Luscher, B. (2001). Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. *Genes Dev* 15(16), 2042-7.

Bouchard, C., Marquardt, J., Bras, A., Medema, R.H. & Eilers, M. (2004). Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. *Embo J* 23(14), 2830-40.

Bowen, H., Biggs, T.E., Baker, S.T., Phillips, E., Perry, V.H., Mann, D.A. & Barton, C.H. (2002). c-Myc represses the murine Nramp1 promoter. *Biochem Soc Trans* 30(4), 774-7.

Brauch, H., Weirich, G., Hornauer, M.A., Storkel, S., Wohl, T. & Bruning, T. (1999). Trichloroethylene exposure and specific somatic mutations in patients with renal cell carcinoma. *J Natl Cancer Inst* 91(10), 854-61.

Brenner, C., Deplus, R., Didelot, C., Loriot, A., Vire, E., De Smet, C., Gutierrez, A., Danovi, D., Bernard, D., Boon, T., Pelicci, P.G., Amati, B., Kouzarides, T., de Launoit, Y., Di Croce, L. & Fuks, F. (2005). Myc represses transcription through recruitment of DNA methyltransferase corepressor. *Embo J* 24(2), 336-46.

Bruick, R.K. & McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294(5545), 1337-40.

Bryan, T.M. & Cech, T.R. (1999). Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol* 11(3), 318-24.

Capili, A.D., Schultz, D.C., Rauscher, I.F. & Borden, K.L. (2001). Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains. *Embo J* 20(1-2), 165-77.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D. & Keshert, E. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394(6692), 485-90.

Carrano, A.C., Eytan, E., Hershko, A. & Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* 1(4), 193-9.

Chastagner, P., Israel, A. & Brou, C. (2006). Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. *EMBO Rep* 7(11), 1147-53.

Chen, F., Kishida, T., Yao, M., Hustad, T., Glavac, D., Dean, M., Gnarra, J.R., Orcutt, M.L., Duh, F.M., Glenn, G. & et al. (1995). Germline mutations in the von

Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 5(1), 66-75.

Choyke, P.L., Glenn, G.M., Walther, M.M., Patronas, N.J., Linehan, W.M. & Zbar, B. (1995). von Hippel-Lindau disease: genetic, clinical, and imaging features. *Radiology* 194(3), 629-42.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat Rev Mol Cell Biol* 6(1), 79-87.

Ciechanover, A., Heller, H., Elias, S., Haas, A.L. & Hershko, A. (1980). ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. *Proc Natl Acad Sci U S A* 77(3), 1365-8.

Clurman, B.E., Sheaff, R.J., Thress, K., Groudine, M. & Roberts, J.M. (1996). Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev* 10(16), 1979-90.

Coin, F. & Egly, J.M. (1998). Ten years of TFIIH. *Cold Spring Harb Symp Quant Biol* 63, 105-10.

Conaway, J.W., Shilatifard, A., Dvir, A. & Conaway, R.C. (2000). Control of elongation by RNA polymerase II. *Trends Biochem Sci* 25(8), 375-80.

Conaway, R.C., Brower, C.S. & Conaway, J.W. (2002). Emerging roles of ubiquitin in transcription regulation. *Science* 296(5571), 1254-8.

Connell, P., Ballinger, C.A., Jiang, J., Wu, Y., Thompson, L.J., Hohfeld, J. & Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol* 3(1), 93-6.

Corn, P.G., McDonald, E.R., 3rd, Herman, J.G. & El-Deiry, W.S. (2003). Tat-binding protein-1, a component of the 26S proteasome, contributes to the E3 ubiquitin ligase function of the von Hippel-Lindau protein. *Nat Genet* 35(3), 229-37.

Corn, P.G., Ricci, M.S., Scata, K.A., Arsham, A.M., Simon, M.C., Dicker, D.T. & El-Deiry, W.S. (2005). Mxi1 is induced by hypoxia in a HIF-1-dependent manner and protects cells from c-Myc-induced apoptosis. *Cancer Biol Ther* 4(11), 1285-94.

Coscoy, L., Sanchez, D.J. & Ganem, D. (2001). A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J Cell Biol* 155(7), 1265-73.

Covello, K.L., Kehler, J., Yu, H., Gordan, J.D., Arsham, A.M., Hu, C.J., Labosky, P.A., Simon, M.C. & Keith, B. (2006). HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev* 20(5), 557-70.

Cowling, V.H., Chandriani, S., Whitfield, M.L. & Cole, M.D. (2006). A conserved Myc protein domain, MBIV, regulates DNA binding, apoptosis, transformation, and G2 arrest. *Mol Cell Biol* 26(11), 4226-39.

Cowling, V.H. & Cole, M.D. (2007). The Myc transactivation domain promotes global phosphorylation of the RNA polymerase II carboxy-terminal domain independently of direct DNA binding. *Mol Cell Biol* 27(6), 2059-73.

Croce, C.M. (2008). Oncogenes and cancer. *N Engl J Med* 358(5), 502-11.

Crossey, P.A., Richards, F.M., Foster, K., Green, J.S., Prowse, A., Latif, F., Lerman, M.I., Zbar, B., Affara, N.A., Ferguson-Smith, M.A. & et al. (1994). Identification of

intragenic mutations in the von Hippel-Lindau disease tumour suppressor gene and correlation with disease phenotype. *Hum Mol Genet* 3(8), 1303-8.

Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C. & Croce, C.M. (1982). Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci U S A* 79(24), 7824-7.

Dang, C.V., Kim, J.W., Gao, P. & Yustein, J. (2008). The interplay between MYC and HIF in cancer. *Nat Rev Cancer* 8(1), 51-6.

Dang, C.V., O'Donnell, K.A., Zeller, K.I., Nguyen, T., Osthus, R.C. & Li, F. (2006). The c-Myc target gene network. *Semin Cancer Biol* 16(4), 253-64.

Dang, C.V., Resar, L.M., Emison, E., Kim, S., Li, Q., Prescott, J.E., Wonsey, D. & Zeller, K. (1999). Function of the c-Myc oncogenic transcription factor. *Exp Cell Res* 253(1), 63-77.

de la Cova, C., Abril, M., Bellosta, P., Gallant, P. & Johnston, L.A. (2004). Drosophila myc regulates organ size by inducing cell competition. *Cell* 117(1), 107-16.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C. & Chen, Z.J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103(2), 351-61.

