The Transcriptional Function of the c-Myc Oncoprotein and its Regulation by the Ubiquitin/Proteasome Pathway

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Findus planted meatballs in the hope to get more meatballs. You never know unless you have tried.

Für Mama
Hier, dass Du Dich freust!

Abstract

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The c-Myc proto-oncogene encodes a short lived-transcription factor that plays an important role in cellular proliferation, growth and apoptosis. *c-myc* is often rearranged in tumours resulting in deregulated expression. c-Myc both activates and represses transcription of a number of target genes.

This thesis focuses firstly on the mechanism by which c-Myc represses differentiation-induced genes and secondly on the regulation of c-Myc by ubiquitin/proteasome mediated turnover and its consequences for c-Myc function in transcription.

Our results show that differentiation-induced expression of the cyclin-dependent kinase inhibitor (CKI) p21Cip1 is repressed by Myc at the level of transcription. Myc was shown to repress the p21Cip1 core promoter by direct interaction with the initiator binding protein Miz-1.

The rapid turnover of c-Myc is shown to be mediated by the ubiquitin/proteasome pathway and we have identified the phosphorylation site Thr58, which is frequently mutated in Burkitt's lymphoma, as an important recognition site for this process. As a result of Thr58 mutation, c-Myc escapes this regulation which results in Myc protein accumulation. We further show that the E3 ubiquitin ligase SCF^{Skp2} interacts with Myc during G1-S phase transition of the cell cycle and promotes its ubiquitylation and proteasomal degradation. Surprisingly, Skp2 promotes c-Myc-induced S-phase transition and is required for transcriptional activation by Myc. Moreover our data suggest that Skp2 and components of the proteasome is recruited by c-Myc target gene promoters in conjunction with protein ubiquitylation. These results suggest that Skp2 is a transcriptional cofactor for c-Myc.
The thesis suggests an important role for c-Myc at the G1/S transition by transcriptional repression of the CKI p21Cip1and by stimulation of cell cycle genes via Skp2 coactivator function. The thesis also sheds light on the regulation of c-Myc turnover and suggests an important interdependence between transcription and ubiquitylation.

Key words: Myc, Skp2, Miz-1, p21Cip1, transcription, ubiquitin/proteasome pathway, cell cycle

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Appendix

Papers I-III

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

I. Wu S., Cetinkaya C., Munoz-Alonso M.J., von der Lehr N., Bahram F., Beuger V., Eilers M., Leon J., and Larsson L.G. (2003) Myc represses differentiation-induced *p21CIP1* expression via Miz-1-dependent interaction with the *p21* core promoter. *Oncogene* 22, 351-360

II. Bahram F., von der Lehr N., Cetinkaya C., and Larsson L.G. (2000) c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood* 95 (6), 2104-2110

III. von der Lehr N., Johansson S., Wu S., Bahram F., Castell A., Cetinkaya C., Hydbring P., Weidung I., Nakayama K., Nakayama K.I., Söderberg O., Kerppola T.K., and Larsson L.G. (2003) Myc-dependent recruitment of the SCF^{Skp2} E3-ligase complex tp promoters connects transcriptional activation and ubiquitin/proteasome-mediated degradation *Submitted*

Papers I and II were reprinted with permission form the publishers.

Abbreviations

List of selected abbreviations commonly used in the text:

APC	anaphase promoting complex
bHLHZip	basic/helix-loop-helix/leucine zipper
CAK	cdk activating kinase
cdk	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CKI	cyclin dependent kinase inhibitor
CoIP	coimmunoprecipitation
CTD	carboxy terminal domain
DUB	deubiquitylating enzyme
GS	growth signal
GTF	general transcription factors
HAT	histone acetylase
HDAC	histone deacetylase
Inr	initiator
K	lysine
LRR	leucine rich repeat
MB	Myc box
MEF	mouse embryonic fibroblast
PolII	RNA polymerase II
SAGE	serial analysis of gene expression
SCF	Skp1, Cullin, F-box complex
SID	Sin-3 interacting domain
TAD	transcriptional activation domain
TAF	TBP associated factor
TBP	TATA binding protein
Ub	ubiquitin

Introduction

Cell division and development are fundamental processes of life and have to be controlled tightly in order to prevent cancer and other diseases. Cells have evolved a high number of self-guard mechanisms to prevent malfunctioning cells from expanding. In order to understand and treat diseases such as cancer researchers are trying to understand the basic mechanisms underlying the biology of the cell. This will lead to insights about how both "ill" and "healthy" cells are functioning and can hopefully be used as a basis for developing medicines and treatments.

Most processes which take place in a living cell are controlled at the level of gene transcription, where genes are transcribed into RNA which in turn is the template for protein synthesis. Transcription is controlled by a variety of factors, among these are the RNA polymerases and the transcription factors with associated cofactors. c-Myc, which was studied in this thesis, is an essential DNA binding transcription factor that regulates the transcription of many genes that are involved in controlling cell growth, proliferation, differentiation and programmed cell death (apoptosis) and many other fundamental cellular processes. Deregulation of c-Myc by chromosomal translocation or other types of mutations are one of the milestones of cancer development. We therefore try to understand more about the function and regulation of this transcription factor.

Although it is essential that c-Myc and other transcription factors exert their functions it is equally important that they are inactivated once the desired effect has been elicited. A very effective way of controlling the regulatory factors is protein destruction by the ubiquitin/proteasome pathway. Proteins that are aimed for destruction are tagged with a chain of small ubiquitin molecules and thereby recognized by the proteasome, which is a cellular machinery for protein degradation. This pathway has attracted a lot of attention in during recent years and it has been shown that covalent attachment of ubiquitin to proteins (ubiquitylation) not only regulates protein destruction but also several other fundamental cellular processes such as transcription, DNA repair, transport between different cell compartments and programmed cell death.

In this thesis, three aspects of the transcription factor c-Myc were studied. In the first part we addressed the question of the mechanism by which c-Myc represses differentiation induced gene expression, in particular an inhibitor of cell cycle progression called p21Cip1. The second part deals with regulation of c-Myc turnover. We investigated mechanisms and factors involved in ubiquitin-tagging of c-Myc for degradation by the ubiquitin/proteasome pathway. The third part addresses the question whether ubiquitylation also contributes to the function of c-Myc as a transcription factor.

Background

Cancer

Cancer is a complex disease generated by multiple genetic alterations (for review see(Hanahan and Weinberg, 2000). Generally, there are two types of gene families involved in the generation of cancer: tumour suppressor genes and proto-oncogenes. By either overriding the activity of tumour suppressor genes or increasing (or deregulating) the potential of proto-oncogenes mammalian cells get predisposed to tumour development. Nevertheless, only one mutation is not enough to give rise to cancer development since mammalian cells have evolved a number of safeguard mechanisms to avoid transformation of normal cells into highly malignant derivatives. For example, cultured cells from rodents require at least two genetic changes before they become tumourogenetic; cultured human cells require at least three genetic changes (Hahn et al., 1999). *In vivo*, however, about seven steps of genetic alterations are required before tumours arise (Vogelstein and Kinzler, 1993). Thus a succession of genetic changes conferring to different types of growth advantages is needed to transform normal cells into cancer cells. These alterations in malignant cells include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, block of cellular differentiation, evasion of programmed cell death (apoptosis), genetic instability, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. These are summarized in Fig.1 and will be outlined briefly below. Those aspects important to this thesis will be described in more detail in following chapters. It is believed that genetic alterations affecting all or most of these processes are needed in combination to generate malignant, invasive tumours (Hanahan and Weinberg, 2000).

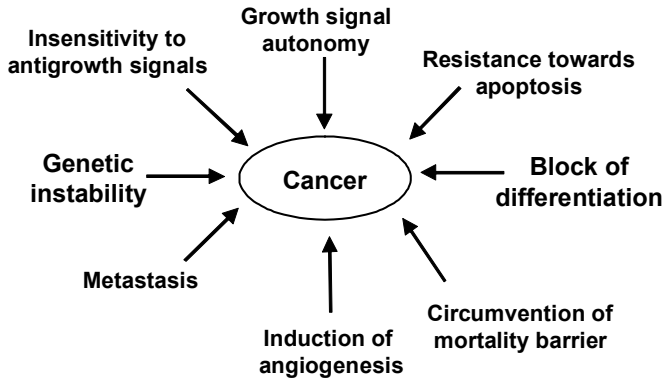


Figure 1: Outline of cellular processes affected by genetic alterations in cancer cells

Growth signal autonomy

Many oncogenes involved in cancer act by mimicking normal growth signalling in one way or another and are therefore independent of other stimuli from their

normal environment. There are many different ways by which independence of mitogeninc signals can be achieved. Some cancer cells acquire the ability to synthesize their own growth factors, a state termed autocrine stimulation. In other types of cancer, cell surface receptors transducing growth-stimulatory signals are targets of genetic lesions. This leads to generation of cells that are hyperresponsive to or independent of growth factors (Slamon et al., 1987). Another mechanism is the ability of cancer cells to switch the types of extracellular matrix (integrins) receptors they express, thereby favouring those that transmit growth signals(Lukashev and Werb, 1998, Giancotti and Ruoslahti, 1999). A more complicated mechanism of acquired growth signal autonomy is the mutation of genes encoding proteins involved in transducing and processing the downstream signals from ligand-activated GF receptors and integrins. One example is the Ras-proteins. These have essential roles in transmitting signals from several crucial signalling pathways that regulate normal cellular proliferation and survival (for recent review see (Downward, 2003). The activation state of Ras is controlled by the cycle of hydrolysis of bound GTP(Campbell et al., 1998). When bound to GTP, Ras is active and can engage downstream target enzymes; when bound to GDP Ras is inactive. The nucleotide exchange between GDP and GTP is catalysed by the guanine nucleotide exchange factors (GEFs) and the nucleotide hydrolysis GTPase activating proteins (GAPs). Once Ras is activated it can affect several effector enzymes. For example, Ras activates the Raf kinase which results in the activation of mitogen-activated protein kinases (MAPKs) which in turn activate transcription factors such as Fos and c-Jun. Fos/Jun heterodimers (Yordy and Muise-Helmericks, 2000) can then stimulate the transcription of key cell cycle regulatory proteins which enable the cells to progress form G1 to S phase. In another pathway, Ras interacts directly with the phosphatidylinositol 3-kinases (PI3Ks) and thereby activate them (Rodriguez-Viciana et al., 1994, Pacold et al., 2000). Activated PI3K controls a large number of downstream enzymes such as Pdk1 and Akt. Akt/Pdk1 has a strong anti-apoptotic function and can thereby signal cell survival (Khwaja et al., 1997). PI3K activation further leads to stimulation of Rac which is involved in the the regulation of transcription factor pathways, leading to cell cycle progression (Lambert et al., 2002, Malliri et al., 2002). Approximately 20% of all human tumours have undergone an activating mutation in one of the *ras* genes (Bos, 1989) and activated Ras contributes significantly to several aspects of the malignat phenotype (Shields et al., 2000).

Insensitivity to antigrowth signals and block of differentiation

Cancer cells must also be able to evade antigrowth signals. In normal cells, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. These signals can either force the cell from their active proliferating state into quiescent (G_0) state or cells may be part of a differentiation programme. Two such antiproliferative signals are exerted by the cytokines TGFβ and interferons (IFNα, β and γ). TGFβ exerts its cellular effects via binding to type I and II serine/threonine kinase receptors and as a result the Smad-signalling pathway is activated. The Smad complexes then translocate into the nucleus where they, in cooperation with coactivators and corepressors, act as transcription factors regulating gene expression. Inappropriate regulation of TGFβ signalling has been

implicated in carcinogenesis (for review see(Moustakas et al., 2001). Interferons have ligand specific type I and type II receptors which bind to the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). These subsequently activate the STAT proteins which translocate into the nucleus as a homodimer and activate transcription of genes negatively regulating the cell cycle progression (for review see(Brivanlou and Darnell, 2002).

Mutations inactivating tumour suppressor genes are common in cancer cells. These are usually involved in the transmission of negative growth signals, inhibition of positive growth signals or acts as negative regulators of cell cycle progression. One important tumour suppressor protein and regulatory factor for cell cycle progression is the retinoblastoma protein (pRb), which controls the activity of the transcription factor E2F. E2F activates genes participating in G1-S phase transition. When pRB is hypophosphorylated it binds to E2F and thereby hinders transcriptional activation (for recent review see (Ho and Dowdy, 2002). Upon mitogenic signalling or other events that signal for cell cycle progression, pRb is hyperphosphorylated and releases E2F which then can activate transcription of genes necessary for G1/S transition. When pRb is inactivated by mutations in cancer cells, E2F is free to activate S-phase genes in the absence of mitogenic stmuli. Other molecules that guard G1/S transition are the cyclin dependent kinase inhibitors (CKIs). They inhibit the cyclin/cyclin dependent kinases (cdks) complexes which activate cell cycle progression and thereby represent another layer of control of the cell cycle (Ho and Dowdy, 2002). These mechanisms will be described more extensively in the next chapter which gives an introduction to the mammalian cell cycle. Another frequent mutation in cancer is the disruption of the *p53* tumour suppressor gene. Both DNA-damaging agents, such as irradiation and chemotherapeutic drugs, and internal aberrations such as deregulation of oncogenes, stabilise and activate p53. p53 is a transcription factor that control a number of genes participating in cell cycle progression, DNA repair and apoptosis. All of these functions of p53 can be seen as a safeguard mechanism of cells to prevent tumourigenesis (for recent review see(Hickman et al., 2002).

Another hallmark of cancer is the lack or block of cellular differentiation (for recent review see (Tenen, 2003). This is particularly evident in among hematopoietic tumours. The decisions to differentiate from self-renewing pluripotent stem cells via immature but committed progenitor cell to mature terminally differentiated are controlled by specific haematopoietic transcription factors. These transcription factors are frequently translocated and deregulated in leukaemia. Additionally, the process of irreversible differentiation is blocked by overexpression of many oncoproteins, e.g. c-Myc which will induce a shift from cell differentiation to cell proliferation (for review see(Grandori et al., 2000). During human colon carcinogenesis, inacativation of the APC/β-catenin pathway blocks the development of enterocytes in the colonic crypts into a differentiated, postmitotic state (Kinzler and Vogelstein, 1996).

Resistance towards apoptosis

Programmed cell death (apoptosis) is another important mechanism to maintain homeostasis. The apoptotic programme is present in latent form in virtually all cell

types of the body. Apoptosis is an energy-dependent process which proceeds through the activation of many downstream effectors and consists of two main pathways, the extrinsic and the intrinsic pathway (for reviews see(Igney and Krammer, 2002, Jesenberger and Jentsch, 2002)). Important components of both these apoptotic pathways are the caspases which consist of a family of cysteine proteases that can be grouped into initiator (caspase-8 and -9) and effector caspases (caspase-3, -6 and -7). Caspase activation requires the proteolytic cleavage to liberate subunits that reconstitute an active caspase heterodimer. The so-called caspase cascade, which is a sequence of subsequent proteolytic caspase cleavages giving rise to the active caspase dimers, can be initiated either through the death receptor Fas (also referred to as the extrinsic pathway) or by the mitochondrial pathway (also referred to as the intrinsic pathway). Activation of the extrinsic pathway is stimulated by binding of a ligand to the death receptor which induces the formation of a death-signalling complex (DISC). This complex is capable of recruiting several procaspase-8 molecules through an adaptor molecule which results in cross activation of caspase-8 and the release of active caspase-8 into the cytosol. Thereafter, if the levels of caspase-8 are high, the effector caspase cascade is activated directly through caspase-3 or, upon low levels of caspase-8, the proapoptotic Bcl-2 family member Bid is cleaved. The product tBid works together with other Bcl-2 family members to trigger the mitochondrial pathway. Members of the Bcl-2 family, which can either block or enhance apoptosis are other important players in the process of apoptosis. The intrinsic pathway is initiated upon stress-induced signals such as DNA-damage and results in the release of cytochrome c and other apoptotic factors from the intermembrane space of the mitochondria. Cytochrome c binds to the adaptor molecule apoptotic protease factor 1 (Apaf-1) and this complex then recruits and activates the initiator caspase-9 which in turn can activate caspase 3. Both ways lead to caspase-3 activation whereafter several signals are triggering apoptosis. The apoptotic process is tightly controlled by various proteins. As mentioned earlier, the tumour suppressor protein p53 has important pro-apoptotic functions. p53 is activated by stress-response and activates both the extrinsic and intrinsic apoptotic pathway (for review see(Igney and Krammer, 2002).

Genetic instability

The role of genetic instability in tumour formation has been a matter of debate for a considerable amount of time. It is now well established that tumours accumulate genetic alterations, including subtle changes in DNA sequences, as a result of genomic instability as well as cytogenetically visible changes, such as chromosome losses, gains and translocations, due to chromosomal instability (Lengauer et al., 1998). One of the best understood genetic instability is arising from the inactivation of DNA mismatch repair (MMR) genes such as *msh2* or *mlh1*. The inactivation of these genes in tumours gives rise to instability at the nucleotide level as naturally occurring replication errors cannot be repaired effectively (for review see(Cahill et al., 1999). Genetic instability can also be the result of the loss of the normal mitotic checkpoint pathway (Cahill et al., 1998).

Another important pathway that impacts genetic instability involves the p53 tumour suppressor gene (Livingstone et al., 1992, Yin et al., 1992). As described earlier, the activation of p53 in normal cells results in cell cycle arrest or apoptosis. Disabling the p53 pathway enables cells to enter and proceed through the cell cycle under conditions that increase the frequencies of aneuploidy and large-scale structural alterations such as gene amplification, deletion and translocation (Livingstone et al., 1992). Both inactivation of the p53 dependent apoptotic response and the increase in genetic instability that accompany loss of the p53 pathway are highly selected during cancer progression (Hollstein et al., 1991). Mutations that precede p53 inactivation such as those that activate oncogenes might create a genome-destabilising environment that also selects for loss of p53 function (Eischen et al., 1999).

Circumvention of mortality barrier, induction of angiogenesis and metastasis

Cells growing in culture have a limited replicative potential (Hayflick, 1997), ensuring that cells having gone through a certain amount of cell doublings (50 for human cell cultures) will stop growing. This is ensured by shortening of the telomeres after each division of a cell, eventually leading to the inability of protection of chromosomal DNA which results in cell death (Counter et al., 1992). Malignant cells show upregulation of the telomerase enzyme which ensures telomere maintenance (Bryan et al., 1995). Alternatively, malignant cells can activate a mechanism which maintains telomeres through recombination-based interchromosomal exchanges of sequence information (Bryan et al., 1995).

In order for cells to function and survive they must be supplied with oxygen and nutrients. Therefore neoplastic cells must develop angiogenic abilities in order for tumours to progress to larger size (Bouck et al., 1996, Hanahan and Folkman, 1996). This ability seems to be acquired in discrete steps during tumour development via an “angiogenic switch” from vascular quiescence to angiogenesis. This is achieved by a change in the balance of angiogenesis inducers and inhibitors, e.g. by increased expression angiogenesis inducers such as growth factors and decreased expression of endogenous inhibitors. Further, proteases which can control the availability of angiogenic activators and inhibitors can be deregulated (Hanahan and Weinberg, 2000).

The development of most tumours leads to tissue invasion and metastasis. Primary tumour cells evade the tissue and migrate to other sites where they can form new colonies. Metastases are the major cause for human cancer deaths (Sporn, 1996). The mechanism underlying invasion and metastasis is poorly understood. Proteins involved in adherence seem to play a central role, further also proteins acting as suppressors of invasion and metastasis seem to be eliminated or downregulated (reviewed in(Hanahan and Weinberg, 2000).

The mammalian cell cycle

The mammalian cell cycle is divided into four phases, namely gap1 (G1), DNA-synthesis (S), gap 2 (G2) and mitosis (M) (for review see (Johnson and Walker, 1999)). In the G1 phase the cells prepare for the process of DNA replication. Mitogenic and growth inhibitory signals are integrated and the cell decides whether to pause, exit or proceed the cell cycle. This occurs at an important G1 checkpoint which has been identified both in yeast and mammalian cells. At this point the cell becomes irreversibly committed to DNA replication. During S phase DNA synthesis occurs whereas in G2 the cell prepares for the process of cell division. At another checkpoint in G2 phase before the onset of M-phase the cell responds to DNA damage and causes a delay to allow DNA repair before the cell enters mitosis. In M phase the replicated chromosomes are separated into separate daughter nuclei and two daughter cells are formed. An additional checkpoint in M ensures that the chromosomes are attached correctly to the spindle. The term G_0 is used for cells that have exited the cell cycle and have become quiescent. The main mechanisms of cell cycle control described here are summarized in a simplified view as shown in Fig. 2.

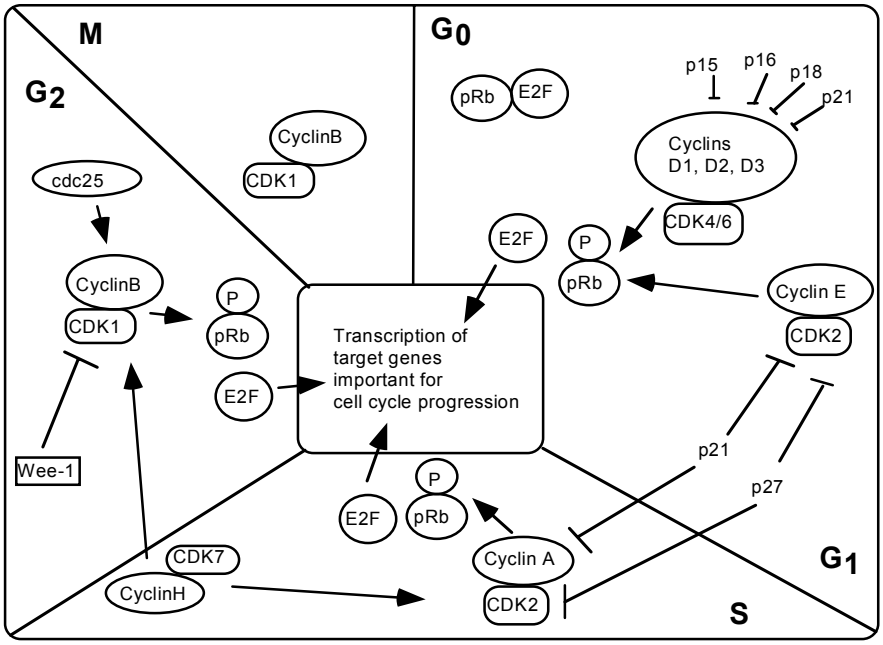


Figure 2: A simplified view of the mammalian cell cycle. Printed with permission of Anna Dimberg

Like many other processes in the mammalian cell, transition from one cell cycle phase to another is tightly regulated. One of the first genes to be identified as an important regulator of the cell cycle was the yeast *cdc2/cdc28* (Hartwell, 1978, Nurse and Bissett, 1981). Activation of *cdc2/cdc28* requires association with a regulatory subunit referred to as cyclin(Rosenthal et al., 1980 which were first

identified as proteins whose accumulation and degradation oscillated during the cell cycle. The sequential activation and inactivation of cyclin-dependent kinases by cyclins provide the primary means of cell cycle regulation. In mammalian cells, nine cdks (referred to as cdk1-9) and at least 16 cyclins have been identified {Johnson, 1999 #184). All cyclins contain a homologous region which is referred to as the cyclin box which is a domain to bind and activate cdks. Other key players in the mammalian cell cycle are the proteins pRb and E2F. The E2F family of transcriptional activators form dimeric complexes consisting of one E2F subunit and one DP partner. There are six E2Fs, E2F1-6 and two DPs, DP1 and 2. pRb interacts mainly with E2F1-4 dimerized with DP1. Further, pRb is member of a protein family that includes the pRb related proteins p107 and p130. These preferentially bind to other E2F family members and DPs. p130 is the predominant E2F regulator in cells that have entered quiescent (G_0) state whereas p107 is associated with E2F primarily in S-phase (reviewed in(Trimarchi and Lees, 2002)).