Denicourt, C. & Dowdy, S.F. (2004). Cip/Kip proteins: more than just CDKs inhibitors. *Genes Dev* 18(8), 851-5.

Dey, A., Kim, L. & Li, W. (1999). Gamma interferon induces expression of Mad1 gene in macrophage, which inhibits colony-stimulating factor-1-dependent mitogenesis. *J Cell Biochem* 72(2), 232-41.

Duan, D.R., Humphrey, J.S., Chen, D.Y., Weng, Y., Sukegawa, J., Lee, S., Gnarra, J.R., Linehan, W.M. & Klausner, R.D. (1995). Characterization of the VHL tumor suppressor gene product: localization, complex formation, and the effect of natural inactivating mutations. *Proc Natl Acad Sci U S A* 92(14), 6459-63.

Dvir, A., Conaway, J.W. & Conaway, R.C. (2001). Mechanism of transcription initiation and promoter escape by RNA polymerase II. *Curr Opin Genet Dev* 11(2), 209-14.

Eberhardy, S.R. & Farnham, P.J. (2001). c-Myc mediates activation of the cad promoter via a post-RNA polymerase II recruitment mechanism. *J Biol Chem* 276(51), 48562-71.

Eberhardy, S.R. & Farnham, P.J. (2002). Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. *J Biol Chem* 277(42), 40156-62.

Eilers, M. & Eisenman, R.N. (2008). Myc's broad reach. *Genes Dev* 22(20), 2755-66.

Eischen, C.M., Roussel, M.F., Korsmeyer, S.J. & Cleveland, J.L. (2001). Bax loss impairs Myc-induced apoptosis and circumvents the selection of p53 mutations during Myc-mediated lymphomagenesis. *Mol Cell Biol* 21(22), 7653-62.

Elledge, S.J. & Harper, J.W. (1998). The role of protein stability in the cell cycle and cancer. *Biochim Biophys Acta* 1377(2), M61-70.

Elsasser, S., Chandler-Militello, D., Muller, B., Hanna, J. & Finley, D. (2004). Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. *J Biol Chem* 279(26), 26817-22.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N.,

Hamilton, D.L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. & Ratcliffe, P.J. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107(1), 43-54.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. & Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69(1), 119-28.

Faiola, F., Liu, X., Lo, S., Pan, S., Zhang, K., Lymar, E., Farina, A. & Martinez, E. (2005). Dual regulation of c-Myc by p300 via acetylation-dependent control of Myc protein turnover and coactivation of Myc-induced transcription. *Mol Cell Biol* 25(23), 10220-34.

Fang, S. & Weissman, A.M. (2004). A field guide to ubiquitylation. *Cell Mol Life Sci* 61(13), 1546-61.

Fanidi, A., Harrington, E.A. & Evan, G.I. (1992). Cooperative interaction between c-myc and bcl-2 proto-oncogenes. *Nature* 359(6395), 554-6.

Featherstone, M. (2002). Coactivators in transcription initiation: here are your orders. *Curr Opin Genet Dev* 12(2), 149-55.

Felsher, D.W. & Bishop, J.M. (1999). Transient excess of MYC activity can elicit genomic instability and tumorigenesis. *Proc Natl Acad Sci U S A* 96(7), 3940-4.

Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T. & Johnston, S.A. (2001). The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. *Mol Cell* 7(5), 981-91.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A. & Amati, B. (2003). Genomic targets of the human c-Myc protein. *Genes Dev* 17(9), 1115-29.

Flinn, E.M., Busch, C.M. & Wright, A.P. (1998). myc boxes, which are conserved in myc family proteins, are signals for protein degradation via the proteasome. *Mol Cell Biol* 18(10), 5961-9.

Frank, S.R., Schroeder, M., Fernandez, P., Taubert, S. & Amati, B. (2001). Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. *Genes Dev* 15(16), 2069-82.

Freytag, S.O., Dang, C.V. & Lee, W.M. (1990). Definition of the activities and properties of c-myc required to inhibit cell differentiation. *Cell Growth Differ* 1(7), 339-43.

Gallant, P., Shiio, Y., Cheng, P.F., Parkhurst, S.M. & Eisenman, R.N. (1996). Myc and Max homologs in Drosophila. *Science* 274(5292), 1523-7.

Gao, P., Zhang, H., Dinavahi, R., Li, F., Xiang, Y., Raman, V., Bhujwalla, Z.M., Felsher, D.W., Cheng, L., Pevsner, J., Lee, L.A., Semenza, G.L. & Dang, C.V. (2007). HIF-dependent antitumorigenic effect of antioxidants in vivo. *Cancer Cell* 12(3), 230-8.

Glenn G, S.C., Sgambati M, Choyke P, Hurley K, Peterson J, Manolatos C, et al (1999). New mutations versus silent-carrier parent as source of first generation diagnoses in a hereditary neoplastic disorder: von Hippel-Lindau disease. *Proc Am Assoc Cancer Res* 40(464)

Gnarra, J.R., Tory, K., Weng, Y., Schmidt, L., Wei, M.H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F.M. & et al. (1994). Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat Genet* 7(1), 85-90.

Gnarra, J.R., Ward, J.M., Porter, F.D., Wagner, J.R., Devor, D.E., Grinberg, A., Emmert-Buck, M.R., Westphal, H., Klausner, R.D. & Linehan, W.M. (1997). Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. *Proc Natl Acad Sci U S A* 94(17), 9102-7.

Goda, N., Ryan, H.E., Khadivi, B., McNulty, W., Rickert, R.C. & Johnson, R.S. (2003). Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol* 23(1), 359-69.

Goldstein, G. (1974). Isolation of bovine thymin: a polypeptide hormone of the thymus. *Nature* 247(5435), 11-4.

Goldstein, G. (1975). The isolation of thymopoietin (thymin). *Ann N Y Acad Sci* 249, 177-85.

Gomez-Roman, N., Grandori, C., Eisenman, R.N. & White, R.J. (2003). Direct activation of RNA polymerase III transcription by c-Myc. *Nature* 421(6920), 290-4.

Gonzalez, F., Delahodde, A., Kodadek, T. & Johnston, S.A. (2002). Recruitment of a 19S proteasome subcomplex to an activated promoter. *Science* 296(5567), 548-50.

Gordan, J.D., Bertout, J.A., Hu, C.J., Diehl, J.A. & Simon, M.C. (2007a). HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell* 11(4), 335-47.

Gordan, J.D. & Simon, M.C. (2007). Hypoxia-inducible factors: central regulators of the tumor phenotype. *Curr Opin Genet Dev* 17(1), 71-7.

Gordan, J.D., Thompson, C.B. & Simon, M.C. (2007b). HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. *Cancer Cell* 12(2), 108-13.

Grandori, C., Cowley, S.M., James, L.P. & Eisenman, R.N. (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu Rev Cell Dev Biol* 16, 653-99.

Grandori, C., Gomez-Roman, N., Felton-Edkins, Z.A., Ngouenet, C., Galloway, D.A., Eisenman, R.N. & White, R.J. (2005). c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* 7(3), 311-8.

Grant, P.A., Schieltz, D., Pray-Grant, M.G., Yates, J.R., 3rd & Workman, J.L. (1998). The ATM-related cofactor Tra1 is a component of the purified SAGA complex. *Mol Cell* 2(6), 863-7.

Greasley, P.J., Bonnard, C. & Amati, B. (2000). Myc induces the nucleolin and BN51 genes: possible implications in ribosome biogenesis. *Nucleic Acids Res* 28(2), 446-53.

Grewal, S.S., Li, L., Orian, A., Eisenman, R.N. & Edgar, B.A. (2005). Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. *Nat Cell Biol* 7(3), 295-302.

Gross-Mesilaty, S., Reinstein, E., Bercovich, B., Tobias, K.E., Schwartz, A.L., Kahana, C. & Ciechanover, A. (1998). Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway. *Proc Natl Acad Sci U S A* 95(14), 8058-63.

Guardavaccaro, D. & Pagano, M. (2004). Oncogenic aberrations of cullin-dependent ubiquitin ligases. *Oncogene* 23(11), 2037-49.

Hammel, P.R., Vilgrain, V., Terris, B., Penfornis, A., Sauvanet, A., Correas, J.M., Chauveau, D., Balian, A., Beigelman, C., O'Toole, D., Bernades, P., Ruszniewski, P. & Richard, S. (2000). Pancreatic involvement in von Hippel-Lindau disease. The Groupe Francophone d'Etude de la Maladie de von Hippel-Lindau. *Gastroenterology* 119(4), 1087-95.

Hanahan, D. & Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100(1), 57-70.

Harris, C.C. (1996). p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis* 17(6), 1187-98.

Harvat, B.L., Seth, P. & Jetten, A.M. (1997). The role of p27Kip1 in gamma interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. *Oncogene* 14(17), 2111-22.

Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N. & Nakayama, K.I. (2001). U box proteins as a new family of ubiquitin-protein ligases. *J Biol Chem* 276(35), 33111-20.

Henriksson, M., Bakardjiev, A., Klein, G. & Luscher, B. (1993). Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. *Oncogene* 8(12), 3199-209.

Herbst, A., Hemann, M.T., Tworkowski, K.A., Salghetti, S.E., Lowe, S.W. & Tansey, W.P. (2005). A conserved element in Myc that negatively regulates its proapoptotic activity. *EMBO Rep* 6(2), 177-83.

Hergovich, A., Lisztwan, J., Barry, R., Ballschmieter, P. & Krek, W. (2003). Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. *Nat Cell Biol* 5(1), 64-70.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., Dang, C.V., Sedivy, J.M., Eick, D., Vogelstein, B. & Kinzler, K.W. (2000). Identification of CDK4 as a target of c-MYC. *Proc Natl Acad Sci U S A* 97(5), 2229-34.

Herold, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukkala, T., Syvaoja, J., Saluz, H.P., Haenel, F. & Eilers, M. (2002). Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* 10(3), 509-21.

Hershko, A., Ciechanover, A., Heller, H., Haas, A.L. & Rose, I.A. (1980). Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proc Natl Acad Sci U S A* 77(4), 1783-6.

Hershko, A., Heller, H., Elias, S. & Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* 258(13), 8206-14.

Hodawadekar, S.C. & Marmorstein, R. (2007). Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. *Oncogene* 26(37), 5528-40.

Hoffman, M.A., Ohh, M., Yang, H., Klco, J.M., Ivan, M. & Kaelin, W.G., Jr. (2001). von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet* 10(10), 1019-27.

Honda, R. & Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *Embo J* 18(1), 22-7.

Hoppe, T. (2005). Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. *Trends Biochem Sci* 30(4), 183-7.

Hsu, T., Adereth, Y., Kose, N. & Dammai, V. (2006). Endocytic function of von Hippel-Lindau tumor suppressor protein regulates surface localization of fibroblast growth factor receptor 1 and cell motility. *J Biol Chem* 281(17), 12069-80.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B. & Simon, M.C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 23(24), 9361-74.

Huang, L.E. (2008). Carrot and stick: HIF-alpha engages c-Myc in hypoxic adaptation. *Cell Death Differ* 15(4), 672-7.

Huibregtse, J.M., Scheffner, M., Beaudenon, S. & Howley, P.M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* 92(7), 2563-7.

Hurlin, P.J. & Huang, J. (2006). The MAX-interacting transcription factor network. *Semin Cancer Biol* 16(4), 265-74.

Hurlin, P.J., Queva, C. & Eisenman, R.N. (1997). Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev* 11(1), 44-58.

Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K.J., Finley, D. & Dikic, I. (2008). Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* 453(7194), 481-8.

Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J. & Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* 102(4), 463-73.

Iliopoulos, O., Ohh, M. & Kaelin, W.G., Jr. (1998). pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A* 95(20), 11661-6.

Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y. & Takahashi, R. (2001). An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 105(7), 891-902.

Japan, C.R.G.f.V.i. (1995). Germline mutations in the von Hippel-Lindau disease (VHL) gene in Japanese VHL *Human Molecular Genetics* 4(12), 2233-2237.

Jiang, J., Ballinger, C.A., Wu, Y., Dai, Q., Cyr, D.M., Hohfeld, J. & Patterson, C. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *J Biol Chem* 276(46), 42938-44.

Joazeiro, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T. & Liu, Y.C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* 286(5438), 309-12.

Johnson, D.G. & Walker, C.L. (1999). Cyclins and cell cycle checkpoints. *Annu Rev Pharmacol Toxicol* 39, 295-312.

Jonsson, Z.O., Jha, S., Wohlschlegel, J.A. & Dutta, A. (2004). Rvb1p/Rvb2p recruit Arp5p and assemble a functional Ino80 chromatin remodeling complex. *Mol Cell* 16(3), 465-77.