The D-type cyclins are induced upon mitogenic signalling and associate with cdk4 and cdk6 whereafter they phosphorylate the retinoblastoma protein (pRb) (for review see(Sherr and Roberts, 1999)). Additional phosphorylation of pRb by cyclin E/Cdk2 releases pRb from E2F, which in turn can activate transcription of genes necessary for DNA replication. Among these are cyclin E and A, both required for the G1/S transition. Cyclin E regulates cdk2 to enforce pRb phosphorylation, thus creating a positive feedback loop. Additionally, cyclin D/Cdk4 can sequester the cdk inhibitors (CKIs) p21 and p27. Cyclin E/Cdk2 can further phosphorylated p27 on Thr187 and thereby induce its degradation. These changes reduce the cell's dependency on growth signals and the cells enter S phase irreversibly.

The cyclin/Cdk complexes are in turn themselves regulated by the cdk inhibitors (CKIs). The CKIs can be divided into two families based on their structure and cdk targets. The first family is referred to as the INK4 proteins since they inhibit the catalytic subunits of Cdk4 and 6. Proteins belonging to this family are p16Ink4a, p15Ink4b, p18Ink4c and p19Ink4d. The second family is referred to as the Cip/Kip family and consists of p21Cip1, p27Kip1 and p57Kip2. These can inhibit the cyclin-Cdk1 and -Cdk2 complexes (Vidal and Koff, 2000). CKIs of the Cip/Kip family bind to both the cyclin and the cdk in the complex, thereby either distorting the active site of the cdk or inhibiting enzymatic activity by insertion into the ATP-binding sites. Binding of these CKIs to cyclin/Cdk complexes also inhibit the stimulatory phosphorylation of Cdk by CAK (see below).

p21Cip1 is involved in p53-dependent DNA-damage induced G1 arrest. The amount of p21Cip1 protein increases following exposure to DNA damaging agents. p21Cip1 also associates with PCNA, an elongation factor for DNA replication and inhibits DNA replication. p21Cip1 is often upregulated upon differentiation (Vidal and Koff, 2000). p27Kip1 is critical to the maintenance of G_0 and plays also a role in cyclin E activation at the restriction point. p27Kip1 degradation is dependent on phosphorylation by cyclin E/Cdk2 complexes. It has further been demonstrated that p27Kip1 is a downstream effector of pRb mediated senescence, indicating that p27Kip1 plays a critical role in mediating cell cycle exit rather than the restriction point in cycling cells (Ho 2002). p57Kip2 has a role in the control of the

commitment/withdrawal decision as well as differentiation and apoptosis in particular tissues (Vidal and Koff, 2000).

Cyclins and cdks can also have other function than regulation of the cell cycle. These include regulation of transcription, DNA repair, differentiation and apoptosis (Dynlacht, 1997). Cyclin C/Cdk8, cyclin T/Cdk9 and cyclin H/Cdk 7 have been found to be components of the basal transcription machinery. Cyclin H/Cdk7, also referred to as CAK (cdk activating kinase), activates the Cdks by phosphorylating a conserved threonine residue (Thr 114) whereas the Wee1 kinase can inhibit the cdks by adding phosphorylations on two tyrosine (Tyr 14 and 15) residues. There are thus two ways of regulating cdk activity. To obtain full cdk activation the negative phosphorylations have to be eliminated by the family of cdc25 phosphatases and the threonine has to be phosphorylated by CAK.

The cell cycle regulatory factors also need to be tightly regulated since they are responsible for key events in cell cycle progression. It has been shown that both cyclins, cdks and CKIs are degraded by the ubiquitin-proteasome pathway which will be described in more detail in another chapter. By ensuring rapid degradation of these regulatory factors an additional level of the control of cell cycle progresion is exerted (for review see(Koepp et al., 1999).

Overview of transcriptional regulation

The control of gene expression is one of the most fundamental processes in biology and contains many layers of complexity. Regulation of gene transcription is exerted by specific DNA binding transcriptional activators and repressors and their cofactors, the RNA polymerase I-III complexes , associated general transcription factors (GTFs), and chromatin-structure regulatory factors. RNA polymerase II (PolII) transcribes protein-encoded genes whereas RNA polymerase I and III transcribe rRNA and 5S RNA/tRNA, respectively. The mechanisms underlying transcriptional regulation by PolII will be outlined briefly in this chapter.

Initiation of transcription by activators and RNA polymerase II

The promoter region of a gene contains different sequence elements to allow for regulatory molecules to bind in order to exert their transcriptional activity. Core promoter elements include the TATA-box, the initiator (Inr) and the downstream promoter element (DPE). Core promoter elements direct the start of transcription by RNA polymerase II, a multiprotein complex that will be described below. Promoter-proximal elements usually are bound by transcription factors such as Sp1 that enable a basal level of transcription. Gene- and tissue-specific, signal-responsive upstream promoter or distal enhancer elements interact with different types of DNA-binding transcription factors that can be either activators or repressors of transcription (reviewed in (Lemon and Tjian, 2000)). The DNA-binding transcription factors possess at least two domains of importance: a DNA-binding domain and a transactivation domain (TAD) in order to function as activators. The negatively acting transcription factors possess a domain that is capable of repressing recruitment of PolII or that can interact with corepressors.

The DNA-binding domains of transcription factors (TFs) can have different motifs that recognise a specific DNA-sequence and TFs can therefore be classified into groups depending on which motif(s) they possess. The helix-turn-helix motif contains two α-helices that are connected by a short chain of amino acids (the "turn"). Among these are the homeodomain proteins which are a special class of the helix-turn helix proteins. DNA-binding can further be mediated by zinc-finger motifs which consist of an α-helix and a β-sheet which are held together by zinc. Clusters of zinc-finger motifs can be arranged one after another so that each α-helix can contact the major groove of DNA and thereby form a continuous stretch of α-helices along the groove. The number of zinc-finger repeats dictates the strength and specificity of the DNA-protein interaction. Another DNA-binding motif is the leucine zipper linked to a basic region which mediates DNA binding through protein dimerisation. Binding of DNA by heterodimers enables TFs to expand their repertoire of DNA sequences recognised and also exerts combinatorial control on TFs. The helix-loop-helix motif (HLH) consists of a short α-helix which is connected by a loop to a second, longer α-helix. The first helix of this dimerisation motif is usually continuous with a connected DNA-binding basic region.

After binding to specific DNA elements, one role of many activators is to recruit RNA polymerase II to the promoter. Transcription by PolII is a multistep process that requires the assembly of a complex of initiation (basal) factors at promoters. This complex is also referred to as the preinitiation complex (PIC) and consists of 5 basal factors, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. A sixth factor, TFIIA potentiates the magnitude of transcription (Orphanides et al., 1996, Roeder, 1996, Hampsey, 1998). The first step is promoter recognition by TFIID which is a multisubunit complex which contains the TATA-binding protein (TBP) and at least 14 tightly associated factors among these the TBP-associated factors (TAFs). TBP binds to the TATA-box and leads to the formation of a bent DNA complex which creates a platform for the interaction of the remaining factors. One of the TAF subunits of TFIID is $TAF_{II}250$ which possesses a variety of activities that are likely to contribute to the initial steps of PolII transcription. Shortly, $TAF_{II}250$ provides a scaffold for the assembly of other TAFs and TBP into TFIID and binds to activators present on the promoter to recruit TFIID. $TAF_{II}250$ also regulates the binding of TBP to DNA. It further binds to core promoter initiator elements and possesses various enzymatic activities that are important in the regulation of histone modification. These will be described later. The activities of $TAF_{II}250$ is to achieve two goals; firstly to aid in positioning and stabilising of TFIID at a particular promoter and secondly to alter chromatin structure at a particular promoter to allow assembly of GTFs (for review see (Wassarman and Sauer, 2001)).

Once TFIID has bound to either the TATA box or to the initiator element, TFIIB binds and stabilises TFIID at the promoter by contacting TFIID and flanking sequences. TFIIB further recruits the TFIIH complex which contains helicase and kinase activities that catalyse the ATP-dependent melting of the promoter at the transcriptional start site. The kinase activity consists of the cyclin H/Cdk7 complex which phosphorylates the C-terminal domain (CTD). TFIIH activity is dependent on TFIIE incorporation into the PIC. TFIIF and TFIIH are required for promoter

escape and progression into elongation phase of transcription (reviewed by (Reese, 2003)). The transition of PolII from initiation to elongation is accompanied by hyperphosphorylation of the heptapeptide repeats in the CTD of the largest polymerase subunit (reviewed in (Conaway et al., 2000)). Two classes of elongation factors responsible for transcription exist, a class of negatively acting factors that inhibit transcription and a second class of positively acting factors that overcome this inhibition. P-TEFb is a postive acting factor and is composed of Cdk9 and one of several cyclins including T1, T2 and K. CTD phosphorylation by P-TEFb is required to prevent arrest of elongating PolII. NELF is a negative factor and is a multiprotein complex composed of polypeptides with potential RNA-binding activity. Another negatively acting factor is DSIF which is a heterodimer. The precise mechanism by which DSIF, NELF and P-TEFb act together in the elongation by PolII is unclear. It has been suggested that DSIF and NELF exert their negative effect on elongation through interactions with polymerase containing a hypophosphorylated CTD. Phoshorylation of the PolII CTD by P-TEFb might thus promote elongation by preventing the binding of the negative factors DSIF and NELF (review (Conaway et al., 2000)). Additionally a diverse collection of proteins, including TFIIF, ELL and Elongin, suppress pausing of the PolII.

Coactivators and corepressors

Animal cells utilise from 2000-3000 different transcriptional regulators. This explains the need for specialised adaptors employed by different classes of activators and repressors to interface with a limited number of targets within the general transcription apparatus. Some of these regulators may have similar types of activation or repression domains but structural features of these domains contribute to a higher order of complexity. Studying the mechanisms by which coactivators and corepressors interface with gene regulators and the transcription machinery has become essential to understanding transcriptional regulation in eukaryotes. Transcriptional cofactors can be divided into five classes depending on their activity (reviewed in (Lemon and Tjian, 2000). How these affect transcription is shown schematically in Fig. 3 and 4. The first class includes those cofactors that are intrinsic or very tightly associated with the basal transcription machinery. One example would be the TAFs of TFIID. A second class of cofactors includes those that are associated with activator or repressor molecules at the promoter. These include cellular factors such as OCA-B, Groucho, Notch, CtBP, HCF and the viral coregulators E1A and VP16 (summarized in (Lemon and Tjian, 2000).

The third class of cofactors consists of multisubunit coactivators. Examples include the yeast mediator (Thompson et al., 1993, Kim et al., 1994) which was initially purified as an activity that helped to stimulate activator-dependent transcriptional activity in reconstituted transcription reactions. The mediator was found to interact with the CTD of PolII and thereby to stimulate TFIIH dependent phosphorylation of CTD. Components of the mediator include a subset of *SRB* polypeptides and several previously uncharacterised proteins (Med1–Med7) (Thompson et al., 1993, Kim et al., 1994, Koleske and Young, 1994, Myers et al., 1998). Additionally, the yeast mediator complex was found to copurify with a proposed subcomplex containing the Srb8, Srb9, Srb10/Cdk8 and Srb11/ cyclin C

polypeptides, which have been suggested to be involved in negative regulation of gene activity (Song et al., 1993, Kim et al., 1994, Tabtiang and Herskowitz, 1998). One form of the mediator complex which lacks Srb8–Srb11 supports activated transcription in a yeast in vitro transcription system (Kim et al., 1994). Metazoan counterparts of the yeast mediator have been subsequently identified: (i) CRSP and PC2 (Ryu and Tjian, 1999, Malik et al., 2000), (ii) ARC/DRIP/TRAP (Gu et al., 1999, Ito et al., 1999, Naar et al., 1999) and (iii) NAT/SMCC/Srb-Mediator (Sun et al., 1998, Gu et al., 1999). The human mediator-like complexes bind to the underphosphorylated form of the largest PolII subunit and stimulate CTD phosphorylation but are unable to interact with the CTD directly (Sun et al., 1998, Gu et al., 1999). It is likely that the mediator is multifunctional and may contribute to transcriptional activation at several rate-limiting steps or promote activation in different ways at distinct genes (reviewed in (Naar et al., 2001)). The NAT (negative regulator of activated transcription) and SMCC complexes inhibit activated transcription which might be related to the presence of Srb10(Cdk8) and Srb11(cyclin C) in this complexes. The CRSP coactivator complex only has two subunits that exhibit homology to the yeast mediator, the other seven polypeptides are novel. This complex has been implied in the activation of transcription by Sp1 but the precise role of CRSP in transcription is not very clear. The PC2 cofactor was found to be very similar to CRSP in size and subunit composition and it is therefore possible that CRSP and PC2 represent highly similar or identical cofactor complexes (Kretzschmar et al., 1994, Malik and Roeder, 2000). The activator-recruited cofactor (ARC) interacts with the activation domain of the sterol-regulated transcription factor SREBP-1a and was shown to be required for activated transcription on chromatin templates (Naar et al., 1998, Naar et al., 1999). Nuclear receptors have also been found to interact with coactivator complexes termed TRAP and DRIP. These complexes, along with ARC, CRSP and PC2, share a large number of subunits and appear to represent a family of metazoan multisubunit complexes distantly related to the yeast Mediator (Rachez et al., 1998, Sun et al., 1998, Yuan et al., 1998, Ito et al., 1999, Naar et al., 1999, Malik et al., 2000).

The cofactors that belong to class IV include those that covalently modify nucleosomes. The histone acetylases CBP/p300, GCN5, P/CAF and the SRC-1-related p160 family belong to the coactivators while the histone deacetylases HDAC1 and 2 (rpd3 in yeast) and Sir2 belong to the corepressors. The class V cofactors include those that hydrolyse ATP in catalytic reactions to reorganise chromatin structure represented by the SWI/SNF containing chromatin remodelling complexes. The activities of class IV and V cofactors will be outlined below in connection with chromatin structure.

Alteration of chromatin structure by histone acetylation and deacetylation

Genomic DNA is organised into chromatin in which DNA is packaged into nucleosomes which represent the basic repeating unit of chromatin. Nucleosomes consist of two superhelical turns of DNA wrapped around an octamer of core histone proteins formed by four histone partner, one H3-H4 tetramer and two H2-H2B dimers. Histones are small basic proteins consisting of a globular domain and

a more flexible and charged NH_2-terminus (the "tail") that protrudes from the nucleosomes (reviewed in (Jenuwein and Allis, 2001)). The tail can be modified by various enzymatic activities which will be described below. Arrays of nucleosomes contain the linker histone H1 that twists and folds the chromatin fibre into increasingly more compact filaments and thereby gives rise to a defined higher order structure. In order for transcription to take place (i.e. to allow for binding of regulatory factors and PIC formation), the higher ordered and highly condensed chromatin structure has to be altered. Tightly packed and therefore inaccessible chromatin is referred to as heterochromatin whereas chromatin that has a more loose structure and is therefore accessible is referred to as euchromatin. This is achieved by a variety of enzymatic activities which will be described briefly.

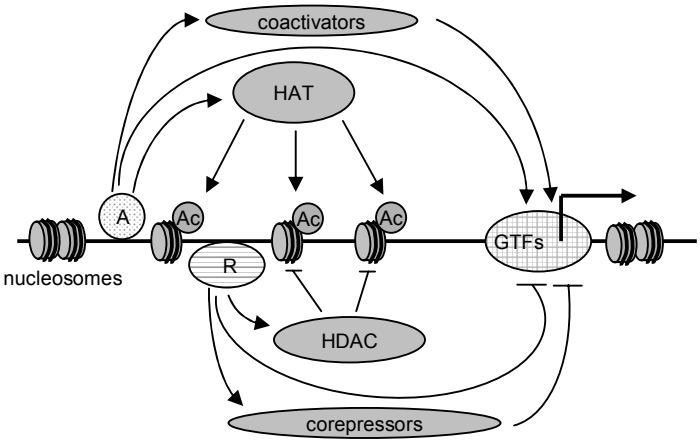


Figure 3: Overview of the effects of coactivators and corepressors on transcriptional initiation. A: activator, R: repressor, Ac: acetyl-moiety, GTFs: general transcription factors, HAT: histone acetyl transferases, HDAC: histone deacetylase transferases.

As mentioned above, the class IV cofactors include histone acetyltransferases (HATs) and histone deacetylase transferases (HDACs). Histone acetylation has been associated with actively transcribed genes whereas histone deacetylation has been associated with silenced genes. The initial evidence linking HAT activity and transcriptional co-activator functions came with the identification of the *Tetrahymena* histone acetyltransferase A (HAT A) which was found to be homologous to Gcn5 which is a genetically defined transcriptional co-activator in yeast (Brownell et al., 1996). The tails of core histones are acetylated by HATs on specific lysine residues. Acetylation of histone tails is suggested to lead to a more "open" chromatin structure and therefore increased accessibility to GTFs, the PolII holoenzyme and to additional gene regulatory proteins. There are two models how acetylation of lysines in the histone tails by HATs alters the structure of chromatin and thereby activates transcription. The addition of an acetyl-group neutralises the positive charge of the lysine residue, which decreases the interaction with the negatively charged DNA backbone. This is suggested to loosen up the rigid structure of packed chromatin, thereby allowing activator proteins to bind and to recruit the transcriptional machinery. Another possibility is that the added acetyl-groups present a platform for the binding of regulatory proteins. Examples for the

latter model will be given below in connection with other chromatin modifications. In addition to chromatin, HATs can also acetylate proteins which will be discussed in more detail below. A number of transcriptional coactivators have been demonstrated to possess HAT activity. This includes the CREB-binding protein CBP and the related protein p300 and the p160 family of coactivators represented by SRC-1, TIF2 and pCIP which are targeted by nuclear receptors and STATs. The previously described subunit of TFIID, $TAF_{II}250$, has also been described to contain intrinsic HAT activity (Mizzen et al., 1996). HATs are often associated with other proteins in large coactivator complexes. In yeast, the HAT Gcn5 has been found in two complexes, The Spt-Ada-Gcn5-acetyltransferase (SAGA) and the adaptor (ADA) coactivators (Horiuchi et al., 1995, Grant et al., 1997). Gcn5 has two mammalian homologues, hGCN5 and P/CAF, both were identified in high-molecular-weight complexes containing mammalian Ada and Spt homologues (Candau et al., 1996, Yang et al., 1996, Wang et al., 1997). Yeast and mammalian GCN5 type co-activator complexes contain a Tra1/TRRAP polypeptide which belongs to the ATM/PI3-kinase superfamily and has been shown to interact with specific activators, including c-Myc (McMahon et al., 1998, Saleh et al., 1998) (McMahon 1998, Saleh 1998). The Coactivators CBP and p300 have also been found to interact with P/CAF (Glass and Rosenfeld, 2000) (Glass 2000).

HDACs in general exert their effect by removing acetyl groups from histone tails. This regains the positive charge of lysine and reverse the effects of histone acetylation described above. At the chromatin level, localised histone deacetylation can stabilise nucleosomal structure and internucleosomal histone-histone interactions to inhibit the DNA accessibility of transcriptional activators and the general transcription machinery. For example, Rpd3 in yeast represses transcription by inhibiting recruitment of SWI/SNF, SAGA and TBP to promoters (Deckert and Struhl, 2002). Histone deacetylation can further potentiate transcriptional silencing and thereby promote the association of silencers. In yeast, Sir3 binds preferentially to non-acetylated histone H3 tails and interacts with Sir2/Sir4. Histone-binding ability is coupled with histone deacetylation activity and a “silencing module” for heterochromatin spreading is formed (Moazed, 2001). It has also been observed that deacetylation of TAF_I68 inhibits PolI-dependent transcription, thus the general transcription machinery might also be targeted by deacetylation (Muth et al., 2001). Additional examples of histone deacetylation in combination with other chromatin modifications will be described below.

Many proteins have been shown to possess intrinsic HDAC activity (for review see (Grozinger and Schreiber, 2002). HDACs can be grouped into three classes based upon sequence similarity to the yeast HDACs reduced potassium dependency 3 (Rpd3), histone deacetylase 1 (Hda1), and silent information regulator (Sir2). The most extensively studied group of HDACs is those of the mammalian class I enzymes that are highly homologous to Rpd3. These include HDAC1, 2, 3 and 8. These can assemble in different corepressor complexes. HDAC1/2 binds are the catalytic core of the Sin3, Mi2/NuRD and CoREST complexes. HDAC3 is the catalytic subunit of the N-CoR and SMRT complexes (Grozinger and Schreiber, 2002, Yang and Seto, 2003). HDAC1-3 can also cooperate with other chromatin and transcriptional regulators. The NuRD complex possesses intrinsic activity to remodel chromatin which is required for

deacetylation of nucleosomal histones (Cress and Seto, 2000, Ng and Bird, 2000, Grozinger and Schreiber, 2002). The Sin3 and N-CoR/SMRT complexes associate with components of the hSWI/SNF complexes (which will be described below) (Battaglioli et al., 2002). Further HDAC1 and 2 associate with DNA methyltransferases (DNMTs) and transcriptional repressors recruit both HDACs and DNMTs to repress transcription (Fuks et al., 2001, Burgers et al., 2002). Additionally HDAC1 and 2 interact with both histone methyltransferases (HMTs) (also described in more detail below) and topoisomerases which aid in the process of transcriptional repression. HDAC1-3 also cooperate with other transcriptional repressors.