Juin, P., Hueber, A.O., Littlewood, T. & Evan, G. (1999). c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. *Genes Dev* 13(11), 1367-81.

Juin, P., Hunt, A., Littlewood, T., Griffiths, B., Swigart, L.B., Korsmeyer, S. & Evan, G. (2002). c-Myc functionally cooperates with Bax to induce apoptosis. *Mol Cell Biol* 22(17), 6158-69.

Kaelin, W.G., Jr. (2002). Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer* 2(9), 673-82.

Kamura, T., Hara, T., Kotoshiba, S., Yada, M., Ishida, N., Imaki, H., Hatakeyama, S., Nakayama, K. & Nakayama, K.I. (2003). Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. *Proc Natl Acad Sci U S A* 100(18), 10231-6.

Kanazawa, S., Soucek, L., Evan, G., Okamoto, T. & Peterlin, B.M. (2003). c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. *Oncogene* 22(36), 5707-11.

Kato, G.J., Barrett, J., Villa-Garcia, M. & Dang, C.V. (1990). An amino-terminal c-myc domain required for neoplastic transformation activates transcription. *Mol Cell Biol* 10(11), 5914-20.

Keller, U.B., Old, J.B., Dorsey, F.C., Nilsson, J.A., Nilsson, L., MacLean, K.H., Chung, L., Yang, C., Spruck, C., Boyd, K., Reed, S.I. & Cleveland, J.L. (2007). Myc targets Cks1 to provoke the suppression of p27Kip1, proliferation and lymphomagenesis. *Embo J* 26(10), 2562-74.

Kim, J.W., Gao, P., Liu, Y.C., Semenza, G.L. & Dang, C.V. (2007). Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol Cell Biol* 27(21), 7381-93.

Kim, J.W., Zeller, K.I., Wang, Y., Jegga, A.G., Aronow, B.J., O'Donnell, K.A. & Dang, C.V. (2004). Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. *Mol Cell Biol* 24(13), 5923-36.

Kim, S.Y., Herbst, A., Tworkowski, K.A., Salghetti, S.E. & Tansey, W.P. (2003). Skp2 regulates Myc protein stability and activity. *Mol Cell* 11(5), 1177-88.

Kleine, H., Poreba, E., Lesniewicz, K., Hassa, P.O., Hottiger, M.O., Litchfield, D.W., Shilton, B.H. & Luscher, B. (2008). Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. *Mol Cell* 32(1), 57-69.

Knudson, A.G., Jr. & Strong, L.C. (1972). Mutation and cancer: neuroblastoma and pheochromocytoma. *Am J Hum Genet* 24(5), 514-32.

Kodadek, T., Sikder, D. & Nalley, K. (2006). Keeping transcriptional activators under control. *Cell* 127(2), 261-4.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U. & Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* 96(5), 635-44.

Kohl, N.E., Kanda, N., Schreck, R.R., Bruns, G., Latt, S.A., Gilbert, F. & Alt, F.W. (1983). Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* 35(2 Pt 1), 359-67.

Kominsky, S., Johnson, H.M., Bryan, G., Tanabe, T., Hobeika, A.C., Subramaniam, P.S. & Torres, B. (1998). IFNgamma inhibition of cell growth in glioblastomas correlates

with increased levels of the cyclin dependent kinase inhibitor p21WAF1/CIP1. *Oncogene* 17(23), 2973-9.

Kornberg, R.D. (2005). Mediator and the mechanism of transcriptional activation. *Trends Biochem Sci* 30(5), 235-9.

Kornberg, R.D. (2007). The molecular basis of eukaryotic transcription. *Proc Natl Acad Sci U S A* 104(32), 12955-61.

Koshiji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C. & Huang, L.E. (2004). HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *Embo J* 23(9), 1949-56.

Koshiji, M., To, K.K., Hammer, S., Kumamoto, K., Harris, A.L., Modrich, P. & Huang, L.E. (2005). HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. *Mol Cell* 17(6), 793-803.

Kowalik, T.F., DeGregori, J., Schwarz, J.K. & Nevins, J.R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J Virol* 69(4), 2491-500.

Krude, T., Jackman, M., Pines, J. & Laskey, R.A. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* 88(1), 109-19.

Kuras, L., Rouillon, A., Lee, T., Barbey, R., Tyers, M. & Thomas, D. (2002). Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. *Mol Cell* 10(1), 69-80.

Land, H., Parada, L.F. & Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304(5927), 596-602.

Latchman, D.S. (1998). *Eukaryotic Transcription Factors*. 3rd edition. Academic press.

Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F.M., Orcutt, M.L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L. & et al. (1993). Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260(5112), 1317-20.

Lee, T.C., Li, L., Philipson, L. & Ziff, E.B. (1997). Myc represses transcription of the growth arrest gene gas1. *Proc Natl Acad Sci U S A* 94(24), 12886-91.

Li, W. & Ye, Y. (2008). Polyubiquitin chains: functions, structures, and mechanisms. *Cell Mol Life Sci* 65(15), 2397-406.

Liotta, L.A. & Stetler-Stevenson, W.G. (1991). Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res* 51(18 Suppl), 5054s-5059s.

Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C. & Krek, W. (1998). Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45(SKP2): evidence for evolutionary conservation in the subunit composition of the CDC34-SCF pathway. *Embo J* 17(2), 368-83.

Liu, J. & Nussinov, R. (2008). Allosteric effects in the marginally stable von Hippel-Lindau tumor suppressor protein and allostery-based rescue mutant design. *Proc Natl Acad Sci U S A* 105(3), 901-6.

Lonser, R.R., Glenn, G.M., Walther, M., Chew, E.Y., Libutti, S.K., Linehan, W.M. & Oldfield, E.H. (2003). von Hippel-Lindau disease. *Lancet* 361(9374), 2059-67.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S. & Weissman, A.M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A* 96(20), 11364-9.

Lovering, R., Hanson, I.M., Borden, K.L., Martin, S., O'Reilly, N.J., Evan, G.I., Rahman, D., Pappin, D.J., Trowsdale, J. & Freemont, P.S. (1993). Identification and preliminary characterization of a protein motif related to the zinc finger. *Proc Natl Acad Sci U S A* 90(6), 2112-6.

Lu, Z., Xu, S., Joazeiro, C., Cobb, M.H. & Hunter, T. (2002). The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol Cell* 9(5), 945-56.

Lum, J.J., Bui, T., Gruber, M., Gordan, J.D., DeBerardinis, R.J., Covello, K.L., Simon, M.C. & Thompson, C.B. (2007). The transcription factor HIF-1alpha plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev* 21(9), 1037-49.

Luscher, B. & Larsson, L.G. (1999). The basic region/helix-loop-helix/leucine zipper domain of Myc proto-oncoproteins: function and regulation. *Oncogene* 18(19), 2955-66.

Lutterbach, B. & Hann, S.R. (1994). Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. *Mol Cell Biol* 14(8), 5510-22.

Maher, E.R., Iselius, L., Yates, J.R., Littler, M., Benjamin, C., Harris, R., Sampson, J., Williams, A., Ferguson-Smith, M.A. & Morton, N. (1991). Von Hippel-Lindau disease: a genetic study. *J Med Genet* 28(7), 443-7.

Maher, E.R., Webster, A.R., Richards, F.M., Green, J.S., Crossey, P.A., Payne, S.J. & Moore, A.T. (1996). Phenotypic expression in von Hippel-Lindau disease: correlations with germline VHL gene mutations. *J Med Genet* 33(4), 328-32.

Maher, E.R., Yates, J.R., Harries, R., Benjamin, C., Harris, R., Moore, A.T. & Ferguson-Smith, M.A. (1990). Clinical features and natural history of von Hippel-Lindau disease. *Q J Med* 77(283), 1151-63.

Malik, S. & Roeder, R.G. (2005). Dynamic regulation of pol II transcription by the mammalian Mediator complex. *Trends Biochem Sci* 30(5), 256-63.

Mandal, M., Bandyopadhyay, D., Goepfert, T.M. & Kumar, R. (1998). Interferon-induces expression of cyclin-dependent kinase-inhibitors p21WAF1 and p27Kip1 that prevent activation of cyclin-dependent kinase by CDK-activating kinase (CAK). *Oncogene* 16(2), 217-25.

Marhin, W.W., Chen, S., Facchini, L.M., Fornace, A.J., Jr. & Penn, L.Z. (1997). Myc represses the growth arrest gene gadd45. *Oncogene* 14(23), 2825-34.

Martens, J.A. & Winston, F. (2003). Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr Opin Genet Dev* 13(2), 136-42.

Marti, A., Wirbelauer, C., Scheffner, M. & Krek, W. (1999). Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. *Nat Cell Biol* 1(1), 14-9.

Massague, J., Blain, S.W. & Lo, R.S. (2000). TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 103(2), 295-309.

Mateyak, M.K., Obaya, A.J., Adachi, S. & Sedivy, J.M. (1997). Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. *Cell Growth Differ* 8(10), 1039-48.

Matsuoka, M., Nishimoto, I. & Asano, S. (1999). Interferon-gamma impairs physiologic downregulation of cyclin-dependent kinase inhibitor, p27Kip1, during G1 phase progression in macrophages. *Exp Hematol* 27(2), 203-9.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R. & Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399(6733), 271-5.

Maynard, M.A., Qi, H., Chung, J., Lee, E.H., Kondo, Y., Hara, S., Conaway, R.C., Conaway, J.W. & Ohh, M. (2003). Multiple splice variants of the human HIF-3 alpha locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. *J Biol Chem* 278(13), 11032-40.

McMahon, S.B., Van Buskirk, H.A., Dugan, K.A., Copeland, T.D. & Cole, M.D. (1998). The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. *Cell* 94(3), 363-74.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M. & Cyr, D.M. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat Cell Biol* 3(1), 100-5.

Menssen, A. & Hermeking, H. (2002). Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci U S A* 99(9), 6274-9.

Meyer, N., Kim, S.S. & Penn, L.Z. (2006). The Oscar-worthy role of Myc in apoptosis. *Semin Cancer Biol* 16(4), 275-87.

Miliani de Marval, P.L., Macias, E., Rounbehler, R., Sicinski, P., Kiyokawa, H., Johnson, D.G., Conti, C.J. & Rodriguez-Puebla, M.L. (2004). Lack of cyclin-dependent kinase 4 inhibits c-myc tumorigenic activities in epithelial tissues. *Mol Cell Biol* 24(17), 7538-47.

Miltenberger, R.J., Sukow, K.A. & Farnham, P.J. (1995). An E-box-mediated increase in cad transcription at the G1/S-phase boundary is suppressed by inhibitory c-Myc mutants. *Mol Cell Biol* 15(5), 2527-35.

Molinari, E., Gilman, M. & Natesan, S. (1999). Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo. *Embo J* 18(22), 6439-47.

Moreno, E. & Basler, K. (2004). dMyc transforms cells into super-competitors. *Cell* 117(1), 117-29.

Morgan, D.O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* 13, 261-91.

Muratani, M. & Tansey, W.P. (2003). How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol* 4(3), 192-201.

Murre, C., McCaw, P.S. & Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 56(5), 777-83.

Mymryk, J.S., Shire, K. & Bayley, S.T. (1994). Induction of apoptosis by adenovirus type 5 E1A in rat cells requires a proliferation block. *Oncogene* 9(4), 1187-93.

Naar, A.M., Lemon, B.D. & Tjian, R. (2001). Transcriptional coactivator complexes. *Annu Rev Biochem* 70, 475-501.

Nakayama, K., Nagahama, H., Minamishima, Y.A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., Kitagawa, M. & Hatakeyama, S. (2000). Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *Embo J* 19(9), 2069-81.

Nakayama, K., Nagahama, H., Minamishima, Y.A., Miyake, S., Ishida, N., Hatakeyama, S., Kitagawa, M., Iemura, S., Natsume, T. & Nakayama, K.I. (2004). Skp2-mediated degradation of p27 regulates progression into mitosis. *Dev Cell* 6(5), 661-72.

Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F. & Minna, J.D. (1985). L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* 318(6041), 69-73.

Nesbit, C.E., Tersak, J.M. & Prochownik, E.V. (1999). MYC oncogenes and human neoplastic disease. *Oncogene* 18(19), 3004-16.

Neumann, H.P. & Bender, B.U. (1998). Genotype-phenotype correlations in von Hippel-Lindau disease. *J Intern Med* 243(6), 541-5.

Neumann, H.P., Eggert, H.R., Scheremet, R., Schumacher, M., Mohadjer, M., Wakhloo, A.K., Volk, B., Hettmannsperger, U., Riegler, P., Schollmeyer, P. & et al. (1992). Central nervous system lesions in von Hippel-Lindau syndrome. *J Neurol Neurosurg Psychiatry* 55(10), 898-901.