Class I and II HDACs are structurally and functionally quite different despite having similar catalytic activities. Class II mammalian HDACs share the catalytic domain with yeast Hda1 and can be further divided in two subclasses: class IIa (HDAC4, 5,7 and 9) and class Iib (HDAC6 and 10) (Bertos et al., 2001). Class Iia HDACs interact with one or more DNA-binding transcription factors (including MEF2, BCL6, PLZF and TR2), with transcriptional corepressors such as N-CoR, SMRT, BcoR and CtBP and with the methyllysine-binding protein HP1 (Bertos et al., 2001, Fischle et al., 2001, Khochbin et al., 2001).

The class III HDACs which are Sir2-like require NAD^+ for anzymatic activity. In *Drosophila* there are five sirtuins whereof dSir2 is the one which is most similar to yeast Sir2. dSir2 localises to both euchromatic and heterochromatic loci and is required for centromeric silencing (Rosenberg and Parkhurst, 2002). dSir2 interacts with several members of the Hairy/Enhancer of split proteins which are known to bind the Groucho/TLE family of corepressors (Courey, 2001). This interaction suggests that dSir2 may cooperate with Groucho to inhibit transcription. Sirtuins are conserved in mammals and seven members of this family (SIRT1-7) have been identified (Grozinger and Schreiber, 2002). Mammalian sirtuins also deacetylate other proteins than histones which is also an important layer of controlling transcriptional activity. For example, human SIRT1 bind and deacetylate p53, thereby inhibiting the ability of p53 to bind to DNA and to induce cellular senescence and apoptosis.

Other chromatin modifications

Apart from modification by histone acetylation and deacetylation described above, the chromatin structure can also be altered in other ways. These include direct modification by phosphorylation, methylation and ubiquitylation. Modification by ubiquitylation will not be described extensively in this chapter but instead taken up in the next chapter which deals with control of biological processes by ubiquitin. The combination of different modification on the same histone tail has been proposed to function as a “histone code “ (Jenuwein and Allis, 2001) which extends the information content of the genome past the genetic code. Epigenetics, which is imposed at the level of histones, is thought to be a critical feature of a genome wide mechanism of information storage and retrieval. It has been recognised that chromatin structure plays an important regulatory role since multiple signalling pathways converge at histone tails. There are two models which explain the relationship between histone tail modification and gene control. The

first model suggests that histone modifications affect chromatin structure directly as described above and the second model is that different modifications modulate a special surface for interaction with other proteins. These models are non-exclusive and might operate simultaneously (for reviews see (Jenuwein and Allis, 2001, Berger, 2002).

Histone phosphorylation of Ser10 of histone H3 has been described both in transcriptional activation and in chromosome condensation (Cheung et al., 2000). The notion that the same modification is involved in two opposing alterations of chromatins gives rise to the speculation that this modification is serving as a binding surface for other regulatory proteins rather than altering chromatin structure directly (see below). Earlier studies showed that histone phosphorylation has a role in transcriptional induction of immediate early genes in mammalian cells such as the *c-Fos* gene (Mahadevan et al., 1991).

There are two types of histone methylations which target either arginine or lysine residues. Histone arginine methylation is involved in gene activation and methylases are recruited to promoters as coactivators. These comprise the CARM1/PRMT1 family of histone methyl transferases (HMTs) and they predominantly target either H3 or H4 (Chen et al., 1999, Wang et al., 2001a) respectively. Methylation of lysine residues is known to occur on histone H3 (K4, K9 and K27) and H4 (K20). The first histone methyltransferase (HMT) to be discovered was the SUV39 protein (Rea 2000). The methyltransferase activity of SUV39 is directed against K9 of histone H3 and its catalytic domain has a very highly conserved structure, the SET domain. Based on the identification of this domain other HMTs could be identified (73 in humans and 6 in yeast) and can be grouped into four families. These are the SUV39, the SET1, the SET2 and the RIZ family. Methylation at arginines occurs within the tails of histone H3 (R2, R17, R26) and H4 (R3). Arginines can be either mono-methylated or dimethylated. The latter can be either symmetric or asymmetric and enzymes are classified according to their ability to carry out one or the other reaction. Five arginine methyltransferases are known and they have a highly conserved catalytic domain (reviewed in (Kouzarides, 2002).

Another interesting example of histone modfication is linked to cell cycle control. In addition to binding of pRB to E2F in the absence of growth stimulatory signals pRb can inhibit E2F responsive genes through another, possibly two-step, mechanism (Harbour and Dean, 2000, Klochendler-Yeivin et al., 2002, Trimarchi and Lees, 2002). The pRb/E2F complex binds to the promoters of E2F responsive genes and facilitates a more “active” repression by recruiting the histone deacetylases (HDACs). This leads to alterations in the chromatin structure by deacetylation lysine 9 (K9) of the histone 3 tail and subsequent nucleosome packing. Alternatively (or following), The histone H3 methylase SUV39H1 is recruited to the same place and methylates K9 which leads to transcriptional silencing by binding of the methyl binding protein HP1 (Harbour and Dean, 2000, Nielsen et al., 2001). It has further been suggested that Rb cooperation with the SWI/SNF complex downregulates E2F activity (Zhang and Dean, 2001).

As mentioned earlier, modification of histones might also provide binding sites for effector proteins. In agreement with this, both bromo- and chromodomain

containing proteins have been found to interact with modified chromatin. The bromodomain is present in many transcriptional regulators which have intrinsic HAT activity. One example is the TFIID subunit $TAF_{II}250$ which contains two bromodomains. It preferentially binds to diacetylated histone peptides presenting acetyl-lysine moieties that are appropriately spaced (Jacobson et al., 2000). Chromodomains appear to be targeting molecules that have methylation marks. The chromodomain of HP1 binds to methylated H3 at L9 but no or very little binding is observed at H3 containing a methylated K4(Bannister et al., 2001). Suvar 39 HMTs family members also contain a chromodomain whose integrity is critical for silencing in vivo(Nakayama et al., 2001).

Specific modification of histones seems to correlate with specific transcriptional states. Different patterns of combinatorial modifications have been observed, one example is histone H3. H3 can be modified at K4, K9 and K14 by acetylation, at S10 by phosphorylation and at R17 by methylation. Around K9/S10/K14 there appears to be specific patterns for activity and inactivity. The inactive state of H3 is characterised by deacetylation at K14 which is precedes methylation at K9 (Noma et al., 2001). On the other hand, acetylation at K14 is preceded by and dependent upon phosphorylation of S10. *In vivo* the Snf1 histone kinase and Gcn5 are a linked pair of enzymes that operate in this sequence (Lo et al., 2000). The "on"-state of histone H4 is dictated by R3 methylation prior to p300-mediated acetylation at K8 and K12 (Wang et al., 2001b).

In addition to histones, regulatory proteins are also modified by acetylation, phosphorylation, methylation and ubiquitylation (for review see (Freiman and Tjian, 2003). These have been postulated to be equally important in directly modulating transcription. Further these modifications occur at lysine residues and thereby proteins can be marked for carrying out specific functions. Different modifications might also be competitive or protect from one another. Modification of regulatory proteins, in particular of transcription factors, will be discussed in more detail in the next chapter.

Chromatin remodelling

Another way of altering chromatin structure is the use of protein complexes that utilise the energy derived from hydrolysis of ATP to perturb or reorganise chromatin structure by destabilisation and displacement of histone-DNA contacts. Two such complexes will be described here, the SWI/SNF and the ISWI complexes. The SWI/SNF complex was first identified in yeast. Its hallmark subunit, SWI2/SNF2, possesses intrinsic DNA-stimulated ATPase activity and subsequently eukaryotic ATP-dependent remodelling complexes have been found to contain homologues subunits. Brahma (Brm) is the Drosophila homologue of SWI2/SNF2 and human homologues of Brahma, hBRM/hSNF2a and BRG1/hSNF2b have subsequently been identified which have been shown to be components of larger multisubunit complexes. SWI/SNF-type complexes disrupt nucleosomes in vitro and facilitate transcription factor binding in an ATP-dependent manner. Two DNA binding motifs present in SWI/SNF subunits have recently been identified that seem to be important for SWI/SNF function. One domain of importance seems to be the high mobility group (HMG) in BAF57, one

of the subunits of the human SWI/SNF complex. Studies have shown that the HMG possesses DNA-bending activity and that this might be important for BAF57 function (Papoulas et al., 2001, Chi et al., 2002). Another domain of importance is the SANT domain which can be found in the SWI/SNF subunit Swi3. Deletion of this domain results in defective SWI/SNF complexes (Boyer et al., 2002) and it has been suggested that this motif contributes to interactions between the Swi/Snf complexes and DNA. Other motifs found in Swi/Snf subunits that may contribute to DNA binding include AT-rich interaction domain motifs and zinc-finger motifs (Angus-Hill et al., 2001, Wilsker et al., 2002).

SWI/SNF complexes can also function as repressors of transcription as has been demonstrated by recent studies by the demonstration of physical presence of SWI/SNF components at repressed promoters as described above (Battaglioli et al., 2002, Martens and Winston, 2002, Wang et al., 2002, Zhang et al., 2002) for review see(Martens and Winston, 2003)). Targeted repression by SWI/SNF appears to require its recruitment by regulatory proteins, similarly to what has been shown for SWI/SNF activation. For example, the potential tumour suppressor prohibitin which is capable of repressing E2F activation, was shown to recruit SWI/SNF to particular E2F-dependent promoters (Wang et al., 2002). The mechanism by which SWI/SNF activates at some promoters and represses at others is believed to involve to existence of distinct forms of SWI/SNF complexes. In human cells some SWI/SNF complexes have been identified that contain components of the Sin3 histone deacetylase complex (Sif et al., 2001). Further differential use of the same complex may occur at promoters that are activated or repressed by SWI/SNF as has been shown for the repression of yeast SER3 (Sif et al., 2001).

A second family of ATP-remodelling complexes is the ISWI complex. This complex was first identified in metazoans and contains homologues of the *Drosophila* imitation switch (ISWI) ATPase (Elfring et al., 1994, LeRoy et al., 1998). The first identified ISWI-containing complex, was the nucleosome remodelling factor (NURF) which was found to enhance sequence-specific DNA binding by the GAGA transcription factor to nucleosomal templates in an ATP-dependent manner (Elfring et al., 1994, LeRoy et al., 1998). Other ISWI containing complexes include *Drosophila* ATP-utilising chromatin assembly and remodelling factor (ACF), *Drosophila* chromatin-accessibility complex (CHRAC), human remodelling and spacing factor (RSF) and yeast ISWI1-containing complexes. These complexes exhibit the ability to reorganise or space nucleosomes; ACF is also capable of facilitating nucleosomes assembly (Ito et al., 1997, Varga-Weisz et al., 1997, LeRoy et al., 1998, Tsukiyama et al., 1999). NURF and CHRAC remodel chromatin catalytically by nucleosome sliding (Hamiche et al., 1999, Langst et al., 1999).

Regulation of biological processes by ubiquitin

Ubiquitin is a small protein of 76 amino acids that is evolutionary conserved throughout eukaryotes. From yeast to human only three amino acid changes occur. The covalent conjugation to other proteins (ubiquitylation) is one of many ways of

protein modification. Ubiquitylation has been recognized as an important cellular process and much research during the past decade has focused on elucidating the precise function of this modification (for reviews see(Hershko and Ciechanover, 1998, Weissman, 2001). The first identified function was degradation of proteins which is important not only for protein turnover but also for the generation of peptides for antigen presentation. Recently more functions have been ascribed to the ubiquitin-proteasome pathway linking it to many cellular processes such as transcription, DNA repair, protein secretion and trafficking and apoptosis (for recent review see(Aguilar and Wendland, 2003). Ubiquitylation thus seems to be essential for most cellular processes. In this chapter an outline of the different functions of ubiquitylation will be given, the areas relevant to this thesis will be described in more detail.

Ubiquitin conjugation and degradation by the proteasome

Ubiquitylation (the conjugation of proteins with ubiquitin) is a multistep process which involves at least three enzymes referred to as E1, E2 and E3 (reviewed in (Hershko and Ciechanover, 1998, Pickart, 2001, Weissman, 2001)). The E1 enzyme activates ubiquitin by forming a thiol-ester bond with the C-terminal glycine of ubiquitin. This is an ATP-dependent process. A ubiquitin conjugating or carrier enzyme (E2) accepts ubiquitin from E1 by a *trans*thiolation reaction (which also involves the C-terminus of ubiquitin). Then a ubiquitin protein ligase (E3) recognises the the substrate specifically and "presents" it to the E2 ligase. Thereby the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue on the substrate is catalysed. Once a single ubiquitin is attached to a lysine of a protein, further ubiquitins can be linked to one of the lysines of ubiquitin resulting in a poly-ubiquitin chain.

Ubiquitylation is a dynamic and reversible process. The deubiquitylating enzymes (DUBs) have many functions and are thus important regulators of the ubiquitin pathway. DUBs cleave ubiquitin from proteins and disassemble multi-ubiquitin chains. Further they are important for the processing of immature ubiquitin which is encoded on multiple genes and is often translated as fusion protein either with other ubiquitin molecules or as N-terminal component of two small ribosomal subunits (Weissman, 2001).

The first recognized and best described consequence of ubiquitylation is degradation by the 26S proteasome which is shown in Fig. 4**.** In addition to proteasomal degradation, other degradation mechanisms exist, for example the lysosomal pathway which will be described later.

The discovery of ubiquitin and the conjugating pathway stemmed from the observation by Avram Hershko in 1971 (Hershko and Tomkins, 1971) of a process of energy dependent intracellular protein breakdown . The 26S proteasome carries out non-lysosomal protein destruction in an ATP-dependent manner (Hershko and Ciechanover, 1998) and is composed of two distinct subunits: the 20S proteolytic cylinder and the 19S regulatory caps. The 19S particle is further divided into the "base" and the "lid". The base contains six AAA ATPases and binds to the end of the 20S cylinder and renders proteolysis ATP-dependent. The lid contains eight proteins (Rpn in yeast) which assemble together and bind to the base.

Ubiquitylated substrates are tethered to the assembled 26 proteasome via interactions of the ubiquitin chain with components of the lid followed by

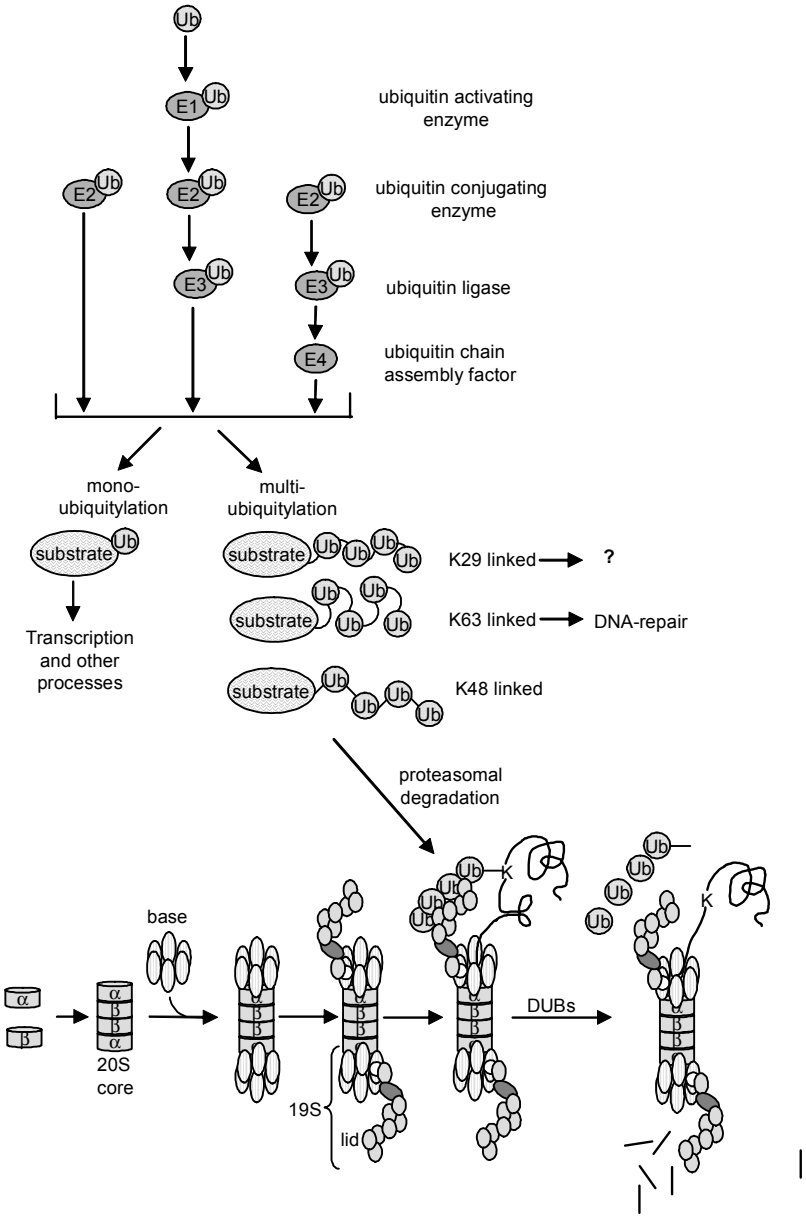


Figure 4: Overview of ubiquitylation, proteasome assembly and proteasome mediated degradation. Ub: ubiquitin, K: lysine, DUBs: deubiquitylating enzymes.

unfolding of the substrate by the ring of ATPases and proteolysis in the inner chamber of the 20S subunit (reviewed by (Verma and Deshaies, 2000)). The ubiquitin chain is released from the substrate prior to its degradation by a deubiquitylating enzyme that is a part of the 26S proteasome. Inhibition of DUBs leads to a stabilisation of the ubiquitylated protein, thus demonstrating the importance of this mechanism. Recently the Rpn11 subunit of the "lid" was identified as the DUB responsible for ubiquitin cleavage prior to proteasomal processing of ubiquitylated substrates (Verma et al., 2002). The process of ubiquitylation and subsequent degradation by the proteasome is shown in Fig. 4.

Protein ubiquitylation and degradation by the proteasome has been recognized as an important regulatory pathway rather than simply being a waste bin of the cell. Regulatory proteins such as cyclins, transcription factors, oncoproteins, tumour suppressors etc have been shown to be degraded by this mechanism. The rationale behind this is simple: Once a regulatory protein has performed its task it must usually be removed from the cell rapidly in order to allow for the next step in the cellular process to take place. Mutations leading to the deregulation of ubiquitylation and subsequent proteasomal degradation can give rise to cellular abnormalities that result in cancer or other diseases.

The minimal targeting signal for degradation is a tetraubiquitin chain (Thrower et al., 2000). Ubiquitin chain assembly via lysines is thus an essential process at least for the mediation of proteasomal degradation and is has been suggested that an enzyme, referred to as E4 can be responsible for this process after the addition of the first ubiquitin by the E3 ligase (Koegl et al., 1999). The choice of which lysine in ubiquitin is used for the building of ubiquitin-chains seems to determine the fate of the ubiquitylated protein. Chains involving links of K11, K29, K48 and K63 have been observed (reviewed by (Hicke, 2001b)). Of these K48 seems to play a role in the process of degradation. The other chains seem to be of importance for non-proteolytic functions of ubiquitin which will be described in more detail below.

E3 ligases

While there is only one E1 enzyme and a few E2 enzymes (about 25 mammalian members whereof some carry out other functions than forming thiol-esters with ubiquitin) there is a vast amount of E3 ligases which determines the specificity of substrate recognition. The E3 ligases can be grouped into three subfamilies: the HECT-domain, U-box and Ring-finger families based upon structural elements or common elements.

The first E3 enzyme to identified by was E6-AP which belongs to the HECT-type of E3 ligases. In transformed cells, interaction between the human papilloma virus gene product E6 and the tumour suppressor p53 results in proteasomal degradation of p53 (Scheffner et al., 1990). E6-AP catalyses the E6-dependent ubiquitylation of p53 (Scheffner et al., 1993). This E3 enzyme forms a thiol intermediate with a ubiquitin moiety transferred from an E2 to a conserved catalytic cysteine in the HECT-domain near its C-terminus. Ubiquitin is

subsequently transferred to either a lysine residue of p53 or a the growing end of a ubiquitin chain which ultimately leads to the proteasomal degradation of p53 (Huibregtse et al., 1995, Scheffner et al., 1995) reviewed by (Weissman, 2001)). At least ten proteins in different species have been identifies that carry the HECT-domain. Another feature of most HECT E3 ligases is the WW domain which is involved in protein-protein interactions and has a role in targeting substrates for ubiquitylation. This motif forms a hydrophobic pocket for proline-rich sequences as well as certain phosphoserine and phosphothreonine-containing sequences. Additionally WW-domain HECT E3 ligases also have a N-terminal C2 domain that mediates translocation to the plasma membrane in response to increases in intracellular Ca^{2+}.

The largest family of E3 ligases is the RING-finger family. Ring-finger E3s function as molecular scaffolds that bring proteins of the ubiquitylation machinery together by binding to E2s (Borden, 2000). There are hundreds of Ring-finger proteins but it is not clear how many of them function as Ring-finger E3s (reviewed by(Pickart, 2001)). Ring fingers include eight metal-binding residues that coordinate two zinc-ions which are arranged in an interleaved pattern (Freemont, 2000). Members of this family can be divided into single and multisubunit E3 ligases. The single-subunit E3s contain the substrate recognition element and the Ring-finger within the same protein. Examples of single subunit Ring-family are the E3 ligase Mdm2 which mediates ubiquitylation of p53 and the proto-oncoprotein c-Cbl which ubiquitylates growth factor receptors. Another example of this is the tumour suppressor protein Brca1. The multisubunit E3s all contain a ring-finger protein, a member of the cullin family, an adaptor protein and a protein that recognises the substrate specifically (reviewed in (Weissman, 2001)). Subfamilies of the multisubunit E3s are the SCF, VCB and APC complexes which are shown in Fig. 5.

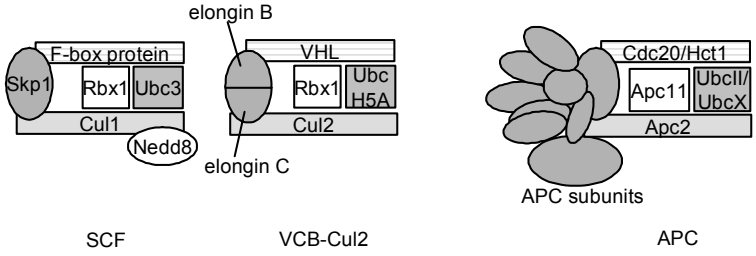


Figure 5: Components and architecture of multisubunit Ring-finger E3 ligases

The most studied class of the three multisubunit E3 ligases is the SCF complexes. The name stems from the components that were first identified: Skp1, Cullin and F-box protein. Further components are the Ring-finger protein Rbx1 and/ Rub1/Nedd8. All components of the SCF complex are evolutionary highly conserved and can be found in metazoans, yeast and plants. Specific domains within the proteins mediate the formation of the SCF complex. Skp1 is a critical scaffold protein that binds an F-box protein and a cullin (Bai et al., 1996, Kipreos et al., 1996). Up to date seven cullins have been identified and at least Cul1, Cul2

and APC2 are involved in proteasomal degradation by being part of a SCF, VCB or APC complex, respectively (Weissman, 2001). In the context of E3 ligases, the cullin family proteins interact with linker proteins such as Skp1 that recruit the substrate-recognition components. Cul1 has three domains that mediate its association with other components of the SCF complex (reviewed in (Deshaies, 1999)). The least conserved domain among the members of the cullin family is the N-terminal region where Cul1/Skp1 binding is mediated. The most highly conserved domain is present in the extreme C-terminus and mediates the attachment of a ubiquitin-like protein called Nedd8. The ubiquitin-ligase activity of some SCF ligases is enhanced by the covalent attachment of Nedd8 to Cul1 (this process is also referred to as neddylation) (Ohh et al., 2000). Downstream of the Skp1-binding region a third domain called the cullin homology (CH) or Cdc34/Ring-H2 domain is situated. This domain is conserved among all cullins and binds to Roc1/Rbx1. Apart from binding to Cul1, Roc1/Rbx1 also associates with either Ubc3, Ubc4 or Ubc5 which are E2 ubiquitin-conjugating enzymes (Seol et al., 1999, Skowyra et al., 1999). The F-box proteins comprise a large family of proteins that contain the conserved F-box which is named after the first protein where it was identified, cyclin F (Bai et al., 1996). Binding to Skp1 and thereby the rest of the SCF complex is mediated by the F-box. Members of this family can be grouped into three clusters based onto the domain that can mediate protein-protein interaction: the Fbws which contain WD-40 repeats, Fbls, which contain leucine-rich repeats (LRR) and Fbxs which lack a known protein-protein interaction domain (reviewed in (Winston et al., 1999, Ilyin et al., 2000, Kipreos and Pagano, 2000)). Apart from the F-box these proteins are not very homologoues to each other. Each family of F-box proteins is however evolutionary conserved and orthologues of mammalian F-box proteins can be found in many organisms. F-box proteins often recognise phosphorylated proteins (Skowyra et al., 1997). The substrates of most F-box proteins remain to be identified. Some well studied examples of F-box proteins in humans are β-TRCP, Skp2, NFB42 and cyclinF (reviewed in (DeSalle and Pagano, 2001)). In this thesis the SCF complex containing the F-box protein Skp2 was studied and will be therefore described in more detail below.

The anaphase-promoting complex (APC) includes at least 12 distinct subunits in yeast and at least 10 subunits in mammals. The APC complex seems to control two important events in mitosis, sister chromosome separation and exit from telophase into G1. APC substrates are mitotic cyclins such as cyclin A and B, spindle proteins and mitotic kinases (reviewed in (Koepp et al., 1999)). Phosphorylation and dephosporylation are important regulators of the activity of APC (Page and Hieter, 1999).

The VCB complexes consist of the Ring-finger protein Rbx1, Cul2,elongin B/C and an F-box-like component recognising the substrate. A well studied example of a VCB complex is VHL-VCB where VHL interacts with the substrate, hypoxia-inducible factor 1α (HIF1α), which leads to its ubiquitylation. HIF1α positively regulates vascular endothelial growth factor (VEGF) which explains the highly vascular nature of the clear cell carcinomas seen in VHL disease (Cockman et al., 2000, Kamura et al., 2000, Ohh et al., 2000) (. In von Hippel-Lindau disease,

mutations in the VHL gene generate VHL proteins that cannot assemble with the VBC core, resulting in the development of malignancies .

A very recently identified family of E3 ligases is the U-box family (for review see (Hatakeyama and Nakayama, 2003)). The U-box is a domain of approximately 70 amino acids and is present in proteins from yeast to humans. The first U-box protein was yeast Ufd2 which was originally identified as a ubiquitin chain assembly factor (E4) cooperating with E1, E2 and an E3 to catalyse the formation of a ubiquitin chain on an artificial substrate (Koegl et al., 1999). U-box proteins can mediate ubiquitylation in the absence of HECT or RING-finger E3s (Hatakeyama et al., 2001). It has been proposed that the E4 activity is a specialized type of E3 activity based on the observation that Ufd2 targets oligoubiquitylated artificial fusion proteins as substrates. An example of U-box proteins in mammalians is the carboxy-terminus of Hsc70 interacting protein (CHIP), which was originally identified as a co-chaperone of Hsc70. CHIP has both a tetratricopeptide repeat (TPR) motif and an U-box domain. The TPR associates with Hsc70 and Hsc90 while the U-box possesses ubiquitin ligase activity. Unfolded or misfolded proteins are recognized by chaperones such as Hsp70 and Hsp90 and then either refolded in an ATP-dependent manner or ubiquitylated by CHIP which leads to proteasomal degradation. Thus CHIP functions as a "quality control E3" involved in selective ubiquitylation of target proteins (Hatakeyama et al., 2001, Murata et al., 2001).

SCF^{Skp2}

Skp2 is one of the most studied F-box proteins. Skp2 binds the other components of the SCF complex through its F-box (Lisztwan et al., 1998), thus forming SCF^{Skp2}. The crystal structure of this complex has recently been solved and shows that that Skp2 binds to the substrate in such a way that the lysine of the substrate is "presented" to the E2 enzyme. This model of SCF^{Skp2} structure is the result of information form several structural studies(Schulman et al., 2000, Zheng et al., 2002b). Another feature of the SCF^{Skp2} complex is binding of the Cdk-associated protein Cks1 to the complex. Cks1 enhances the interaction between the substrate p27Kip1 and Skp2. It is, however, at present unclear how exactly Cks1 operates(Ganoth et al., 2001, Spruck et al., 2001), reviewed by (Harper, 2001)).

Suggested Skp2 substrates include p27Kip1, cyclin E, E2F, Orc1, the pRb related protein p130, cyclin D1 and Cdk9 (Yu et al., 1998, Marti et al., 1999, Sutterluty et al., 1999, Tsvetkov et al., 1999, Carrano and Pagano, 2001, Kiernan et al., 2001, Yeh et al., 2001, Mendez et al., 2002, Tedesco et al., 2002). The most established substrates are p27 and cyclin E. Binding and subsequent ubiquitylation of p27 by Skp2 is mediated by the phosphorylation of threonine 187 (T187) of p27 by Cdk2 which has been demonstrated both in vivo and in vitro. Further, stabilisation of endogenous p27 and cyclin E has been observed in Skp2-/-cells (Nakayama et al., 2000). Skp2 binding and ubiquitylation of E2F on the other hand does not seem to be phosphorylation dependent and turnover is not influenced in Skp2-/-cells (Marti et al., 1999, Nakayama et al., 2000).

Skp2 (along with Skp1) was originally identified as an essential protein interacting with the S-phase promoting kinase cyclin A/cdk2 (Zhang et al., 1995).

Hence the name Skp2: S-phase associated kinase associated protein 2. Skp2 seems to play an important role in the progression of the mammalian cell cycle. The expression of Skp2 can be detected first at the G1/S transition, amounts accumulate during S/G2 transition and drop as cells proceed through M phase (Lisztwan et al., 1998). Evidence that Skp2 is required for G1/S transition is accumulating. For example, microinjection of antibodies against Skp2 in cultured cells inhibits S-phase entry (Zhang et al., 1995). Overexpression of Skp2 by adenoviral vectors in serum deprived fibroblasts leads to p27 degradation, cyclin A expression, cyclin/cdk2 expression and S-phase entry. Expression of a p27 mutant that is resistant to degradation leads to a suppression of Skp2-mediated S-phase transition, demonstrating that degradation of p27 by Skp2 is a key event in the progression of the cell cycle (Sutterluty et al., 1999).

Skp2 expression is itself regulated by ubiquitylation and proteasome-mediated degradation by an autocatalytic mechanism (Wirbelauer et al., 2000). The physiological function of Skp2 has also been analysed in a mouse-knockout model (Nakayama 2000). Skp2-/- mice are smaller than their littermates and Skp2-/- cells show accumulation of p27 and free (not bound to cdk) cyclin E. The knockout mice exhibit enlarged nuclei with polyploidy and multiple centrosomes. These findings imply Skp2 in the control of chromosome replication and centrosome duplication.

Skp2 has been suggested to be an oncogene since it is overexpressed in transformed cells (Zhang et al., 1995). Although Skp2 overexpression per se is not sufficient to transform cells, more recent studies indicate that Skp2 and activated Ras cooperate in vitro transformation assays. Additionally, injection of cells overexpressing Skp2 and activated Ras into nude mice induce tumour formation in vivo (Gstaiger et al., 2001, Latres et al., 2001). Skp2 also cooperates with Ras in an in vivo model of lymphogenesis (Gstaiger et al., 2001, Latres et al., 2001). A substantial amount of evidence is accumulating that the downregulation of p27 and upregulation of Skp2 is involved in a variety of cancer forms (Gstaiger et al., 2001, Kudo et al., 2001, Latres et al., 2001, Chiarle et al., 2002, Lim et al., 2002, Signoretti et al., 2002, Shim et al., 2003) (. Further, Skp2 is a target of the extra-cellular matrix signalling that controls cell proliferation and adhesion-independent cell cycle progression is potentiated by coexpressing Skp2 with cyclin D1. This suggests a role for Skp2 in the adhesion-independent ability of tumour cells to grow (Carrano and Pagano, 2001).

Ubiquitin-like modifications

In addition to ubiquitylation other ubiquitin-like modifications of proteins have been described. The ubiquitin-like proteins (UBLs) are a family of proteins that are distantly related to ubiquitin in their amino acid sequence and share the same structural fold. These proteins are also conjugated covalently to lysine residues on their substrates by isopeptide bonds through their C-terminus.

Neddylation is a ubiquitin-like modification that has been described in the context of the SCF complex. Conjugation of Nedd8 to Cul1 enhances the ligase activity of the SCF complex. It has for example been shown that Nedd8 and enzymes catalysing Nedd8 conjugation to proteins are required for the efficient

degradation of p27 in cell extracts (Podust et al., 2000). Modification occurs by conjugation of a single moiety. The majority of Cul1 can be found in a complex with Cand1 and Roc1 independent of Skp1 and the F-box protein Skp2 as described earlier. Neddylation of Cul1 or the presence of Skp1 and ATP causes Cand1 dissociation from Cul1 (Liu et al., 2002, Zheng et al., 2002a) (. The proteasome-like COP9 signalosome cleaves Nedd8 from Cul1 by activities of the Jab1/Csn5 subunit. The Jab1/MPN domain metaloenzyme (JAMM) motif was found to underlie this enzymatic activity and evolutionary conserved JAMM motifs can be found in the Rpn11 subunit of the "lid" of the proteasome (Cope et al., 2002) which also possesses DUB activity as described earlier.

Another ubiquitin-like modification is sumoylation. The small ubiquitin-like modifier (SUMO) has been shown to be conjugated to many substrates, many of them being important cellular regulators. Sumoylation is a reversible process and has a variety of functions. One described function is that sumoylation regulates the location of proteins to sites in or around the nucleus. Since sumoylation occurs at the same lysines than ubiquitylation it has also been proposed that sumoylation regulates multi-ubiquitylation negatively by occupying lysine sites otherwise modified with ubiquitin and thereby preventing ubiquitylation and degradation. Sumoylated proteins are often found in nuclear bodies. This is exemplified by the promyelocytic leukaemia gene product PML which has been detected in subnuclear structures called PML oncogenic domains (PODs) or nuclear bodies. The disruption of nuclear bodies observed in acute promyelocytic leukaemia suggests that the nuclear bodies perform an important function in protecting against certain forms of leukaemia. By retaining a number of sumoylated transcription factors nuclear bodies can regulate the activity and function of transcription factors, thus SUMO seems to be an important regulator of transcriptional activation as will be outlined in the next chapter.

Another pathway of protein degradation is the process of macroautophagy (reviewed in (Ohsumi, 2001)). A portion of the cytosol is sequestered by a so-called isolation membrane which results in the formation of a double-membrane structure which is called the autophagosome. This fuses subsequently with the lysosome/vacuole. The analysis of the mechanism underlying macroautophagy has revealed two ubiquitin-like systems, the Apg12 and Apg8 conjugation systems. These resemble the ubiquitin system by their ability to from bonds with their target proteins.

Other ubiquitin-like modifications are also conjugated to lysine residues on substrates by E2 and E3 enzymes. These usually regulate function in a non-proteolytic manner. These include HUB, homologues to ubiquitin; ISG15, interferon stimulated gene 15; and URM1, ubiquitin related modifier 1; (reviewed in (Hochstrasser, 2000, Weissman, 2001))

The role of ubiquitylation, sumoylation and other protein modifications in transcriptional activation

Although ubiquitin-proteasome mediated degradation of transcription factors is an important regulatory mechanism for the cell to maintain homeostasis, studies during recent years have however appointed out a new, direct positive link between ubiquitylation and transcription. (for reviews see (Tansey, 2001, Conaway et al., 2002, Ottosen et al., 2002, Muratani and Tansey, 2003)). I will here give an overview of recent advances on the role of ubiquitin in transcription.

The first observation which suggested that ubiquitylation and transcriptional potential might be coupled was that the potencies of transcriptional activation domains (TADs) of transcription factors often correlate with the rate at which these factors are turned over in the cell (Molinari et al., 1999). Mutations that abolished transcriptional activation also rendered the transcription factor resistant to proteasome-mediated degradation. It was further observed that the regions of transcription factors that mediate transcription (TADs) and those mediating degradation (so-called "degrons") often overlap. This was first shown for the transcription factor c-Myc (Salghetti et al., 1999). Subsequently other unstable transcription factors which contain an acidic transactivation domain and are degraded by the ubiquitin-proteasome pathway were identified that had overlapping TADs and degrons (Salghetti et al., 2000). The same study also showed that degrons from the yeast cyclins Cln2 and Cln3 fused to the Gal4 DNA binding domain (DBD) could function as TADs and activate transcription. Another subsequent study investigated the connection between the VP16 TAD and its E3 ligase, SCF^{Met30}. As would be expected, in yeast strains which lack Met30, VP16 cannot be degraded. Importantly VP16 also fails to activate transcription under these conditions. This could, however, be circumvented by the fusion of one ubiquitin-moiety to the VP16 activator. These observation demonstrate that ubiquitylation is important in transcriptional activation by VP16 and might serve as a dual signal for both transcriptional activation and subsequent proteasome-mediated destruction (Salghetti et al., 2001). These observations have lead to "licensing" hypothesis (Tansey, 2001) where transcriptional activators have to be conjugated with ubiquitin and therefore be marked for destruction in order to be transcriptionally active (see figure 6).

Ubiquitylation of transcription factors can also play non-proteolytic roles. One example is the yeast transcription factor Met4 which activates the expression of many genes, including a set of genes encoding enzymes for sulfur amino acid bisoynthesis (*MET* genes) and a distinct set of genes required for the production of S-adenosyl-methionine (*SAM* genes). The consequences of SCF^{Met30}-mediated ubiquitylation of Met4 depend heavily on the cellular environment. When yeast cells are grown in minimal media, exposure to high methionine leads to rapid degradation of Met4 by SCF^{Met30} and corresponding depletion of Met4 from *MET* promoters. In rich media, ubiquitylated Met4 is stable and concentrated within the nucleus but is selectively excluded from *MET* promoters but recruited to *SAM promoters* (Kaiser et al., 2000, Rouillon et al., 2000, Kuras et al., 2002).

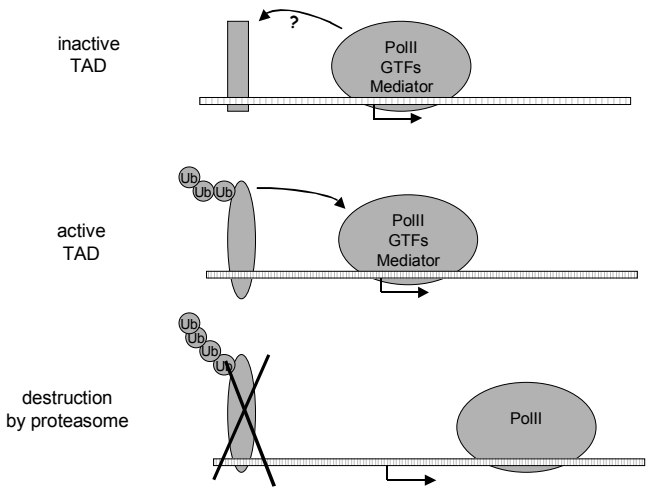


Figure 6: The licensing hypothesis. See text for details.

As described in the previous chapter, histones can be modified in a number of ways. Histone H2A was in fact the first ubiquitylated protein that was identified (Goldknopf et al., 1975) but the function of this modification was not at all understood at the time. It has now been suggested that ubiquitylation is part of to the histone code (for review see (Jenuwein and Allis, 2001)). Studies of the yeast E2 enzyme Rad6 show that Rad6 mono-ubiquitylates histone H2B at lysine K123 through the E3 ligase Bre1 (Robzyk et al., 2000, Wood et al., 2000, Turner et al., 2002, Hwang et al., 2003) which is a prerequisite for the methylation of histone H3 at lysines K4 and K79 through the histone methyltransferase (HMT) COMPASS (Briggs et al., 2002, Dover et al., 2002, Ng et al., 2002, Sun and Allis, 2002). The latter modifications result in gene silencing (Rice 2001). Ubiquitylation resulting in gene activation has also been observed. Mono-ubiquitylation of histones is not associated with histone destruction since histones are in fact very stable, and poly-ubiquitylated histones have not yet been described. Instead the main role of the modification by ubiquitin-conjugation seems to be modulation of chromatin structure and possibly the recruitment of HMTs or other factors to chromatin (for review see (Freiman and Tjian, 2003)). It has already been mentioned that the TBP associated factor $TAF_{II}250$ possesses a variety of enzymatic activities such as intrinsic protein kinase and histone acetylase activities (reviewed in (Wassarman and Sauer, 2001)). It has additionally been shown that $TAF_{II}250$ possesses both E1 and E2 ubiquitin-activating/conjugating activities and can ubiquitylate the linker histone H1 in drosophila. The function of this ubiquitylation is not clear but might contribute to transcriptional activation (Pham and Sauer, 2000). Histone deubiquitylation might also play a role in histone modification since ubiquitin-specific proteases are associated with components of the SIR4 silencing and the SAGA chromatin remodelling complex (Moazed and Johnson, 1996, Sanders et al., 2002).

Another ubiquitylation target is the RNA polymerase II itself. Ubiquitylation of PolII is important for the process of transcription-coupled repair (TCR). The

elongating polII has a unique pattern of phosphorylation on its C-terminal tail. When elongating polII encounters a damaged DNA segment, it stalls and recruits the ubiquitin ligase Rsp5 to ubiquitylate the largest subunit of polII in a CTD-phosphorylation-dependent manner which results in the degradation of at least one subunit of the polymerase which leads to polII disassembly. Subsequently the DNA repair machinery can be recruited and the DNA integrity can be restored (Lee et al., 2002, Svejstrup, 2002, Woudstra et al., 2002).

The polII complex is also implicated in other ways in ubiquitylation. Many proteins involved in ubiquitylation of transcriptional regulators are integral parts of preinitiation complexes and/or the RNA polII holoenzyme (Chi et al., 2001, Brower et al., 2002). The yeast transcription factor Gcn4 is tightly regulated by the E3 ligase SCF^{Cdc4}. Phosphorylation of Gcn4 by the Cdks Pho85 or Srb10, which are part of cyclin H/cdk7 and the mediator, respectively, seems to be sufficient to mediate Gcn4 ubiquitylation (Chi et al., 2001). It has been proposed that Srb10 ubiquitylates Gcn4 during the transcriptional activation, thereby giving further support to the "licensing model" described above. Further, a component of the mammalian mediator complex, mMed8, was found to be an elongin BC-box protein that can form a VHL-like complex together with elongins B and C, Cul2 and Rbx1 (Brower et al., 2002). The E3 subunits were also found to cofractionate with mMed8 and other mediator subunits as a multisubunit complex with associated ubiquitin ligase activity. Substrates for this complex have not yet been identified but one possible function might be to recruit ubiquitin ligase activity directly to the mediator. This could then target transcriptional activators, other subunits or PolII and the general initiation factors.

Conversely, the proteasome is also involved in transcription by PolII. The 19S regulatory particle of the proteasome includes several AAA ATPases (the APIS complex) which function in part by promoting the unfolding of the substrate prior to proteasomal degradation. Genetic studies had suggested a role for the AAA ATPase subunit Sug1 in transcriptional activation and it was recently found that the 19S particle of the proteasome is capable of activating PolII transcriptional elongation in vitro by a mechanism that is independent of proteolysis (Ferdous et al., 2001). It was further shown that the APIS complex but not other 19S subunits needed for proteasome function was recruited to promoters during transcription (Gonzalez et al., 2002). Sug1 has also been shown to interact with other TFs such as TBP, TFIIH (Melcher and Johnston, 1995, Weeda et al., 1997, Makino et al., 1999), nuclear receptors (Masuyama and MacDonald, 1998) and c-Fos (Wang et al., 1996). These interactions could potentially recruit the APIS-complex with or without other subunits of the proteasome to the transcriptional machinery.