Neumann, H.P. & Wiestler, O.D. (1991). Clustering of features of von Hippel-Lindau syndrome: evidence for a complex genetic locus. *Lancet* 337(8749), 1052-4.

O'Connell, B.C., Cheung, A.F., Simkevich, C.P., Tam, W., Ren, X., Mateyak, M.K. & Sedivy, J.M. (2003). A large scale genetic analysis of c-Myc-regulated gene expression patterns. *J Biol Chem* 278(14), 12563-73.

Oberg, F., Larsson, L.G., Anton, R. & Nilsson, K. (1991). Interferon gamma abrogates the differentiation block in v-myc-expressing U-937 monoblasts. *Proc Natl Acad Sci U S A* 88(13), 5567-71.

Ogawa, H., Ishiguro, K., Gaubatz, S., Livingston, D.M. & Nakatani, Y. (2002). A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* 296(5570), 1132-6.

Olschwang, S. (1999). Germline mutation and genome instability. *Eur J Cancer Prev* 8 Suppl 1, S33-7.

Orian, A., van Steensel, B., Delrow, J., Bussemaker, H.J., Li, L., Sawado, T., Williams, E., Loo, L.W., Cowley, S.M., Yost, C., Pierce, S., Edgar, B.A., Parkhurst, S.M. & Eisenman, R.N. (2003). Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. *Genes Dev* 17(9), 1101-14.

Oster, S.K., Mao, D.Y., Kennedy, J. & Penn, L.Z. (2003). Functional analysis of the N-terminal domain of the Myc oncoprotein. *Oncogene* 22(13), 1998-2010.

Osthus, R.C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L.A. & Dang, C.V. (2000). Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem* 275(29), 21797-800.

Peng, J., Schwartz, D., Elias, J.E., Thoreen, C.C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. & Gygi, S.P. (2003). A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* 21(8), 921-6.

Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F. & Eilers, M. (1997). An alternative pathway for gene regulation by Myc. *Embo J* 16(18), 5672-86.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* 70, 503-33.

Pickart, C.M. & Cohen, R.E. (2004). Proteasomes and their kin: proteases in the machine age. *Nat Rev Mol Cell Biol* 5(3), 177-87.

Pickart, C.M. & Eddins, M.J. (2004). Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta* 1695(1-3), 55-72.

Pines, J. (1999). Four-dimensional control of the cell cycle. *Nat Cell Biol* 1(3), E73-9.

Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M. & Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 8(1), 9-22.

Privalsky, M.L. (2004). The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu Rev Physiol* 66, 315-60.

Pulverer, B.J., Fisher, C., Vousden, K., Littlewood, T., Evan, G. & Woodgett, J.R. (1994). Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. *Oncogene* 9(1), 59-70.

Qin, X.Q., Livingston, D.M., Kaelin, W.G., Jr. & Adams, P.D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc Natl Acad Sci U S A* 91(23), 10918-22.

Rankin, E.B., Biju, M.P., Liu, Q., Unger, T.L., Rha, J., Johnson, R.S., Simon, M.C., Keith, B. & Haase, V.H. (2007). Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *J Clin Invest* 117(4), 1068-77.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S. & White, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc Natl Acad Sci U S A* 89(16), 7742-6.

Rathmell, W.K., Hickey, M.M., Bezman, N.A., Chmielecki, C.A., Carraway, N.C. & Simon, M.C. (2004). In vitro and in vivo models analyzing von Hippel-Lindau disease-specific mutations. *Cancer Res* 64(23), 8595-603.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L. & Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol* 25(13), 5675-86.

Raveh, T., Hovanessian, A.G., Meurs, E.F., Sonenberg, N. & Kimchi, A. (1996). Double-stranded RNA-dependent protein kinase mediates c-Myc suppression induced by type I interferons. *J Biol Chem* 271(41), 25479-84.

Reese, J.C. (2003). Basal transcription factors. *Curr Opin Genet Dev* 13(2), 114-8.

Reid, G., Hubner, M.R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J. & Gannon, F. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell* 11(3), 695-707.

Reynisdottir, I., Polyak, K., Iavarone, A. & Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* 9(15), 1831-45.

Richards, F.M., Schofield, P.N., Fleming, S. & Maher, E.R. (1996). Expression of the von Hippel-Lindau disease tumour suppressor gene during human embryogenesis. *Hum Mol Genet* 5(5), 639-44.

Roitt, I., Brostoff, J. & Male, D. (1998). *Immunology*. 5th edition. Mosby.

Rotin, D., Staub, O. & Haguenauer-Tsapis, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J Membr Biol* 176(1), 1-17.

Rubin, D.M., Coux, O., Wefes, I., Hengartner, C., Young, R.A., Goldberg, A.L. & Finley, D. (1996). Identification of the gal4 suppressor Sug1 as a subunit of the yeast 26S proteasome. *Nature* 379(6566), 655-7.

Russell, S.J., Reed, S.H., Huang, W., Friedberg, E.C. & Johnston, S.A. (1999). The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. *Mol Cell* 3(6), 687-95.

Russell, S.J., Sathyanarayana, U.G. & Johnston, S.A. (1996). Isolation and characterization of SUG2. A novel ATPase family component of the yeast 26 S proteasome. *J Biol Chem* 271(51), 32810-7.

Saleh, A., Schieltz, D., Ting, N., McMahon, S.B., Litchfield, D.W., Yates, J.R., 3rd, Lees-Miller, S.P., Cole, M.D. & Brandl, C.J. (1998). Tra1p is a component of the yeast Ada.Spt transcriptional regulatory complexes. *J Biol Chem* 273(41), 26559-65.

Salghetti, S.E., Caudy, A.A., Chenoweth, J.G. & Tansey, W.P. (2001). Regulation of transcriptional activation domain function by ubiquitin. *Science* 293(5535), 1651-3.

Salghetti, S.E., Kim, S.Y. & Tansey, W.P. (1999). Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. *Embo J* 18(3), 717-26.

Salghetti, S.E., Muratani, M., Wijnen, H., Futcher, B. & Tansey, W.P. (2000). Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc Natl Acad Sci U S A* 97(7), 3118-23.

Santamaria, D., Barriere, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., Caceres, J.F., Dubus, P., Malumbres, M. & Barbacid, M. (2007). Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* 448(7155), 811-5.