Modification by sumoylation also has impacts on transcriptional activity. As mentioned earlier, sumoylation of some activator proteins correlates with their entry into nuclear bodies where they are retained and inactivated. Further p53 is also modified by SUMO as a consequence of UV irradiation of cells. Lysine 386 of p53, which is known to regulate the DNA-binding activity of the protein, is sumoylated as a response to irradiation. Mutation of K386 leads to an impaired apoptotic potential of p53, indicating that sumoylation is an essential process in the induction of p53-dependent apoptosis (Muller et al., 2000). Several other transcription factors, e.g. c-Jun, the androgen receptor AR, heat shock transcription

factors 1 and 2 and Sp3 have been identified as targets for sumoylation (Ross et al., 2002) reviewed in (Kim et al., 2002)) implying an important role for modification by Sumo in the regulation of transcription.

Apart from modification by ubiquitylation and sumoylation, regulatory proteins can also be modified by acetylation or methylation as mentioned above. All these modifications take place on lysine residues and it has been suggested that control of transcription and/or the activity of this activators can be fine-tuned by this mechanism (for review see (Freiman and Tjian, 2003)). For example, sumoylation of a lysine residue in IκBα has shown to block ubiquitylation of this identical residue which protects IκBα from degradation by the proteasome (Desterro et al., 1998). Acetylation of HIF-1α by ARD1 has been suggested to enhance the interaction of HIF-1α with VCB-VHL and HIF-1α ubiquitylation, thereby implicating that acetylation of HIF-1α by ARD1 is critical to proteasomal degradation (Jeong et al., 2002). On the contrary, acetylation of the Smad7 transcriptional regulator has recently been shown to protect Smad7 from ubiquitylation which suggests a competition between these two modifications at one critical lysine residue (Gronroos et al., 2002). Lysine residues therefore serve as critical molecular switches that can respond to signals in specific ways. Many transcription factors contain many lysine residues and might therefore undergo multiple modifications either sequentially or simultaneously.

Other non-proteolytic roles of ubiquitin and the proteasome

Ubiquitin has now been implicated in an increasing number of biological processes and seems to be a universal cellular control mechanism. Ubiquitin has been found to play an important role in the protein trafficking machinery on a variety of levels. Ubiquitin participates in the targeting of proteins to endosomal compartments either from the plasma membrane or from the trans-Golgi network. Further, ubiquitin seems to be involved in protein sorting from endosomes to multivesicular bodies (MVBS) and in the delivery of transmembrane proteins to the interior of the lysosome/vacuolar compartment (Hicke, 2001a). Ubiquitin also mediates the transport through nuclear pores, both ubiquitin and Sumo seem to play a role in this process. As shown in Fig. 4, K63 linked ubiquitin chains are needed for ubiquitin to carry out these functions.

Another mechanism where K63 linked ubiquitin chains are required for ubiquitin to carry out its function is the process of DNA repair. It has recently been demonstrated that PCNA is ubiquitylated (Hoege et al., 2002) when it is carrying out its function. Upon DNA damage, the PCNA trimer encircles DNA and binds to DNA-replicating enzymes (polymerases). Rad 18 and Rad6 then attach a single ubiquitin to a specific lysine of PCNA whereafter a second ubiquitin-conjugating complex consisting of Rad5, Mms2 and Ubc13 extends the polyubiquitin chain from the first ubiquitin. The modified PCNA can then promote error free post-replicative DNA repair. If the same lysine residue is however sumoylated, DNA repair is inhibited which indicates that the antagonism between ubiquitin and Sumo seems to much more broader than anticipated.

Ubiquitin-binding proteins

The now evolving impact on many biological processes by protein modification by ubiquitylation postulates the existence of downstream effector proteins that are able to interact with ubiquitylated proteins specifically. A number of ubiquitin-interacting proteins have been identified (reviewed in (Buchberger, 2002)). These proteins are found in members of many different protein families and seem to be linked to ubiquitin-related processes by an internal ubiquitin-binding domain.

The ubiquitin-associated domain (UBA) consists of 40 amino acid residues and was initially identified in E2s, E3s and other proteins linked to the process of ubiquitylation (Hofmann and Bucher, 1996). Thus ubiquitin binding was proposed to be a function of the UBA domain which has been demonstrated in yeast. UBA domain containing proteins in yeast include the DNA repair protein Rad23/Rhp23, the DNA-damage-induced protein Ddi/Mud1 and Dsk2/Dph1 protein which is involved in spindle-pole duplication (Bertolaet et al., 2001, Chen et al., 2001, Wilkinson et al., 2001, Funakoshi et al., 2002). Rad23 possesses two UBA domains, one at the C-terminus and one in the central region. In vitro studies show that the UBA domain binds to mono, di, tetra and multi ubiquitin chains which are linked by K48. The affinity for mono compared to tetrachains is two orders of magnitude lower. In vivo, however, mono or di-ubiqutin displays the best affinity for UBA domains and K29 chains seem to be involved in this process, thereby giving an example of the importance of how the ubiquitin chain is build up.

The ubiquitin-interacting motif (UIM) consists of a stretch of about 20 amino acid residues which probably from an alpha helix that can be embedded in different protein folds. The UIM was identified in the S5a/Rpn10 subunit of the 26S proteasome where it has been shown to function as a receptor for ubiquitin chains and for proteins carrying a ubiquitin-like domain (Young et al., 1998). Homology searches reveals the presence of UIMs in members of a variety of protein families, including proteins that are involved in ubiquitylation, ubiquitin metabolism and receptor-mediated endocytosis (reviewed in (Buchberger, 2002)). The UIM occurs often in tandem or triplet arrays. An example for the funtion of UIMs is the internalisation of the epidermal growth factor (EGFR). Several proteins involved in the process of internalisation of plasma membrane proteins contain UIMs, suggesting that these factors play an important role in the internalisation of ubiquitylated proteins (Polo et al., 2002). In response to EGF these proteins are themselves monoubiquitylated.

The ubiquitin domain (UBD) consists of a stretch of 45-80 residues with significant homology to ubiquitin. The are often found at or close to the N-terminus of proteins. A general property of UBDs is the ability to bind to the 26S proteasome, possibly through the UIM of the S5a/Rpn10 subunit of the 19S regulatory subcomplex. Most UBD proteins possess functions related to the ubiquitin-proteasome pathway which can be interpreted as an involvement of UBDs in the organisation of proteasomal supercomplexes.

The UBX domain consists of 80 amino acid residues and is typically found at the C-terminus of proteins. It has been identified in a variety of proteins. The structure of UBX reveals a close structural relationship to ubiquitin despite the lack of

significant homology (Yuan et al., 2001, Buchberger, 2002). No general function of the UBX domain is yet known, proteins which have an UBX domain can be divided into five families based on evolutionary conservation. The UBX domain of p47 interacts with p97/VCP (which is an AAA ATPase) and thereby mimicks mono-ubiquitylated substrates of this chaperone. Some members of the UBX family also possess an UBA domain.

The CUE domain binds directly to monoubiquitin. This domain was identified in a yeast screen for monoubiquitin binding proteins and one of the identified proteins was Vps9 which possesses an internal CUE domain thus mediating intramolecular monoubiquitylation (Shih et al., 2003).

The protein motifs described above have another interesting feature which is the combination of several motifs in the same protein. For example, the yeast proteins Rad23, Ddi1 and Dsk2 possess an N-terminal UBD and a C-terminal UBA domain. N-terminal UBA and a C-terminal UBX domain are combined in several UBX domain protein family motifs.

There are different models of how these motifs carry out their cellular function. In the first model which is based upon yeast Rad23, the UBD and UBA domains of the protein are responsible for recruitment of mono- or oligo-ubiquitylated substrates which are then released at the proteasome (where the UBD can bind) where ubiquitin chain elongation and subsequent degradation can take place. This has been confirmed by the use of a Rad6 mutant that lacks the UBD which stabilises model substrates more than overexpression of wild-type Rad 23. The other model (which does not entirely exclude the first model) proposes that the UBD and UBA domains recruit multi-ubiquitylated proteins for delivery to the 26S proteasome. The chain length of the ubiquitylated substrates might be substrate specific and depend on the availibilty of ubiquitin related enzymes, the UBD/UBA domain proteins and the proteasome.

The c-Myc transcription factor

The *c-myc* proto-oncogene has been the subject of intense study for more than two decades. The broad interest in this gene comes from the notion that c-Myc seems to be involved in a number of fundamental cellular processes and that deregulation of c-Myc expression often results in tumours (Dang et al., 1999, Nesbit et al., 1999, Grandori et al., 2000). The most prominent example is Burkitt's lymphoma where a translocation involving the *c-myc* and *Ig* loci deregulates c-Myc expression giving rise to a constitutive active myc gene. c-Myc deregulation has also been detected in wide range of other human cancers and is often associated with aggressive and poorly differentiated tumours. Such cancers include breast, colon, cervical, small-lung carcinomas, osteosarcomas, glioblastomas, melanoma and myeloid and lymphoblastic leukaemia and lymphomas (Dang et al., 1999, Nesbit et al., 1999). *myc* was originally identified as an oncogene (*v-myc*) transduced by a number avian retroviruses capable of potently inducing neoplastic disease. Subsequently *c-myc*, the cellular homologue was identified and shown to be a member of the larger family of proto-oncogenes consisting also of N-*myc,* L-*myc,* B-*myc* and other *myc*-family genes. The N- and L-*myc* genes were discovered as

amplified or highly expressed genes in the childhood tumour neuroblastoma (Kohl et al., 1983, Schwab et al., 1983) and in small lung cancer (Nau et al., 1985), respectively. c-*myc* and N-*myc* encodes essential genes, embryos of mice carrying a deletion for the *c-myc* gene die before birth at day E10.5 (Davis et al., 1993). c-*myc* is evolutionary conserved and has been identified in all vertebrates including zebrafish (Schreiber-Agus et al., 1993, Langenau et al., 2003) but also in *Drosophila* (Gallant et al., 1996, Johnston et al., 1999, Orian et al., 2003), and sea star *Asterias vulgaris* (Walker et al., 1992) and these can therefore be used as model organisms for the study of some functions of Myc.

c-Myc is broadly expressed during embryogenesis and in tissue compartments of the adult that possess a high proliferative capacity. The expression of c-Myc strongly correlates with proliferation and in cell cultures c-Myc is rapidly induced in response to a variety of mitogenic stimuli. Other functions of c-Myc is to inhibit terminal differentiation and to induce apoptosis. c-Myc can be seen as a functional module that integrates external signals in order to mediate specific programs of gene expression (Eisenman, 2001). This chapter is giving a brief overview of c-Myc function in several biological processes.

c-Myc structure and the Myc-Max-Mad network

The discovery of the basic/helix-loop-helix/zipper (bHLHZip) at the C-terminus (Murre et al., 1989) and the transactivation domain (TAD) in c-Myc (Kato 1990) a decade ago lead to the hypothesis that *c-myc* functions as a transcription factor (Henriksson and Luscher, 1996). The Myc family thus belong to the bHLHZip family of transcription factors. The structure of the c-Myc protein is shown in Fig. 7. The bHLHZip domain, which is positioned at the C-terminus of the protein mediates protein-protein interaction and DNA-binding (Luscher and Larsson, 1999). The N-terminus of the protein which harbours the TAD contains two evolutionary conserved regions, Myc box 1 and 2 (MB1 and 2). These regions are believed to be important for c-Myc function as will be described later in this chapter. The protein contains further a nuclear localisation site (NLS) which is important for import into the nucleus and a central acidic domain that harbours several phosphorylation sites that might be important for function. c-Myc however cannot activate transcription on its own. Like many other bHLHZip transcription factors it need to dimerise with a partner prior to DNA binding. The identification of the bHLHZip protein Max (Blackwood et al., 1992, Prendergast and Ziff, 1992)

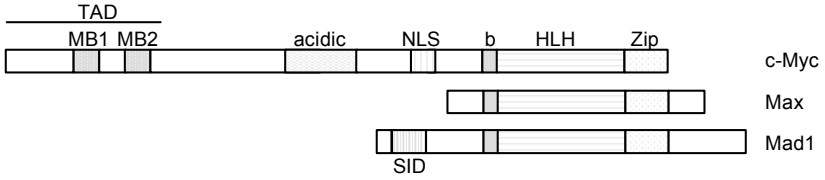


Figure 7: Structure of c-Myc, Max and Mad. Domains of functional importance are indicated. TAD: transcriptional activation domain, MB: Myc box, NLS: nuclear localisation sequence, b: basic region, HLH; helix-loop-helix, Zip: leucine zipper, SID: Sin3 interaction domain

was a major breakthrough in Myc research. Max and c-Myc form heterodimers through interaction of their HLHZip domains and the dimer can then interact with DNA through the basic domains of the two proteins. Myc/Max dimers bind to E-boxes with the consensus core sequence CACGTG and this interaction is of importance both for the activation of transcription and the biological functions of c-Myc (Amati et al., 1993).

. The identification of the bHLHZip protein Max brought about a search for additional bHLHZip proteins that could dimerise with Max and bind to E-boxes. The Mad-family was subsequently discovered as Max-binding partners. Together with Myc these protein define the Myc-Max-Mad network (shown in Fig. 8) where Max is placed in the middle and can promote to different cellular responses dependent on whether it is bound to Myc or Mad. The Mad proteins possess a Sin3-interaction domain (SID) which mediates transcriptional repression. Additional Max-partners, Mnt and Mga have also been identified (Hurlin et al., 1997, Hurlin et al., 1999). Further Mlx, a Max-like protein that can heterodimerise with Mad1, Mad4 and Mnt has been described (Billin et al., 1999, Meroni et al., 2000), the function of this protein in the context of the Myc-Max-Mad network is not very clear. Mlx interacts additionally with the proteins MondoA and WBSCR14. Further, c-Myc can also interact with additional proteins as shown in Fig. x. Interaction with other proteins can occur both in the N- and C-terminus of c-Myc. Examples of proteins that interact with the N-terminus of c-Myc include p107 (Beijersbergen et al., 1994, Gu et al., 1994), Bin1 (Sakamuro et al., 1996), TRRAP (McMahon et al., 1998) and TBP (Hateboer et al., 1993) while some proteins that interact with the C-terminus are YY1 (Shrivastava et al., 1993), AP2 (Gaubatz et al., 1995), TFII-I (Roy et al., 1993), Miz1 (Peukert et al., 1997), Nmi (Bao and Zervos, 1996) and Brca1 (Wang et al., 1998b). Miz1, YY1, AP2 and

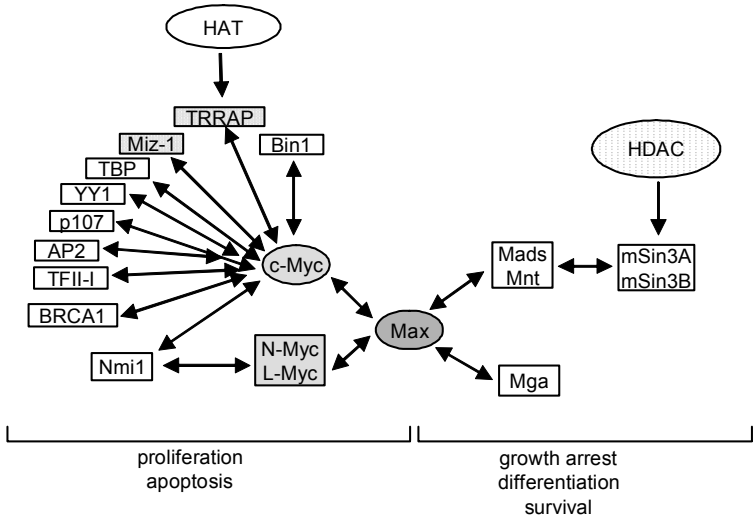


Figure 8: The Myc-Max-Mad network and some associated proteins

TFII-I are transcriptional activators and interaction with c-Myc leads to their inhibition. Miz1 will be discussed in more detail below. Bin1, Brca1 and the pRb-

related protein p107 seem to be negative regulators of c-Myc and inhibit c-Myc dependent transactivation and Ras cotransformation (Sakamuro et al., 1996, Wang et al., 1998b, Elliott et al., 1999). Some of these protein-protein interactions seem to be important for c-Myc function and some of them relevant to this thesis will be described in more detail in the following sections of this chapter.

The cocrystal structures of the Myc/Max and the Mad/Max dimers has recently been solved (Nair and Burley, 2003). These studies reveal that although all the involved proteins contain similar repeated leucine motifs which pack together stably to form a helical coiled structure the hydrogen bonds of the Max/Max dimer do not form as tightly as those of the Myc/Max and Mad/Max dimers due differences in amino acid composition of the leucine zipper. This leads to flaring of the leucine zipper which explains the previous observation that Myc/Max and Mad/Max interact with DNA with higher affinity compared to Max/Max. Myc, Max and Mad hetero- or homodimers make essentially identical protein-DNA contacts with the E-box. Four sequence-specifying contacts are made between each basic region and selected DNA bases. Additional contacts have been observed between residues specific to c-Myc and the phosphate backbone. Interestingly, Myc/Max seems to form a bivalent heterotetramer by the tight interaction of two heterodimers head to tail. This observation explains why Myc/Max can bind to promoters that contain several E-boxes which are widely separated. The Myc/Max heterotetramers which are bound to two cognate sequences thereby stabilises DNA-looping. Another function of the heteroteramer might be the formation of a substantial platform for assembly of additional protein factors such as Miz1, Nmi and Brca1 which bind to the bHLHZip region of Myc.

Taken together the important domains of the proteins of the Myc-Max-Mad network are the bHLHZip for heterodimerisation as a prerequisite for DNA-binding, the TAD of Myc proteins to activate transcription and the SID of Mad proteins which mediate transcriptional repression. These findings place the involved proteins in a network where Max/Myc and Max/Mad have antagonistic functions. Indeed, Mad proteins are expressed preferentially in differentiating cells whereas Myc is expressed in proliferating cells. It has also been reported that overexpression of Mad proteins inhibit Myc-activated reporter genes, interfere with transformation of rat embryo fibroblasts and prevent apoptosis (Henriksson and Luscher, 1996, Grandori et al., 2000).

Regulation of c-Myc by upstream signalling events

Upon mitogenic signalling *c-myc* is induced rapidly and it is believed that this is due primarily an immediate type early response. However, the pathways underlying this rapid upregulation are understood poorly. Signalling through the Scr kinase has been implicated (Barone and Courtneidge, 1995) in *c-myc* upregulation and this notion is supported by recent findings that Src, through a Rac-dependent pathway, mediates PDGFR signalling to *c-myc* (Chiariello et al., 2001). Further it has been suggested that Smad3, a direct mediator of the TGFβ receptor, can mediate the transcriptional repression of *c-myc*. This is achieved by Smad3 complex formation with the transcription factors E2F4/5 and DP1 and the corepressor p107. This complex has been suggested to bind to Smad4 which

recognises a Smad-E2F site on the *c-myc* promoter, thereby mediating repression (Chen et al., 2002). *c-myc* has further been shown to be upregulated by NFκB signalling since inhibition of PI3K-dependent growth coincided with a block of nuclear import of NFκB/c-Rel dimers and a failure to upregulate *c-myc* (Grumont et al., 2002). The Wnt pathway has also been implicated in the regulation of *c-myc*, upon upregulation of TCF4 c-Myc expression was also increased. This is believed to function as a switch between differentiation and proliferation in colorectal cancer cells since the upregulation of c-Myc further leads to a repression of the p21Cip1 promoter (van de Wetering et al., 2002).

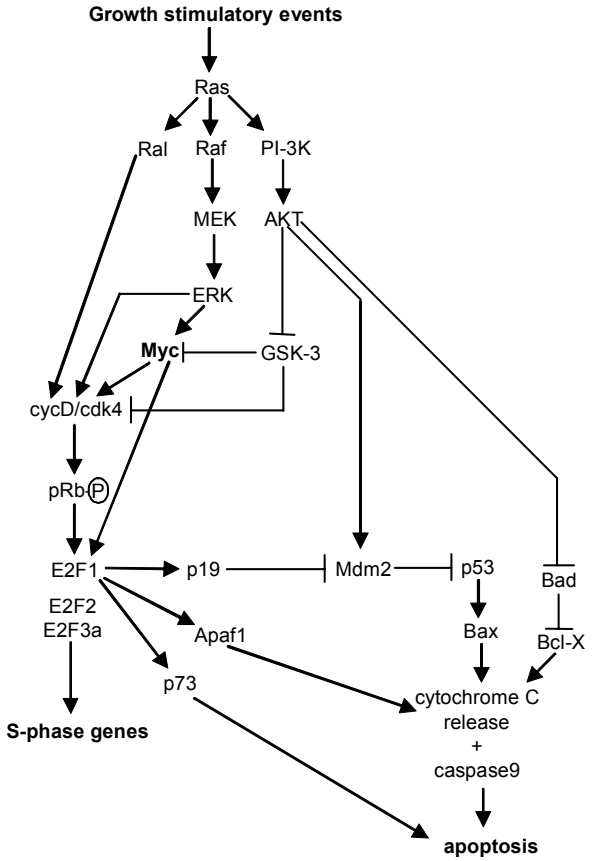


Figure 9: The link between c-Myc and Ras signalling events upstream and downstream of Myc

Another important level of c-Myc control is exerted by post-translational modification by phosphorylations. c-Myc is a highly phosphorylated protein with three clusters of phosphorylation sites. They are positioned (i) in the TAD, more specifically in the region of MB1, (ii) in the central acidic domain and (iii) near the basic region. Although it is known that the two latter clusters are phosphorylated by protein kinase CK2 (Luscher et al., 1989), little is known about their regulation and function. The first cluster, positioned in MB1 has been subject of more intense

study. Two sites, Thr58 and Ser62, are of particular importance since these are so-called hot-spot mutations occurring in e.g. Burkitt's lymphoma. Thr58 is phosphorylated in vitro by glycogen synthase kinase (GSK) 3 which is dependent on the prior phosphorylation of Ser62 (Henriksson et al., 1993, Lutterbach and Hann, 1994, Sears et al., 2000) possibly by MAP kinases and cyclin/cdks. The function and connection between these two phosphorylations has been unclear but was recently suggested to integrate GSK3 and Ras signalling affecting the stability of c-Myc (for review see (Sears and Nevins, 2002)). The signalling pathways are depicted in Fig. 9. This subject will be discussed in more detail in the results and discussion section since the connection between Thr58 and c-Myc stability was one of the objectives of this thesis.

Regulation of transcription by c-Myc and Mad

It has been demonstrated the TAD of c-Myc can interact with a number of proteins (for review see Sakamuro and Prendergast 1999), few of these provide clues to the mechanism of c-Myc induced transcriptional activation. Association of c-Myc with TBP does not seem to be sufficient for transcriptional activation. During recent years new findings have suggested that c-Myc might associate with HAT and SWI/SNF complexes (for review see (Amati et al., 2001)). The first finding along this line was that Myc associates with TRRAP through the conserved domain MB2 of c-Myc (McMahon et al., 1998). TRRAP was shown to be part of at least two multisubunit HAT complexes, one of them containing GCN5/PCAF and the other Tip60/NuA4 (Grant et al., 1998, Vassilev et al., 1998, Ikura et al., 2000). Recruitment of GCN5 HAT activity by c-Myc through association with TRRAP has also been demonstrated (McMahon et al., 2000, Park et al., 2001) Further, c-Myc seems to recruit a Tip60 complex through interaction with TRRAP (Fuchs et al., 2001). Two proteins, Tip48 and Tip49 bind to c-Myc and it has been shown that the ATPase activity of TIP49 is essential for c-Myc oncogenic activity (Wood et al., 2000). Tip48 and Tip49 can further interact with BAF53 (Park et al., 2002) which seems to be a component of STAGA and Tip60 and complexes. Additionally, recruitment of both TRRAP and GCN5 activity has been suggested to be mediated by direct binding of the c-Myc N-terminus to the human STAGA (Spt3/TAF/GCN5/Acetylase) coactivator complex which results in enhanced transcriptional activation. This was shown to require both the SPT3/GCN5 interaction domain of TRRAP and the HAT activity of GCN5 and it has been suggested that TRRAP might function as an adaptor within the STAGA complex that helps to recruit GCN5 HAT activity to c-Myc during transcriptional activation (Liu et al., 2003). The ability of c-Myc to recruit HAT activity to promoters and thereby affecting chromatin structure as part of target gene activation has been demonstrated by (Bouchard et al., 2001, Frank et al., 2001). These studies demonstrate that c-Myc recruits TRRAP in a MB2 dependent manner to target genes which results in histone acetylation and subsequent gene expression. Additionally, a differential requirement for TRRAP recruitment for c-Myc mediated activities has been suggested. The recruitment of TRRAP by c- or N-Myc has been shown to be dispensable to for the partial induction of several basically expressed genes in exponentially growing primary and immortalised fibroblasts. Further, although TRRAP recruitment is required for c- or N-Myc mediated

oncogenic transformation TRRAP is not essential for restoration of the growth defect in *myc-/-* fibroblast (Nikiforov et al., 2002).Other factors involved in the regulation of transcription have been reported to bind to c-Myc. c-Myc can interact with INI1/hSNF5 through its C-terminus. INI1/hSNF5 is a component of the multiprotein SWI/SNF complex involved in chromatin remodelling in an ATP-dependent manner (Cheng et al., 1999, Kingston and Narlikar, 1999). Although the recruitment of the SWI/SNF complex is necessary for c-Myc transactivator function in reporter gene assays, the role of this complex for the regulation of chromosome embedded target genes is not yet clear. It has previously been shown that that c-Myc activates transcription of its target gene *cad* at a post-PolII recruitment step and that the c-Myc TAD interacts with a number of Cdk/cyclin complexes (Eberhardy and Farnham, 2001). It has recently been demonstrated that CyclinT1 and Cdk9 binding to c-Myc is sufficient for activation of a synthetic promoter construct, suggesting that c-Myc activates transcription by stimulating elongation (Eberhardy and Farnham, 2002).

The c-Myc antogonist Mad also possesses abilities to alter chromatin structure. Mad contains a Sin3-interaction domain (SID) through which recruitment of HDAC containing complexes is mediated (Alland et al., 1997, Hassig et al., 1997, Laherty et al., 1997, Sommer et al., 1997). This recruitment leads to histone deacetylation and subsequent transcriptional repression. It had been suggested earlier that Myc/Max and Mad/Max complexes form a molecular switch on responsive E-box DNA elements (Ayer and Eisenman, 1993, Ayer et al., 1993). Myc/Max and Mad/Max complexes are expressed mainly in growing and differentiating cells respectively. In concordance with these observations, Myc/Max complexes bound to the promoters of cyclin D2 and hTERT in exponentially growing cells have been shown to be replaced by Mad/Max complexes upon induction of differentiation (Ayer and Eisenman, 1993, Ayer et al., 1993, Bouchard et al., 2001, Xu et al., 2001). It has been demonstrated Myc/Max and Mad/Max recruit HAT and HDAC activity, respectively, to these promoters, thus demonstrating the switch of activation/repression of genes.

While the function of c-Myc as a transcriptional activator has been more studied and evidence for this has steadily accumulated over recent years, the role of c-Myc in transcriptional repression has been less clear. Originally, one of the first biological functions described for c-Myc was the ability to inhibit the differentiation process of pre-adipocytes to adipocytes in culture by repressing the transcription of the c/EBP-α gene (Freytag and Geddes, 1992). It was subsequently shown that this repression was mediated by the core of the c/EBP-α promoter and it was suggested that c-Myc controls transcription directly or indirectly through the core c/EBP-α promoter (Li et al., 1994). c-Myc has been shown to repress a number of promoters of cell cycle/growth arrest genes including gas1, p15Ink4a, p21Cip1, p27Kip1 and gadds (Gartel and Shchors, 2003) .

The initiator (Inr) element of several of these promoters has been suggested to mediate the repressor activity of c-Myc (Li et al., 1994, Mai and Jalava, 1994, Philipp et al., 1994, Lee et al., 1996). Recent studies have shown that the c-Myc interaction partner Miz 1 binds to and activates transcription from Inr elements and inhibits cell cycle progression (Peukert et al., 1997, Seoane et al., 2001, Staller et

al., 2001, Herold et al., 2002). Both of these functions are antagonized by c-Myc by interaction with Miz-1 at Inr elements (Seoane et al., 2001, Staller et al., 2001, Herold et al., 2002). Transcriptional repression of c-Myc via Miz-1 will be discussed in the Results and Discussion section.

Target genes link c-Myc to its biological functions

A number of Myc-target genes have been identified during the last several years (for review see (Levens, 2002)). These target genes are involved in processes controlling cell growth, proliferation, differentiation, transcription, the cell cycle and apoptosis and can be divided into groups based on their function. Some identified target genes of c-Myc include the following: 1. Genes involved in cell cycle progression: *cdc25a* (Galaktionov et al., 1996), *cyclin D2* (Bouchard et al., 1999), *p21Cip1* (Claassen and Hann, 1999), *p15Ink4b* (Warner et al., 1999), *cul1*(O'Hagan et al., 2000a) and *cdk4* ((Menssen and Hermeking, 2002)). 2. Genes involved in apoptosis: *gadd45, p19Ink4d* and *p53* (Reisman et al., 1993, Zindy et al., 1998). 3. Genes involved in growth and metabolism: *cad* (Miltenberger et al., 1995), *eIF-2α* (Rosenwald et al., 1993), *eIF4E* (Rosenwald et al., 1993), *ornithine decarboxylase (ODC)* (Bello-Fernandez et al., 1993), *LDH-A* (Shim et al., 1997), *α-prothymosin* (Eilers et al., 1991), *DHFR* (Mai and Jalava, 1994), *H-ferritin, iron regulatory protein 2 (IRP2)* (Wu et al., 1999b), and *thymidine kinase* (Pusch et al., 1997). 4. Genes involved in immortalization: *hTert* (Wang et al., 1998a, Wu et al., 1999b, O'Hagan et al., 2000b). 5. Genes involved in transcripton: *tip48, c-myc, pcaf* and *polr2f* (RNA polymerase II subunit F) (O'Hagan et al., 2000b, Menssen and Hermeking, 2002).

Different approaches have been used to identify c-Myc target genes (and to validate these), ranging from differential expression screens, promoter analysis and educated guesses (Cole and McMahon, 1999, Dang, 1999, Greasley et al., 2000) to recently developed methods such as microarray profiling, serial analysis of gene expression (SAGE) and chromatin immunoprecipitation (ChIP) (Coller et al., 2000, Guo et al., 2000, Nesbit et al., 2000, Boon et al., 2001, Neiman et al., 2001, Schuhmacher et al., 2001, Schuldiner and Benvenisty, 2001, Menssen and Hermeking, 2002, O'Connell et al., 2003) , some of these are reviewed in(Grandori et al., 2000, Eisenman, 2001, Levens, 2002). The SAGE and microarray techniques have given rise to a vast amount of putative target genes and obviously these have to be further validated to be able to exclude false positives or negatives. For example, a study using the SAGE technique identified 216 genes that were induced and 258 that were repressed by c-Myc (Menssen and Hermeking, 2002). The overall concordance of genes identified using the microarry technique employed by O'Hagan et al. was about one third which demonstrates the need for additional studies. Most of these newly identified target genes crave further validation by chromatin immunoprecipitation (ChIP) to demonstrate the presence of c-Myc at the promoter *in vivo*. Some studies have also addressed the question whether c-Myc and Mad bind to the same target genes by creating c-Myc containing a substitution of the bHLHZip region with the same region of Mad. This chimeric protein could reproduce the growth-promoting activities of c-Myc but not is apoptotic function. This suggests that although Myc and Mad might possess identical *in vitro* DNA-

binding specificities they do not have identical set of target genes in vivo (James 2002). On the contrary, another study suggest that the basic regions, which mediate DNA-binding of the Myc/Mac and Mad/Max dimers, are functionally equivalent (Nikiforov et al., 2003).

One of the problems in the identification of target genes of c-Myc has been to distinguish between induction/repression and background since most experimental set-ups employ cells expressing background levels of endogenous c-Myc and an engineered cell line overexpressing c-Myc. A recent study by (O'Connell et al., 2003) circumvents this problem by comparing a previously established *myc-/-* cell line with a reconstituted *myc-/-* cells which express the conditionally active Myc-estrogen receptor fusion protein (MycER). Other approaches have been made both at the genomic and proteomic level (Shiio et al., 2002, Fernandez et al., 2003, Orian et al., 2003). Analysis and comparison of the global protein expression pattern in *myc-/-* versus *myc*-reconstituted cells suggests a novel cytoskeletal function for c-Myc (Shiio et al., 2002). A large-scale screen for genomic c-Myc-binding sites in live human cells identified that high affinity E-boxes were located within CpG islands, correlating with an open, pre-acetylated state of chromatin (Fernandez et al., 2003). Another genomic screen in *Drospophila* found that the Myc, Max and Mad/Mnt proteins bind to a large number of loci (approximately 15% of the genome) which implicates a genome-wide interaction of the Myc/Max/Mad network (Orian et al., 2003).

Effects of c-Myc on cell cycle progression

A substantial amount of c-Myc's target genes are involved in the control of cell proliferation. Overcoming the G1/S checkpoint is essential for cell proliferation and it seems thus logical that c-Myc should regulate genes involved in this process. In conclusion with this suggested function of c-Myc, it has been reported that c-Myc is sufficient to overcome the checkpoint and to induce S phase in the absence of growth factors (Eilers, 1999). Further support comes from the analysis of Rat1a *myc-/-* cells and MEFs from conditional *c-myc* knockout cells (Mateyak et al., 1997, de Alboran et al., 2001). Rat1a(*myc-/-*) cells exhibit a prolonged doubling time of 50 hours versus 20 hours in wildtype cells and prolonged G1 and G2 phases whereas the c-Myc deficient MEFs proliferate even slower than the Rat1a deficient cells at a rate of 200 hours versus 20 hours in MEF wildtype cells. Some components of the cyclin/cdk network are direct targets of c-Myc, including *cyclin D2*, *cyclin D1* and *cdk4*. Once cyclin D1 and cyclin D2 are upregulated they can sequester the CKI p27Kip1 which is an inhibitor of cyclin E/cdk2 which in turn is essential for overcoming the checkpoint. Further the expression of cyclin E/cdk2 is enhanced by activation of the suggested c-Myc target gene Cdc25a (Galaktionov et al., 1996) which stimulates cyclin E/cdk2 activity (Blomberg and Hoffmann, 1999, Sexl et al., 1999). The CKI p27Kip1 is further controlled by the upregulation of Cul1 (O'Hagan et al., 2000a) which is part of the SCF E3 ligase participating in the proteasomal degradation of p27Kip1. Further p27Kip1 can be repressed directly by c-Myc (Yang et al., 2001). Studies of the *c-myc-/-* support this notion since the heavily impaired ability of these cells to proliferate correlates with the upregulation of the CKI p27Kip1. Other CKIs such as p15Ink4b and p21Cip1 are also repressed directly (Claassen and Hann, 1999, Gartel et al., 2001)) as will be described in

more detail in the Results & Discussion section of this thesis. CKI repression further adds to cell cycle progression. Another important aspect of cell proliferation is the inactivation of pRb which is taken care of by the upregulation of the c-Myc target gene Id2, a HLH protein that inhibits pRb function (Lasorella et al., 2000).

Role of c-Myc in cell growth

An important aspect of cell proliferation for all organisms is the capacity of the cell to increase in size and to coordinate this with growth division (for review see (Neufeld and Edgar, 1998)). It has been shown that c-Myc is able to influence cell growth by binding to elements in the promoters of the rate-limiting translation initiation factors eIF4E and eIF2α (Rosenwald et al., 1993, Jones et al., 1996). Further it has been demonstrated that cells lacking *c-myc* exhibit decreased rates of protein and ribosomal RNA synthesis which results in reduced growth (Mateyak et al., 1997).

Analysis of *dmyc*, the drosophila orthologue of vertebrate myc (Gallant et al., 1996, Schreiber-Agus et al., 1997) has demonstrated a direct role in cell growth (Johnston et al., 1999). The function of *dmyc* is to maintain the normal size of both cells and organs. Overexpression of *dmyc* produces larger cells but neither overexpression nor loss of *dmyc* has a significant effect on cell division rate. These results suggest that the effects of c-Myc on growth are distinct from those on cell division (Johnston et al., 1999). This is exemplified by c-Myc overexpression in primary B-cells where cells are larger at every stage of their development (Iritani and Eisenman, 1999). c-Myc has been shown to augment growth independent of its effect on the cell cycle in a B cell line as well as in fibroblasts (Schuhmacher et al., 1999, Beier et al., 2000). In addition, N-Myc has also been implied in the regulation of cell size (Knoepfler et al., 2002). N-Myc null progenitor cells from conditional knockout mice exhibit severely impaired proliferation, an increase of neuronal differentiation and altered morphology and size (Knoepfler et al., 2002) which is in concordance for the proposed role of Myc in regulating cell size.

Gene expression analyses show an upregulation of genes involved in ribosome biogenesis, energy and nucleotide metabolism and translational regulation (Grandori et al., 2000). A number of these might be secondary effects but nevertheless the nature of these target genes of c-Myc is well in concordance with the proposed role of c-Myc in cell growth. In conclusion with this notion, it has recently been shown that c-Myc binds to TFIIIB which is a polIII-specific general transcription factor (Gomez-Roman et al., 2003). c-Myc thereby directly activates polIII transcription which is involved in generating tRNA and 5S ribosomal RNA which must be produced in large amounts to meet the need for protein synthesis in growing cells.

Role of c-Myc in apoptosis, immortalisation and tumourigenesis

The ectopic expression of c-Myc in cultured fibroblasts in the absence of survival factors leads to apoptosis (Evan et al., 1992). A number of the identified Myc-target genes are indeed involved in the process of programmed cell death, also referred to as apoptosis. One important pathway of inducing apoptosis involves the

release of cytochrome c from mitochondria (for review see (Igney and Krammer, 2002)) as has been described earlier. Cytochrome c and other nuclear-encoded mitochondrial genes are regulated by the TF nuclear respiratory factor-1 (NRF1). c-Myc seems to compete with NRF1 for binding to common regulatory sites of the cytochrome c promoter and thereby cause cytochrome c release (Morrish et al., 2003). It has further been suggested that growth factor withdrawal correlates with a loss of outer mitochondrial membrane permeability, leading to cytochrome c release and apoptosis. The effect of c-Myc on cell growth and metabolism through target genes such as those encoding ODC and LDH-A may be an additional link to mitochondrial function and death. Additionally both p53 and CD95 (Fas) signalling pathways have been implicated in c-Myc-induced cell death (Hueber et al., 1997). Recently, p38 has been implicated in c-Myc-dependent apoptosis (Desbiens et al., 2003). Specific phosphorylation events on c-Myc have also been linked to its apoptotic function. (Chang et al., 2000) suggested that phosphorylation of Ser71 was involved in cytochrome c release . Another study indicated that activation of the c-Jun N-terminal kinase (JNK) by apoptotic stimuli led to phosphorylation on Ser 62 and 71and that c-Myc proteins mutated at these residues failed to stimulate apoptosis (Noguchi et al., 1999). Overexpression of c-Myc has been found to induce accumulation of reactive oxygen species (ROS) and thereby enhance serum-deprived apoptosis in fibroblasts. It has been suggested that this is due to the inhibition of NFκB activity (Tanaka et al., 2002). Further, the nuclear cofactor TRIP49 that had been shown to have functional roles in c-Myc mediated oncogenesis (Park et al., 2002), has been suggested to induce c-Myc mediated apoptosis (Dugan et al., 2002).

c-Myc is further involved in the immortalisation of cells by the upregulation of telomerase activity which prevents the successive shortening of telomeres with each cell division. This process triggers normally cellular senescence as was described earlier. The *hTert* gene encodes the rate limiting enzyme in the telomerase complex and is a target of c-Myc, at least in mammalian epithelial and B-cells (Wang et al., 1998a, Wu et al., 1999a). This overexpression immortalises human fibroblasts; other cell types such as keratinocytes require additional inactivation of tumour suppressor genes to achieve immortality. It is not clear of cells become truly immortal after upregulation of *hTert* by c-Myc or if only the life span is extended.

It has also been suggested that c-Myc is involved in the induction of genetic instability (Felsher and Bishop, 1999, Vafa et al., 2002). Overexpression of c-Myc was shown to induce DNA damage and to additionally disable the p-53 mediated DNA damage reponse. Thereby cells with damaged genomes are enabled to enter the cell cycle and multistage tumour progression is accelerated.

Several conditional mouse models have been developed to study the role of c-Myc in tumour progression. In summary, these studies show that inducible overexpression of c-Myc led to malignant tumour formation that can regress if the overexpression of c-Myc was temporarily switched off. The cells used in these models, i.e. osteogenic sarcoma cells, hematopoietic cells, pancreatic beta cells, and skin epidermal cells then often differentiated into mature cells and thereby the process of tumourigenesis was reversed. Surprisingly, when c-Myc was

overexpressed again shortly, a high amount of the tumourigenetic cells underwent apoptosis (Felsher and Bishop, 1999, Pelengaris et al., 1999, Felsher et al., 2000, Jain et al., 2002, Pelengaris et al., 2002b) for reviews see (Pelengaris et al., 2002a, Weinstein, 2002)). These models provide an important tool for studies of *c-myc* induced tumour progression and mechanisms underlying tumour regression and thus can make important contributions to the development of cancer treatments. Additionally, a transgenic zebrafish in which T cell acute lymphoblastic leukaemia can be induced has been engineered. This transgenic model provides another platform for drug screens and genetic screens aimed at identifying mutations that suppress or enhance *c-myc* induced carcinogenesis (Langenau et al., 2003).

In the first chapter of this thesis the underlying processes that are deregulated in cancer cells were described. c-Myc has been shown to be involved in all of these processes (for review see (Pelengaris et al., 2002a)). The deregulation of c-Myc is thus a potent factor in tumour development and progression and the development of specific therapeutics aimed at targeting c-Myc overexpression or activity will be important for the efficient treatment of cancer.

Aims of this study

This thesis can be divided into two parts. The aim of the first part was to study the ability and mechanism of c-Myc to repress transcription. Since the mechanism of transcriptional repression by c-Myc is not as well understood as the mechanism of transcriptional activation these studies could potentially provide important information about c-Myc function (paper I). The aim of the second part was to increase our understanding of the regulation of c-Myc protein turnover and how components of this regulatory pathway can influence functions of c-Myc such as cell cycle progression and transcriptional activity (paper II and III).

Broadly, the overall aim of this thesis was to increase our understanding of c-Myc function and regulation. By achieving more information about both how c-Myc regulates its target genes and how it is itself regulated after having carried its function more insight into the complicated cellular mechanisms could be gained. Potentially this could lead to the development of new cancer therapeutics.

Specific aims

The specific aims of this thesis were:

1. To investigate the ability of c-Myc to repress transcription of p21 and to elucidate the mechanism behind transcriptional repression by c-Myc (paper I)

2. To study the regulation of c-Myc half-life via the ubiquitin-proteasome pathway and the role of Thr58 mutations predominant in Burkitt's lymphoma (paper II)

3. To identify a specific SCF-complex participating in the ubiquitylation and proteasomal degradation of the c-Myc protein (paper III)

4. To study the influence of this SCF-complex on the regulation of the cell cycle and transcriptional activity by c-Myc (paper III)

Results and Discussion

c-Myc inhibits differentiation-induced expression of p21Cip1 (paper I)

In response to differentiating signals, the expression of c-Myc is downregulated in most cell types, including in vitro differentiation systems representing hematopoietic, neuronal, muscle or epithelial tissues (for review see (Henriksson and Luscher, 1996)). We have here utilised the U937 differentiation model which was established in 1976 from a patient with generalized histiocytic lymphoma (Sundstrom and Nilsson, 1976). U937 cells are arrested at the monoblastic stage and can be induced to differentiate terminally along the monocytic lineage with agents such as phorbol ester (TPA), retinoic acid (RA) and vitamin D3 (VitD3) (Olsson and Breitman, 1982, Olsson et al., 1983, Einat et al., 1985, Larsson et al., 1988, Oberg et al., 1991). Treatment with these differentiating agents lead to induction of G_0/G_1 arrest and upregulation of a number of monocytic differentiation markers. Further *c-myc* mRNA is downregulated and *mad1* mRNA upregulated (Ayer and Eisenman, 1993, Larsson et al., 1994) upon induction of differentiation.

We had observed previously that U-937 cells that constitutively express v-Myc are not able to undergo differentiation in response to stimulation with TPA (Larsson et al., 1988, Bahram et al., 1999) and speculated how c-Myc can be involved in this block of differentiation. Upon treatment of hematopoietic cell lines, including U937, with differentiating agents, the CKI p21Cip1 is rapidly induced (Liu et al., 1996). As mentioned earlier, p21Cip1 belongs to the Cip/Kip family of cdk inhibitors and plays an important role in cell cycle arrest, differentiation, DNA repair, cell senescence and apoptosis (for review see (Sherr and Roberts, 1999)). We therefore hypothesised that v-Myc expression in U937-Myc6 cells might interfere with the upregulation of p21Cip1 upon treatment with differentiating agents.