Sapountzi, V., Logan, I.R. & Robson, C.N. (2006). Cellular functions of TIP60. *Int J Biochem Cell Biol* 38(9), 1496-509.

Scheel, H. & Hofmann, K. (2003). No evidence for PHD fingers as ubiquitin ligases. *Trends Cell Biol* 13(6), 285-7; author reply 287-8.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D. & Howley, P.M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75(3), 495-505.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. & Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63(6), 1129-36.

Schlesinger, D.H., Goldstein, G. & Niall, H.D. (1975). The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochemistry* 14(10), 2214-8.

Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75(2), 163-89.

Schuster-Bockler, B. & Bateman, A. (2008). Protein interactions in human genetic diseases. *Genome Biol* 9(1), R9.

Sears, R., Leone, G., DeGregori, J. & Nevins, J.R. (1999). Ras enhances Myc protein stability. *Mol Cell* 3(2), 169-79.

Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K. & Nevins, J.R. (2000). Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev* 14(19), 2501-14.

Sears, R.C. (2004). The life cycle of C-myc: from synthesis to degradation. *Cell Cycle* 3(9), 1133-7.

Seizinger, B.R., Rouleau, G.A., Ozelius, L.J., Lane, A.H., Farmer, G.E., Lamiell, J.M., Haines, J., Yuen, J.W., Collins, D., Majoor-Krakauer, D. & et al. (1988). Von Hippel-Lindau disease maps to the region of chromosome 3 associated with renal cell carcinoma. *Nature* 332(6161), 268-9.

Semenza, G.L. (2001). HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 107(1), 1-3.

Semple, C.A. (2003). The comparative proteomics of ubiquitination in mouse. *Genome Res* 13(6B), 1389-94.

Seoane, J., Le, H.V. & Massague, J. (2002). Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 419(6908), 729-34.

Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M. & Massague, J. (2001). TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. *Nat Cell Biol* 3(4), 400-8.

Sgambati, M.T., Stolle, C., Choyke, P.L., Walther, M.M., Zbar, B., Linehan, W.M. & Glenn, G.M. (2000). Mosaicism in von Hippel-Lindau disease: lessons from kindreds with germline mutations identified in offspring with mosaic parents. *Am J Hum Genet* 66(1), 84-91.

Shay, J.W. & Bacchetti, S. (1997). A survey of telomerase activity in human cancer. *Eur J Cancer* 33(5), 787-91.

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M. & Clurman, B.E. (1997). Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* 11(11), 1464-78.

Sheiness, D. & Bishop, J.M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. *J Virol* 31(2), 514-21.

Sherr, C.J. & Roberts, J.M. (2004). Living with or without cyclins and cyclin-dependent kinases. *Genes Dev* 18(22), 2699-711.

Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. & Selkoe, D.J. (2001). Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* 293(5528), 263-9.

Sicinski, P., Zacharek, S. & Kim, C. (2007). Duality of p27Kip1 function in tumorigenesis. *Genes Dev* 21(14), 1703-6.

Spence, J., Gali, R.R., Dittmar, G., Sherman, F., Karin, M. & Finley, D. (2000). Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. *Cell* 102(1), 67-76.

Spence, J., Sadis, S., Haas, A.L. & Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol Cell Biol* 15(3), 1265-73.

Spiegelman, B.M. & Heinrich, R. (2004). Biological control through regulated transcriptional coactivators. *Cell* 119(2), 157-67.

Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F. & Eilers, M. (2001). Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* 3(4), 392-9.

Stebbins, C.E., Kaelin, W.G., Jr. & Pavletich, N.P. (1999). Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science* 284(5413), 455-61.

Stolle, C., Glenn, G., Zbar, B., Humphrey, J.S., Choyke, P., Walther, M., Pack, S., Hurley, K., Andrey, C., Klausner, R. & Linehan, W.M. (1998). Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. *Hum Mutat* 12(6), 417-23.

Strahl, B.D. & Allis, C.D. (2000). The language of covalent histone modifications. *Nature* 403(6765), 41-5.

Strasser, A., Harris, A.W., Bath, M.L. & Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature* 348(6299), 331-3.

Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U. & Krek, W. (1999). p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. *Nat Cell Biol* 1(4), 207-14.

Swaffield, J.C., Bromberg, J.F. & Johnston, S.A. (1992). Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4. *Nature* 357(6380), 698-700.

Tanimoto, K., Makino, Y., Pereira, T. & Poellinger, L. (2000). Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. *Embo J* 19(16), 4298-309.

Terrell, J., Shih, S., Dunn, R. & Hicke, L. (1998). A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol Cell* 1(2), 193-202.

Thomas, D. & Tyers, M. (2000). Transcriptional regulation: Kamikaze activators. *Curr Biol* 10(9), R341-3.

Thrower, J.S., Hoffman, L., Rechsteiner, M. & Pickart, C.M. (2000). Recognition of the polyubiquitin proteolytic signal. *Embo J* 19(1), 94-102.

To, K.K., Sedelnikova, O.A., Samons, M., Bonner, W.M. & Huang, L.E. (2006). The phosphorylation status of PAS-B distinguishes HIF-1alpha from HIF-2alpha in NBS1 repression. *Embo J* 25(20), 4784-94.

Tonini, T., Rossi, F. & Claudio, P.P. (2003). Molecular basis of angiogenesis and cancer. *Oncogene* 22(42), 6549-56.

Trimarchi, J.M. & Lees, J.A. (2002). Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 3(1), 11-20.

Trumpp, A., Refaeli, Y., Oskarsson, T., Gasser, S., Murphy, M., Martin, G.R. & Bishop, J.M. (2001). c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* 414(6865), 768-73.

Tsvetkov, L.M., Yeh, K.H., Lee, S.J., Sun, H. & Zhang, H. (1999). p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* 9(12), 661-4.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M. & Wahl, G.M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell* 9(5), 1031-44.

Wagner, A.J., Small, M.B. & Hay, N. (1993). Myc-mediated apoptosis is blocked by ectopic expression of Bcl-2. *Mol Cell Biol* 13(4), 2432-40.

Vale, R.D. (2000). AAA proteins. Lords of the ring. *J Cell Biol* 150(1), F13-9.

Walker, C.W., Boom, J.D. & Marsh, A.G. (1992). First non-vertebrate member of the myc gene family is seasonally expressed in an invertebrate testis. *Oncogene* 7(10), 2007-12.