We first investigated the ability of TPA to induce differentiation in U937 and v-Myc transformed U937-myc6 cells. Differentiation was measured by analysis of expression of the CD11c monocytic differentiation marker and cell cycle progression by the incorporation of ^3H labelled thymidine. U937 cells differentiated as expected as evidenced by the increased expression of CD11c and the reduced incorporation of ^3H-labelled thymidine. In the v-Myc-expressing U937-myc6 cells no induction of CD11c and almost no reduction of ^3H thymidine incorporation was observed, indicating that differentiation and growth inhibition by TPA were blocked in these cells, as previously reported (Larsson 1988). In U937 cells *p21Cip1* mRNA could be detected 4 hours after induction whereas no induction at all could be seen in the U-937-myc6 cells. Similarly, analysis of protein levels of p21Cip1 and c- and v-Myc correlated with these results, showing a rapid increase in p21Cip1 protein upon TPA treatment in U937 but not in U937-Myc6 cells. Levels of c-Myc were initially high but levels declined as p21 levels

increased in U937 and U937-myc6 cells whereas v-Myc levels in U937-myc6 cells was unaltered.

Having established an inverse correlation between the expression of Myc and TPA-induced expression of p21Cip1, we utilised transient transfection assays to investigate whether Myc can repress the p21Cip1 promoter. To begin with, a full-length p21 promoter/reporter construct (0-luc) was transfected into U937 cells together with increasing amounts of c-Myc whereafter cells where treated with TPA or left untreated. Coexpression of c-Myc led to a repression of reporter activity already at low levels of transfected c-Myc both in the absence or presence of TPA. To map the c-Myc-responsive region of the p21 promoter we used a number of p21 promoter/reporter mutants where sequences downstream of the transcriptional start site where deleted up to nucleotide –94 relative to the transcriptional start site. Our analysis showed that deletions of sequences –2326 bp to –94bp relative to the p21Cip1 transcriptional start site did not abolish the ability of c-Myc to repress basal- and TPA-dependent transcription of p21 expression. Further we generated swap mutant constructs by replacing either nucleotides –94/-50 of the p21 promoter (generating CMV/p21Luc) or nucleotides –49/+16 with the corresponding region of the CMV promoter (generating p21/CMVLuc). c-Myc was able to repress the activity of CMV/p21Luc but not p21/CMVLuc. This means that the c-Myc responsive region of the p21 core promoter is situated between nucleotides –49 and +16. This also suggests that the four Sp1/3 binding sites found in the upper region of the promoter were not essential for the repressing effect of c-Myc. These sites were previously found to be required for p21 induction by multiple signals (Gartel and Tyner, 1999). We further analysed which regions of c-Myc were of importance for mediating the effect at the p21 promoter and found that deletion of the conserved MB2, HLH or Zip domain abolished the ability of c-Myc to mediate repression. Deletion of the basic region did not influence the transcriptional repression which indicates that direct DNA-binding of c-Myc is not required.

c-Myc and Miz1 bind to the p21 core promoter (paper I)

The c-Myc binding protein Miz-1 has been shown to bind to the initiator sequence (Inr) at the p15Ink4b core promoter (Seoane et al., 2001, Staller et al., 2001). Since we had observed that the HLH domain of c-Myc, mediating binding to Miz1, was involved in repression of the p21Cip1 promoter, we thus investigated the possibility that c-Myc exerts its effect via Miz-1. First we examined whether Miz-1 can activate the p21 promoter in U937 cells by transient transfection and luciferase assays as before. We found that Miz-1 activated p21Cip1 from the same core promoter region as c-Myc did, and identified three potential Miz-1 binding sites in this region of the promoter. Mutations of these sites abolished the activating effect of Miz-1, suggesting that Miz-1 acted via these sites. Cotransfection of c-Myc inhibited Miz-1-induced transcription in a dose-dependent manner. Further Miz-1 mRNA and protein were shown to be upregulated during the differentiation process in several hematopoietic cell lines, while c-Myc was downregulated.

We next investigated whether Miz-1 and c-Myc were able to bind to the p21Cip1 core promoter *in vitro*. For this analysis DNA oligo pulldown assays were utilised. Cos 7 cells were transfected with expression vectors for Miz-1 and/or c-Myc prior to precipitation with a double-stranded biotinylated oligonucleotide representing nucleotides –49/+16 of the p21Cip1 promoter. Transfected Miz-1 alone was able to bind to this promoter sequence only when the three potential Miz-1- binding sites were intact. c-Myc transfected on its own could not bind to the wt sequence. Upon cotransfections of c-Myc and Miz-1, c-Myc interactions with DNA could be detected indicating that c-Myc binding to the p21Cip1 promoter was Miz-1 dependent. Interstingly we also observed that the ability of Miz-1 to bind to the sequence increased upon cotransfections with c-Myc. The dependence of c-Myc on Miz-1 for DNA-binding in this assay was confirmed by using a point-mutant of c-Myc, MycV394D, with reduced Miz-1 binding but unaltered binding to Max (Herold et al., 2002). This mutant c-Myc protein showed a reduction both in Miz-1- and DNA-binding to approximately 25-30% of that of wildtype c-Myc. Further, a Myc(BR)-Mad mutant where the HLHZip region of c-Myc is replaced with the corresponding region of the c-Myc antagonist Mad, was utilised. This mutant binds to Max and thereby activates transcription from E-boxes but is unable to bind to Miz-1 (Staller et al., 2001). Unlike wt c-Myc, this mutant as well as the Myc V394D mutant, was unable to repress transcription of the p21Cip1 core promoter. K562 leukaemia cell lines containing Zn^{2+} inducible wt c-Myc orMycV394D genes confirmed that TPA induced endogenous p21 expression was repressed by wt c-Myc but not by the MycV394D mutant.

To investigate whether c-Myc and Miz-1 interact with the p21 promoter *in vivo* we employed chromatin immunoprecipitation (ChIP) assays. Chromatin and proteins bound to it were crosslinked by formaldehyde treatment whereafter cells were lysed and the chromatin was sheared to obtain fragments of 300-500bp. The presence of proteins on specific promoters was analysed by immunoprecipitation using specific antisera followed by de-crosslinking and PCR amplification of promoter regions. A PCR product corresponding to the p21Cip1 promoter region (-194 to +88) could be immunoprecipitated with different c-Myc and Miz-1 antisera. The presence of Max at the promoter was also demonstrated. This analysis also showed that the association of c-Myc with the p21Cip1 promoter decreased after TPA-induced differentiation of HL-60 cells while the binding of Max and Miz-1 was only slightly altered. In contrast, in v-Myc transformed U937 cells where p21Cip1 expression is blocked, binding of c-Myc, Miz-1 and Max to the p21 promoter increased after TPA-treatment.

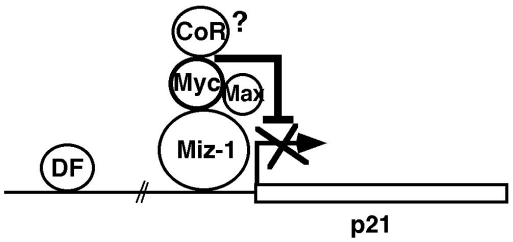
c-Myc and Miz-1 function as a growth/differentiation switch (paper I)

Different models have been proposed previously how c- Myc might mediate transcriptional repression (for review see (Facchini and Penn, 1998, Wanzel et al., 2003)). The first model suggests that c-Myc activates the synthesis of transcriptional repressor proteins and thereby acts indirectly to repress genes. Another model is that the Myc/Max dimer represses transcription by direct DNA-binding. Until now, Myc/Max dimers have not been found to bind directly to core

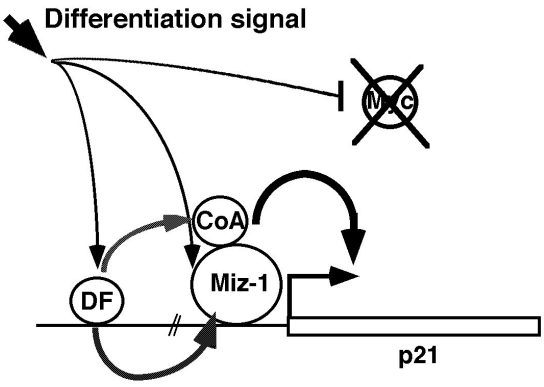
promoters of repressed genes *in vivo*. Other models suggest that c-Myc might sequester an essential component of the basal transcription machinery by interaction with the TFIID subunit TBP and thus interference with the PIC via an active repression mechanism (reviewed by Facchini 1998). Another possibility is the binding of c-Myc to activators such as Sp1, Smads or NFY and thereby preventing activation (Gartel and Shchors, 2003). c-Myc could also be recruited to promoters via interaction with Inr-binding proteins than, such as TFI-II (Roy et al., 1993), YY-1 (Shrivastava et al., 1993) and Miz-1 (Peukert et al., 1997). These proteins are able to bind to Inr elements of promoters and could thereby potentially recruit c-Myc.

The results of this study are in agreement with the last model. c-Myc did not bind to the p21Cip1 promoter directly since deltion of the basic region of c-Myc did not abolish repression of the p21Cip1 promoter. We have no evidence suggeting interaction with components of the basal transcription machinery as explanation for the repressive effect of Myc. Rather, the results from the promoter/reporter, DNA pulldown and ChIP assays suggest that the c-Myc responsive region of the p21 promoter is situated near the transcriptional start site and that c-Myc binds to this region through interaction with Miz1, which associates directly with the promoter. The observation that Myc mutants with reduced binding to Miz-1 showed impaired repression of the p21Cip1 promoter in vitro and in vivo further emphasises the role of Miz-1 in Myc repression. Based on our findings we propose a model where Miz1 plays a dual role at the promoter (see Fig. 10). Miz-1 can act as an activator of the p21 and possibly other differentiation-induced promoters. In addition Miz-1 expression increased during hematopoietic differentiation further suggesting a positive role in this process. On the other hand, upon cooperation with c-Myc, Miz1 can act as a repressor of the same promoter. This is supported by our findings that c-Myc utilises Miz-1 for repression of p21 and that c-Myc enhances the DNA-binding activity of Miz-1 *in vitro* and possibly *in vivo*. Further, c-Myc has also been reported to facilitate the transport of Miz-1 to the nucleus (Peukert et al., 1997). We propose that c-Myc/Miz-1 functions as a growth/differentiation switch which resembles the E2F/pRb switch for G1/S transition.

a Undifferentiated, proliferating cells



b Differentiating cells



c Differentiation blocked by Myc

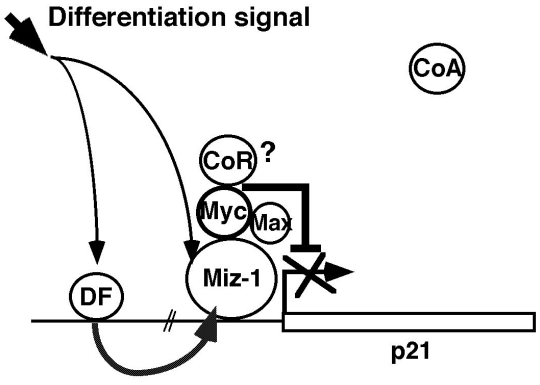


Figure 10: A Myc/Miz-1 switch model for regulation of *p21* gene expression during differentiation. DF: differentiation factor, CoR: corepressor, CoA: coactivator.

Our model is also compatible with other findings from studies regarding Myc/Miz-1 interaction . The p15Ink4b promoter has been shown to be regulated by Miz-1 and c-Myc (Seoane et al., 2001). TGF-β, which induces cell cycle arrest, downregulates expression of *c-myc* and was shown to release Miz-1 from an inhibitory complex with c-Myc. This allowed binding of the coactivator p300 to Miz-1, thereby activating transcription from the p15Ink4b promoter (Staller et al., 2001). Further, TGF-β activates Smad-proteins which were been shown to bind upstream in the promoter to cooperate with Miz-1 in the activation of transcription (Feng et al., 2002, Seoane et al., 2002). In the case of the p21Cip1 promoter, (Herold et al., 2002) found that Miz-1 activity was regulated negatively by association with the topoisomerase II binding protein (TopBP1). Upon UV-irradiation Miz-1 was released from this complex and could bind to the p21Cip1 promoter and activate transcription. c-Myc regulated Miz-1 dependent transcription after UV-irradiation of p21 negatively and facilitated recovery from UV-induced cell cycle arrest through binding to Miz-1. Further, (Seoane et al., 2002) showed that c-Myc-induced repression of the p21Cip1 gene prevented p21Cip1-dependent protection from p53 induced death signals. This suggests that c-Myc dependent repression of p21Cip1 can lead to apoptosis (Seoane et al., 2002). Another recent study describes the regulation of the p21 promoter by the transcription factor TCF via c-Myc and Miz-1 (van de Wetering et al., 2002). TCF4 is a downstream transcription factor of the APC tumour suppressor gene. Upon mutation of APC, TCF4 levels rise and induce transcription of c-Myc which in turn can bind to Miz-1 at the p21 promoter and repress transcription. Our report and the reports above thus agree that c-Myc mediated repression of the p21Cip1 promoter is mediated via Miz-1.

We have limited our analysis to the transcription factors c-Myc and Miz-1 but it is not unlikely that also other transcription factors participate in the early induction of p21Cip1 during differentiation. A number of factors that mediate differentiation and/or growth inhibitory signals bind to upstream elements in the p21Cip1 promoter (Gartel and Tyner, 1999). The p15Ink4b promoter is similar to the p21 promoter in its organisation and it has been shown that, upon TGF-β induced activation, Miz-1 interacts directly with Smads and p300. Smads bind to upstream elements of the promoter and interact also with Sp1 sites. Sp1, Smads and p300 have been implicated in the regulation of the p21 promoter in response to different signals, including those that lead to differentiation (Gartel et al., 2001, Gartel and Shchors, 2003). There are reports suggesting that c-Myc interacts directly with Sp1 and Smads (Gartel et al., 2001, Feng et al., 2002). It was further shown that c-Myc interferes with Miz-1dependent activation of transcription by binding and thereby sequestering Smad2 and 3 (Feng et al., 2002). It is therefore likely that Miz-1 interacts with other transcription factors and coactivators regulated by differentiation signals at the promoter as suggested in Fig. 10b. Nevertheless our data suggest that c-Myc binding to and repression of the p21 promoter relies on binding to Miz-1 and is independent of upstream regulatory elements. Miz-1 therefore seems to be a keyplayer for both negative and positive signals which

regulate the p15 and p21 core promoter. It remains to be investigated whether regulation by Miz-1 could be a general mechanism for c-Myc induced repression of differentiation-induced promoters.

How does Myc transform Miz-1 from an activator to a repressor? In the case of p15 repression, c-Myc prevents binding of p300 to Miz-1 by binding through its HLH domain to the same surface on Miz-1 as p300 (Staller et al., 2001). We do not know whether this might be the case also for p21 repression. However, we have found that apart from the HLH region, both the Zip domain and MB2 are required for c-Myc to be able to repress the p21 promoter. Another possible scenario is therefore the recruitment of a corepressor to the p21 promoter possibly via MB2 as is shown in our model. The Zip domain mediates interaction with Max which is a part of the c-Myc/Miz-1 complex *in vivo* and *in vitro*. The role of Max in this context is not clear. MB2 interacts with TRRAP, TIP48, TIP49 and BAF53 which are components of different HAT-complexes involved in the regulation of chromatin structure (McMahon et al., 1998, McMahon et al., 2000, Bouchard et al., 2001, Frank et al., 2001) for review see (Amati et al., 2001)). HAT-complexes are usually associated with transcriptional activation. GCN5 however has recently been reported to be directly involved in repression of the *ARG1* gene in yeast (Ricci et al., 2002) and it is an open question whether recruitment of HAT-activity via MB2 also plays a role in c-Myc induced repression. MB2 thus seems to play a role in transcriptional repression and elucidation of this mechanism will give further insights into the precise mechanism underlying c-Myc mediated repression of target genes.

Mutation of Thr58 and Pro57 leads to increased c-Myc halflife (paper II)

c-Myc is a short-lived nuclear phosphoprotein and has a half-life of 20-30 minutes in mammalian cells. As mentioned earlier, c-Myc deregulation is often involved in the formation of tumours. In Burkitt's lymphoma c-Myc is translocated to the Ig-locus thereby giving rise to uncontrolled expression. In addition to the translocation the phosphorylation sites Thr58 and Pro57 within MB1 are hot spot mutations in primary tumours and cell lines derived from patients with Burkitt's lymphoma (BL). Mutation of Thr58 of c-Myc increases its transforming potential (Frykberg et al., 1987, Henriksson et al., 1992, Pulverer et al., 1994). Thr58 is suggested to be phosphorylated by GSK3 and the phosphorylation of Pro57 has been shown to be a prerequisite for this phosphorylation (Henriksson et al., 1992, Lutterbach and Hann, 1994, Pulverer et al., 1994). The observation that many transcription factors are degraded by the ubiquitin/proteasome pathway in a phosphorylation-dependent manner (for review see (Hochstrasser, 1996, Hershko and Ciechanover, 1998)) prompted us to investigate the possibility that c-Myc halflife is controlled by the ubiquitin/proteasome pathway via phosphorylation of Thr58.

We first analysed the half-life of c-Myc in different cell lines derived from patients with Burkitt's lymphoma by pulse chase analysis. We observed that those cell lines expressing c-Myc with hot spot mutations (i.e. Thr58 or Pro57) exhibited

a prolonged c-Myc half-life of two hours compared to cell lines expressing wildtype c-Myc with a half-life of c-Myc of 30 minutes. To ensure that this phenomenon was entirely due to the observed c-Myc mutations and not to some other alterations in the cells we transfected wt c-Myc into Raji cells which express a Thr58-mutated c-Myc. The half-life of the transfected c-Myc was 30 minutes as expected whereas endogenous c-Myc half-life was again two hours. We also measured the half-life of both v- and c-Myc in v-Myc transformed U937-Myc6 cells. We could also observe that v-Myc, which contains two mutations whereof one is Thr58 was turned over at a much slower rate (approximately 2 hours) whereas endogenous c-Myc was turned over rapidly (approximately 30 minutes). We next constructed expression vectors for c-MycT58A for analysis of ectopically expressed c-Myc half-life. The T58A mutant showed a longer half-life of over 1 hour compared to ectopically expressed wt c-Myc which had a half-life of 30 minutes. We thus concluded that Thr58 plays a role in the turnover rate of c-Myc.

c-Myc is degraded by the ubiquitin/proteasome pathway via phosphorylation of Thr58 (paper II)

Earlier in vitro studies had suggested that c-Myc is degraded by the ubiquitin/proteasome pathway *in vitro* (Ciechanover et al., 1991). While our study was in progress it was further reported that N-Myc and c-Myc are degraded by the ubiquitin/proteasome pathway *in vivo* (Ciechanover et al., 1991, Bonvini et al., 1998, Gross-Mesilaty et al., 1998). We therefore wanted to clarify the role of Thr58 and Pro57 in this process. We treated U937-myc6, a cell line constitutively expressing v-Myc, with different proteasome inhibitors and observed that endogenous c-Myc was stabilised whereas v-Myc which was stabilised also in untreated cells, was not influenced by proteasome inhibition. The analysis of two BL cell lines further revealed that stabilisation of c-Myc after proteasome inhibition only applied to those cells carrying wt c-Myc. Cells expressing mutated c-Myc exhibited an increased half-life of c-Myc even in the absence of proteasome inhibitors which could not be further stabilised, indicating that this mutation blocked proteasome mediated degradation . To examine whether the proteasome mediated degradation was linked to ubiquitylation of c-Myc we carried out in vivo ubiquitylation assays. c-Myc wt and T58A were cotransfected with an expression vector for His-tagged ubiquitin (His-Ub) into U2OS cells, the proteasome was inhibited and the ubiquitylation status was analysed. Poly-ubiquitin conjugation of substrates results in a high molecular weight smear since chains of different lengths of ubiquitin are attached at different lysine residues. We could observe that cotransfection of His-Ub with wt c-Myc generated a strong smear of ubiquitylated proteins, whereas the smear generated together with c-MycT58A was much weaker. This indicates that ubiquitylation of c-Myc is regulated via phosphorylation of Thr58. However, ubiquitylation did not seem to be entirely dependent on Thr58 since ubiquitylation was not completely abolished using the T58A mutant. We had also observed that deletion of either MB1 (where these phosphorylation sites lie) or MB2 lead to increased half-life of c-Myc (unpublished results). The phosphorylation of Thr58 therefore seems to be an important regulator for ubiquitin/proteasome mediated degradation of c-Myc. This suggests

that not only deregulated gene expression as a consequence of chromosomal translocation but also increased half-life through mutation of a single phosphorylation site in c-Myc contribute to the oncogenesis in Burkitt's lymphoma.

Reports from (Flinn et al., 1998, Salghetti et al., 1999, Gregory and Hann, 2000) have also addressed the question whether c-Myc is degraded by the ubiquitin-proteasome pathway. In addition one report (Sears and Nevins, 2002) studied the influence of phosphorylations of Thr58 and Ser62 on c-Myc stability. These reports are partly in agreement with our findings and partly in conflict.
All reports agree that mutation of the phosphorylation site of Thr58 leads to stabilisation of c-Myc. However, although Gregory & Hann found that c-Myc is often stabilised in BL cell lines they did not find that mutation of Thr58 contributed to the increased stability. Gregory & Hann did not examine the status of mutations in c-Myc in the BL cell lines under study, but based their conclusion on transfection of wt c-Myc into the BL cell line CA46 and found that the transfected wt c-Myc was stabilised. They therefore proposed that c-Myc stabilisation might be due to disturbance of the ubiquitin-proteasome pathway in this cells rather than the c-Myc mutation. This is in direct conflict with our findings since we have demonstrated that the half-life of c-Myc in BL cell lines with mutated Thr58 is extended to two hours versus a half-life of 30 minutes of the wildtype protein. We have further demonstrated that ectopically expressed wt c-Myc is degraded rapidly in Raji cells whereas endogenous Thr58 mutated c-Myc is stable. This result demonstrates that the ubiquitin-pathway in these cells is functional. An explanation for these conflicting results is the use of different cell lines. While the degradation pathway for Myc was shown to be intact in Raji cells it might very well be impaired in CA46 cells. During the course of our study we also observed that vast overexpression of wt c-Myc can lead to stabilisation in different cell lines, including BL cell lines and fibroblasts. We therefore titrated the amount of transfected wt c-Myc in order to exclude stabilisation due to overexpression in the Raji cell line. The study by Sears et al. 2000 suggested that Thr58 and Ser62 were both regulated by mitogen stimulation but have opposing roles in regulating c-Myc stability. Ser62 was shown to stabilise whereas Thr58 destabilised c-Myc. The authors propose a sequential mechanism of phosphorylation which ensures a transient accumulation of c-Myc by Ras-signalling through ERK and PI3K/Akt. GSK3, suggested to phosphorylate c-Myc on Thr 58 can be inhibited through the action of the PI3K/Akt pathway by Ras. However, phosphorylation of Ser62 by ERK is also induced by Ras. The result of Ras-signalling is therefore stabilisation of c-Myc. Eventually, when Akt activities decline, GSK3 is reactivated, phosphorylates Thr58 and c-Myc is degraded. We have not addressed the role of Ser62, but our conclusion on the role of Thr58 is in agreement with those of Sears et al. and highlights the effects of phosphorylation events on c-Myc stability. However, it is not clear whether Thr58 is directly involved in the degradation and ubiquitylation process, for example phosphorylated Thr58 might play an indirect role by for instance facilitating the transport of c-Myc to another cellular compartment prior to ubiquitylation and degradation. GSK3 has been shown to link the cellular localisation and proteasomal degradation of cyclin D1 by phosphorylation (Diehl 1998). A similar

mechanism could possibly also be involved in regulating c-Myc as well. A recent study (Arabi et al., 2003) suggests that both c-Myc and proteasomes accumulate at the nucleoli upon overexpression of c-Myc or proteasome inhibitory treatment and propose a model where sequestration of c-Myc is accompanied by the recruitment of proteasomes leading to subsequent degradation. It is therefore tempting to speculate that protein transport might contribute further to the complexity of c-Myc degradation.