Walther, M.M., Lubensky, I.A., Venzon, D., Zbar, B. & Linehan, W.M. (1995). Prevalence of microscopic lesions in grossly normal renal parenchyma from patients with von Hippel-Lindau disease, sporadic renal cell carcinoma and no renal disease: clinical implications. *J Urol* 154(6), 2010-4; discussion 2014-5.

Walther, M.M., Reiter, R., Keiser, H.R., Choyke, P.L., Venzon, D., Hurley, K., Gnarra, J.R., Reynolds, J.C., Glenn, G.M., Zbar, B. & Linehan, W.M. (1999). Clinical and genetic characterization of pheochromocytoma in von Hippel-Lindau families: comparison with sporadic pheochromocytoma gives insight into natural history of pheochromocytoma. *J Urol* 162(3 Pt 1), 659-64.

Wang, J., Xie, L.Y., Allan, S., Beach, D. & Hannon, G.J. (1998). Myc activates telomerase. *Genes Dev* 12(12), 1769-74.

Vassilev, A., Yamauchi, J., Kotani, T., Prives, C., Avantaggiati, M.L., Qin, J. & Nakatani, Y. (1998). The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. *Mol Cell* 2(6), 869-75.

Vaux, D.L., Cory, S. & Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335(6189), 440-2.

Weissman, A.M. (2001). Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2(3), 169-78.

Welcker, M., Orian, A., Grim, J.E., Eisenman, R.N. & Clurman, B.E. (2004). A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. *Curr Biol* 14(20), 1852-7.

Welcker, M., Singer, J., Loeb, K.R., Grim, J., Bloecher, A., Gurien-West, M., Clurman, B.E. & Roberts, J.M. (2003). Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Mol Cell* 12(2), 381-92.

Vennstrom, B., Sheiness, D., Zabielski, J. & Bishop, J.M. (1982). Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. *J Virol* 42(3), 773-9.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R., 3rd, Koonin, E.V. & Deshaies, R.J. (2002). Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* 298(5593), 611-5.

Verma, R., Oania, R., Graumann, J. & Deshaies, R.J. (2004). Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell* 118(1), 99-110.

Vermeulen, K., Van Bockstaele, D.R. & Berneman, Z.N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* 36(3), 131-49.

Vervoorts, J., Luscher-Firzlaff, J.M., Rottmann, S., Lilischkis, R., Walsemann, G., Dohmann, K., Austen, M. & Luscher, B. (2003). Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP. *EMBO Rep* 4(5), 484-90.

Vidal, A. & Koff, A. (2000). Cell-cycle inhibitors: three families united by a common cause. *Gene* 247(1-2), 1-15.

Vlach, J., Hennecke, S. & Amati, B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *Embo J* 16(17), 5334-44.

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

Won, K.A. & Reed, S.I. (1996). Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *Embo J* 15(16), 4182-93.

Wong, C.W. & Privalsky, M.L. (1998). Transcriptional repression by the SMRT-mSin3 corepressor: multiple interactions, multiple mechanisms, and a potential role for TFIIB. *Mol Cell Biol* 18(9), 5500-10.

Wood, M.A., McMahon, S.B. & Cole, M.D. (2000). An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. *Mol Cell* 5(2), 321-30.

Wu, K.J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J. & Dalla-Favera, R. (1999a). Direct activation of TERT transcription by c-MYC. *Nat Genet* 21(2), 220-4.

Wu, K.J., Polack, A. & Dalla-Favera, R. (1999b). Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC. *Science* 283(5402), 676-9.

Wu, S., Cetinkaya, C., Munoz-Alonso, M.J., von der Lehr, N., Bahram, F., Beuger, V., Eilers, M., Leon, J. & Larsson, L.G. (2003). Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. *Oncogene* 22(3), 351-60.

Wu, X. & Levine, A.J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. *Proc Natl Acad Sci U S A* 91(9), 3602-6.

Xaus, J., Cardo, M., Valledor, A.F., Soler, C., Lloberas, J. & Celada, A. (1999). Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. *Immunity* 11(1), 103-13.

Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K. & Nakayama, K.I. (2004). Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *Embo J* 23(10), 2116-25.

Yamasaki, L. & Pagano, M. (2004). Cell cycle, proteolysis and cancer. *Curr Opin Cell Biol* 16(6), 623-8.

Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M. & Ashwell, J.D. (2000). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 288(5467), 874-7.

Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W.C., Stukenberg, P.T., Shenolikar, S., Uchida, T., Counter, C.M., Nevins, J.R., Means, A.R. & Sears, R. (2004). A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol* 6(4), 308-18.

Yeh, K.H., Kondo, T., Zheng, J., Tsvetkov, L.M., Blair, J. & Zhang, H. (2001). The F-box protein SKP2 binds to the phosphorylated threonine 380 in cyclin E and regulates ubiquitin-dependent degradation of cyclin E. *Biochem Biophys Res Commun* 281(4), 884-90.

Yu, H., Peters, J.M., King, R.W., Page, A.M., Hieter, P. & Kirschner, M.W. (1998a). Identification of a cullin homology region in a subunit of the anaphase-promoting complex. *Science* 279(5354), 1219-22.

Yu, M., Schreek, S., Cerni, C., Schamberger, C., Lesniewicz, K., Poreba, E., Vervoorts, J., Walsemann, G., Grotzinger, J., Kremmer, E., Mehraein, Y., Mertsching, J., Kraft, R., Austen, M., Luscher-Firzlaff, J. & Luscher, B. (2005). PARP-10, a novel Myc-interacting protein with poly(ADP-ribose) polymerase activity, inhibits transformation. *Oncogene* 24(12), 1982-93.

Yu, Z.K., Gervais, J.L. & Zhang, H. (1998b). Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. *Proc Natl Acad Sci U S A* 95(19), 11324-9.

Zbar, B., Kishida, T., Chen, F., Schmidt, L., Maher, E.R., Richards, F.M., Crossey, P.A., Webster, A.R., Affara, N.A., Ferguson-Smith, M.A., Brauch, H., Glavac, D., Neumann, H.P., Tisherman, S., Mulvihill, J.J., Gross, D.J., Shuin, T., Whaley, J., Seizinger, B., Kley, N., Olschwang, S., Boisson, C., Richard, S., Lips, C.H., Lerman, M. & et al. (1996). Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 8(4), 348-57.

Zhang, H., Gao, P., Fukuda, R., Kumar, G., Krishnamachary, B., Zeller, K.I., Dang, C.V. & Semenza, G.L. (2007). HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. *Cancer Cell* 11(5), 407-20.

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