Other regions of importance for functional ubiquitin-proteasome mediated degradation of c-Myc seem to be the conserved Myc-boxes. Deletion of MB1, within which Thr58 is situated, was shown in the study by Flinn et al. (1998) to lead to stabilisation of c-Myc, which is in agreement with our unpublished results. Salghetti et al. (1999) did not utilise a specific MB1 deletion mutant, but suggested that one or more degrons are located within the first 128 amino acids of c-Myc. Gregory & Hann did not find MB1 the first 100 amino acids to be involved in c-Myc stability since a truncated version of c-Myc (c-MycS) lacking this portion was rapidly degraded and ubiquitylated. This is a bit surprising since the same authors find that mutation of Thr58 alone is sufficient to stabilise c-Myc. Flinn et al (1998) and Gregory & Hann observed that deletion of MB2 led to increased c-Myc stability, which was confirmed by us in paper III. . In fact, Flinn et al. show that fusion of either of the Myc-boxes to otherwise stable proteins induces their rapid degradation in yeast (Flinn et al., 1998) which demonstrates the importance of MB1 and MB2 for c-Myc stability. Salghetti et al. (1999) argue against an involvement of MB2 in degron function. They have however not studied the effect of a specific MB2 deletion in the context of a full length c-Myc protein. In paper III we found that binding of c-Myc to the E3 ligase SCF^{Skp2} was mediated via MB2.

Other regions than Thr58, MB1 and 2 were also implicated in c-Myc stability by some of the above mentioned studies. The central acidic region was demonstrated to be important for c-Myc stability bit not for ubiquitylation (Gregory and Hann, 2000). The authors suggest that this region might be important for efficient recognition by the proteasome since it has been suggested that the high degree of negative charge conferred by the acidic residues might be required for unfolding of a protein substrate after docking with the proteasome (Brown et al., 1997). Another recent study identified amino acids 127-189 as an important region for increased c-Myc stability in response to stress induction by TNF-α and MEKK1 (Alarcon-Vargas et al., 2002). Salghetti et al. further suggested that the C-terminus of c-Myc promotes stability. These residues also mediate Miz-1 binding and cotransfection of c-Myc with Miz-1 leads to enhanced stability of c-Myc. This is suggested to relate to the function of Myc/Miz-1 complexes to function as transcriptional repressors (Salghetti et al., 1999). The discrepancies of the different studies might be due several factors. One explanation is that the c-Myc mutants used were not identical. Several different regions of c-Myc might be of importance for regulating c-Myc stability. These different degrons may have different potencies in different cells. The use of different cell lines in the different studies can therefore lead to different conclusions. Further, deletion of certain regions can also have structural consequences which potentially could effect c-Myc stability.

SCF^{Skp2} is involved in ubiquitylation and proteasomal degradation of c-Myc (paper III)

In paper II we showed that c-Myc is ubiquitylated and subsequently degraded by the proteasome. Since we and others had observed that this pathway of degradation seemed to be phosphorylation dependent and an increasing amount of regulatory factors were reported to bind to SCF-complexes prior to their degradation we aimed for identifying a specific E3 ligase, possibly of SCF nature, for c-Myc.

To establish whether c-Myc does bind to a SCF-complex we initiated this study by cotransfections and coimmunoprecipitations of c-Myc and Cul1, one of the common components of the SCFs. We could observe an interaction, which was enhanced in the presence of proteasome inhibitor, between the two proteins. We then tested several F-box proteins for binding to c-Myc and found that the F-box protein Skp2 interacted strongly and reproducibly with c-Myc. Another F-box protein of the LRR-family, Fbl5, also bound weakly to c-Myc. We tested the ability of Skp2, Fbl5 and other F-box proteins these to promote degradation of c-Myc and found that Skp2 promoted degradation of c-Myc together with Cul1 whereas the other tested F-box proteins, including Fbl5, had only minor effects. We therefore continued studying the role of Skp2 in c-Myc degradation and function. Interaction of c-Myc and Skp2 was observed *in vivo* after coexpression in Cos 7 cells, in endogenous cell extracts from HeLa cells and *in vitro* utilising GST-pulldown. In order to map the interaction region of c-Myc we constructed a panel of deletion mutants which either lacked conserved domains of the c-Myc protein such as MB1, MB2 and the bHLHZip domain or were constructed by systematically deleting amino acid sequences from the N- or C-terminus of the protein. The capability of Skp2 to bind those mutants was analysed by cotransfection and coimmunoprecipitation using Cos 7 cells. We concluded that two domains were essential for the interaction with Skp2, namely MB2 and a region within the HLHZip domain. These two domains are essential for c-Myc to be able to carry out its biological functions. Surprisingly MB1 including Thr58 did not seem to be involved in mediating the c-Myc/Skp2 interaction. The results obtained *in vivo* by from the coimmunoprecipitation studies were confirmed by *in vitro* GST-pulldown assays. Further, bimolecular fluorescence complementation assay (BiFC) as described by (Hu et al., 2002) demonstrated the interaction between c-Myc and Skp2 in living cells. This method is based upon the reconstitution of full-length YFP from YFP-N terminal (YN) and YFP-C terminal (YC) fragments which are fused to the proteins which are subject of investigation. When YN and YC fragments are brought together as a consequence of protein-protein interaction the YFP-fragments reassemble to give full-length YFP and thereby fluorescence. Using this method we could also make the important observation that the interaction between c-Myc and Skp2 takes place in the nucleus in living cells.

Having established the interaction between the two proteins we studied the influence of Skp2 on c-Myc turnover and ubiquitylation. The establishment of an *in vitro* system would have been most favourable to demonstrate a direct role of Skp2 in c-Myc degradation as has been demonstrated for some other SCF

substrates (Carrano et al., 1999). We did however not succeed in establishing such a system, possibly due to the lack of post-translational modifications of c-Myc or the lack of some important factor in the process of ubiquitylation and degradation in the cell lysates. We therefore attempted to demonstrate a role for Skp2 in c-Myc degradation by several approaches. We tested the potential of different F-box proteins to promote c-Myc degradation together with Cul1 under conditions where these factors were limiting as mentioned earlier. Another method was to utilise a dominant negative Skp2 mutant (Skp2ΔF) which lacks the F-box and therefore cannot bind to Skp1 and function as an E3 ligase.

Skp2ΔF increased both c-Myc halflife and turnover from 30 minutes to two hours. To demonstrate that the observed effect of Skp2ΔF was a specific dominant effect with respect to SCF^{Skp2} rather than an indirect effect on other degradation sytems, we first stabilised c-Myc by cotransfection with Skp2ΔF and then titrated increasing amounts of Skp2 wt. This led to a reduction in c-Myc levels, further indicating that Skp2 participates in the degradation of c-Myc. We next employed the siRNA technique, which is based upon the interference of small synthetic double-stranded RNA oligos with transcribed mRNA. Transient transfection of HeLa cells with Skp2 siRNA showed an approximate two-fold increase of c-Myc expression. Cycloheximide (CHX) chase experiments showed that the turnover rate of c-Myc in Skp2 siRNA treated cells decreased from one hour to two hours, further indicating a role for Skp2 in c-Myc degradation. c-Myc mutants with diminished or almost abolished binding to Skp2 displayed an increased halflife, indicating that the interaction surfaces required for Skp2 binding also contribute to protein stability. Taken together these results suggest that the E3 ligase activity of SCF^{Skp2} plays an important role in the turnover of c-Myc.

We then investigated the impact of Skp2 on c-Myc ubiquitylation. As observed in paper II, cotransfection of c-Myc wt with His-Ub produced a strong high molecular weight smear of poly-ubiquitylated c-Myc. Coexpression with the dominant-negative Skp2 mutant Skp2ΔF, however, strongly reduced the intensity of the smear indicating that c-Myc ubiquitylation was inhibited under these circumstances. Titration of increasing amount of Skp2 wt in cells cotransfected with Skp2ΔF resulted in re-established ubiquitylation. The analysis of ubiquitylation status of c-Myc mutants deficient in Skp2 binding also showed a diminished ubiquitylation of these mutants compared with wt c-Myc. Taken together, the results from the ubiquitylation assays indicate that Skp2 participates in the ubiquitylation of c-Myc.

The interaction of c-Myc and Skp2 is cell cycle regulated (paper III)

Having established a role of Skp2 in c-Myc degradation and ubiquitylation we addressed the biological function of this interaction. One of the most important functions of c-Myc is to promote G1/S phase transition in the cell cycle. Skp2 has also been implicated in regulating G1/S transition by the ability to mediate rapid degradation of the CKI p27Kip1 (Carrano et al., 1999, Sutterluty et al., 1999). Further both c-Myc and Skp2 have been reported to function as oncogenes

(Gstaiger et al., 2001, Latres et al., 2001). Thus the biological function of the c-Myc/Skp2 interaction seems to be far more complex than a scenario where Skp2 functions as a c-Myc antagonist as is the case in Skp2-mediated degradation of p27 leading to cell cycle transition (Carrano et al., 1999, Sutterluty et al., 1999).

To gain some more insight into the timing of the c-Myc/Skp2 interaction in regard to the cell cycle, we studied c-Myc and Skp2 expression and interaction in human normal peripheral blood lymphocytes (PBLs). Myc expression was very low in resting cells but increased strongly in G1 phase and levels declined as cells enter S-phase. Skp2 expression was upregulated at the G1/S transition after which levels increased during S-phase. The interaction of the two proteins was strongest in S-phase which correlated with strong expression of Skp2 and low steady state levels of c-Myc. The interaction between c-Myc and Skp2 in S-phase thus correlated with increased c-Myc degradation. We further utilised Rat1MycER cells stably expressing an inducible MycER construct which were transiently transfected with Skp2 wt or Skp2ΔF together with pEGFP. GFP-positive cells were sorted by FACS and the S/G1 ratios were determined. Upon activation of MycER by 4-OHT, cells were forced to enter S-phase in concordance with previous reports (Beier et al., 2000). Coexpression of Skp2 wt led to an increased number of cells in S-phase whereas expression of Skp2ΔF led to an inhibition of c-Myc induced S-phase transition. These effects were not due to a reduction of p27 by Skp2-mediated degradation since similar results where obtained performing the same experiment in p27-/- MEF cells expressing MycER. These results suggest that the E3 ligase activity of Skp2 is a positive regulator of c-Myc-dependent S-phase entry.

c-Myc induced transcription is regulated by and dependent on SCF^{Skp2} E3 ligase activity (paper III)

Recently, ubiquitylation has been shown to be important for the regulation of transcription (for reviews see (Tansey, 2001, Conaway et al., 2002, Ottosen et al., 2002, Muratani and Tansey, 2003). This notion and our observation that c-Myc and Skp2 cooperate during the process of G1/S transition prompted us to investigate whether Skp2 affects c-Myc induced transcription.

We first analysed the ability of c-Myc and Skp2 to induce transcription from the α-prothymosin promoter, a well known c-Myc target, utilising a α-prothymosin-promoter/luciferase (α-proT) reporter construct in transient transfections. Reporter activity was activated3.5 fold by c-Myc but not from a similar construct lacking E-boxes as previously reported (Desbarats et al., 1996). Interestingly transfection of Skp2 on its own also induced reporter activity in an E-box dependent fashion. Since the α-proT E-box has been shown to be Myc-specific (Desbarats et al., 1996), this indicates that the activation was mediated via endogenous c-Myc. A c-Myc mutant, MycΔMB2, which is diminished in its ability to bind to Skp2 and cannot bind TRRAP, could not activate transcription of the reporter and further abolished the stimulatory effect of Skp2. The dominant-negative mutant Skp2ΔF did not activate promoter activity indicating that transcriptional stimulation by Skp2 involves the E3 ligase complex. Additionally, cotransfection of c-Myc and Skp2ΔF led to a strong inhibitory effect of transcriptional activity by c-Myc,

whereas cotransfection of c-Myc and Skp2 wt led to an enhancement of reporter activity. These results indicate firstly that SCF^{Skp2} activity is required for efficient transcriptionally activation by c-Myc and secondly that the Skp2-mediated effect on transcription of the c-Myc target gene is mediated via c-Myc.

We further utilised c-Myc and Skp2 negative cells to carry out the same kind of transcriptional assay. In Myc-/- cells, transfected Skp2 failed to activate transcription of the α-proT promoter/reporter construct. In Skp2 -/- cells, ectopic c-Myc expression alone did not activate transcription, whereas ectopic Skp2 expression led to a two-fold activation which was further enhanced by c-Myc. The role of Skp2 in Myc-regulated transcription was investigated by studies of the influence of c-Myc and Skp2 siRNA on the α-proT promoter/reporter activity. These results showed that Skp2 siRNA reduced the activity as much as c-Myc siRNA did. Analysis of the endogenous c-Myc target genes cyclin D2 and ODC by RT-PCR in MycER-expressing p27-/- MEF cells showed a 3-fold increase in mRNA expression of these genes after c-Myc activation with 4-OHT. Skp2 expression further enhanced while Skp2ΔF inhibited the c-Myc induced cyclin D2 and ODC mRNA expression. These results suggest that c-Myc and Skp2 cooperate to activate transcription of c-Myc target genes and that their activities are interdependent. Taken together our results indicate that Skp2 is a coactivator for c-Myc in transcriptional activation.

To further pursue this hypothesis we investigated whether c-Myc might be present together with SCF^{Skp2} at the c-Myc target gene cyclin D2 *in vivo*. Using ChIP analysis, we observed the presence of c-Myc and Max at the cyclin D2 promoter in exponentially growing HeLa cells as expected (Bouchard 2001). Interestingly, Skp2 and Cul1 antisera were also able to precipitate the E-box region of the cyclin D2 promoter. We next asked whether ubiquitylated proteins or proteasomal subunits could be detected at the promoter. Intriguingly, we found that ubiquitin, Sug1/Rpt6 and Rpt3 (both components of the hexameric ring of AAA ATPases (APIS) of the 19S lid particle), Rpn7 and the α2 subunit of the 20S proteasome bound to the cyclin D2 promoter. Performing the same assay in Myc-/- cells showed that neither Skp2 nor ubiquitin bound to the same promoter, indicating that the binding of these proteins was mediated via interaction of c-Myc with E-boxes at the promoter. We were also able to show that Sug1 and c-Myc interacted in solution after cotransfection and coimmunoprecipitation of Cos 7 cells. The Sug1-containing hexameric AAA ATPase ring has been reported to bind to ubiquitylated proteins and has been proposed to participate in transcription (Ferdous et al., 2001, Gonzalez et al., 2002) for reviews see (Conaway et al., 2002, Ottosen et al., 2002, Muratani and Tansey, 2003). We observed an inhibition of transcription of the α-proT promoter/reporter construct in Sug1 siRNA treated cells to the same extend as in cells treated with c-Myc siRNA or Skp2 siRNA. This observation suggests that Sug1 is needed for transcription of the α-proT gene.

Skp2 functions as cofactor for c-Myc function in cell cycle progression and transcriptional activation (paper III)

Addressing the question of the biological significance of the c-Myc/Skp2 interaction and of the Skp2 induced degradation and ubiquitylation of c-Myc, we performed functional cell cycle and transcriptional assays with the expectation that Skp2 would be an antagonist to c-Myc function. Taken together, our results suggest the contrary. Instead of inhibiting or dampening c-Myc activity, Skp2 seems to enhance c-Myc's functions at least as cell cycle regulator and transcriptional activator. Skp2 seems also to stimulate the apoptotic activity of c-Myc (data not shown). Indeed, when the function of Skp2 as an E3 ligase was impaired, c-Myc function also seemed to suffer. During recent years much insight has been gained about a link between ubiquitylation and transcriptional activation and some components of the proteasome and the PolII holoenzyme seem to participate in both processes (Ferdous et al., 2001, Brower et al., 2002, Gonzalez et al., 2002). Our results suggest that Skp2 is an essential cofactor for c-Myc function and propose a model (Fig. 11 based on our present results and on reports by others. According to this model, SCF^{Skp2} is recruited to c-Myc-target promoters by c-Myc and subsequent ubiquitylation of c-Myc and/or other proteins at the promoter occurs through interaction with SCF^{Skp2}. Ubiquitylation of c-Myc leads to the recruitment of the APIS complex (possibly mediated through binding of c-Myc and Sug1) alone or together with the other subunits of the proteasome. We propose that the APIS complex, the proteasome and/or SCF^{Skp2} exert a coactivator function of unknown nature which potentially stimulates target gene transcription. The APIS complex has been suggested to possess chaperone-like activity that may induce remodelling of protein complexes involved in PIC formation, initiation, promoter clearance and/or in transcriptional elongation at the promoter, thereby facilitating transcription (Ferdous et al., 2001, Gonzalez et al., 2002). As a consequence of ubiquitylation and recruitment of the proteasome, c-Myc is rapidly degraded after having activated transcription. Newly synthesised c-Myc can then start the cycle from the beginning. Note that the E3 ligase activity of SCF^{Skp2} might also act on other targets than c-Myc at the promoter. Further, the proposed connection between ubiquitylation and transcriptional activity of c-Myc could also be of non-proteolytic nature which has been demonstrated for the yeast transcription factor Met4 (Kaiser et al., 2000, Kuras et al., 2002). The ability of c-Myc to recruit both coactivators with E3 ligase activity and APIS ATPases to target promoters would add to the complexity by which c-Myc regulates target gene transcription. It has been shown earlier that c-Myc recruits HAT-containing complexes and SWI/SNF complexes with enzymatic activities to target genes (for review see(Amati et al., 2001)). It thus seems that E3 ligase activity is another enzymatic activity in the repertoire of c-Myc in order to regulate gene transcription. One of the future tasks will be to elucidate the link between c-Myc ubiquitylation and transcriptional activity in more detail.

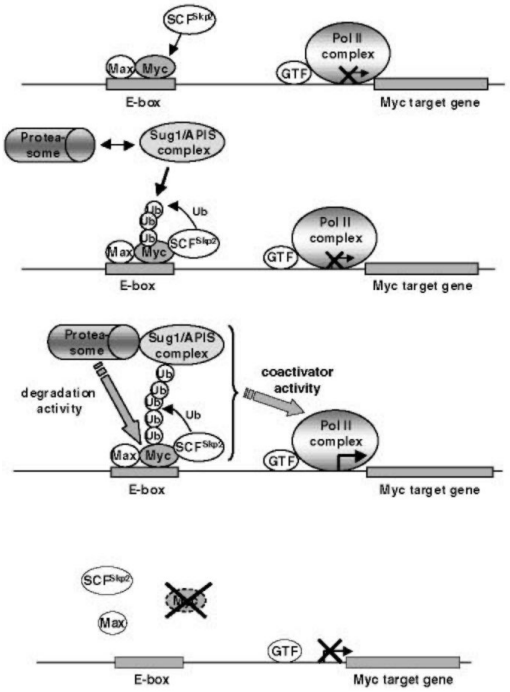


Figure 11: Hypothetical model of transcriptional coactivator and E3 ligase function of SCF^{Skp2} on c-Myc target genes

Additional degradation mechanisms for c-Myc might exist (paper II and III)

We and others (Salghetti et al., 1999, Gregory and Hann, 2000, Sears et al., 2000, Flinn et al., 2002) have shown that Thr58 and also other regions are important in the process of mediating ubiquitylation and proteasomal degradation of c-Myc (paper II). It has also been suggested that the adjacent Ser62 plays a role for c-Myc half-life (Sears et al., 2000).

These different studies and our own notion that Thr58, although an important phosphorylation site regulating ubiquitylation, does not seem to be involved in Skp2 dependent ubiquitylation and degradation. This indicates that c-Myc degradation is controlled by multiple pathways. The existence of multiple SCF E3 ligases has been demonstrated for a number of substrates. For example, p27Kip1 is ubiquitylated by a Skp2-independent mechanism (Hara et al., 2001) and also cyclin E has been found to be regulated by other F-box proteins than Skp2 (Strohmaier et al., 2001). This could also be the case for c-Myc; multiple degradation mechanisms could for example control turnover in other cell cycle phases than S-phase and/or control turnover upon signalling events. Considering the variety of biological processes involving c-Myc and the need for tight

regulation of this oncoprotein c-Myc it would be rather surprising if there were not multiple mechanisms of c-Myc turnover. It has neither been demonstrated if c-Myc is modified by ubiquitin-like proteins such as Sumo. Potentially modifications by other ubiquitin-like proteins might further contribute to c-Myc function.

In paper III we investigated the impact of SCF^{Skp2} on ubiquitylation and degradation of c-Myc. These studies are mostly based on the use of a dominant-negative Skp2 mutant or Skp2 siRNA or were performed under circumstances where c-Myc is first stabilised and can then be destabilised by the addition of Skp2 wt. Although this suggests that SCF^{Skp2} promotes c-Myc degradation, the lack of an *in vitro* system has rendered it impossible for us to study any direct influence of factors such as Skp2 on c-Myc ubiquitylation and degradation. We can therefore not exclude the possibility that the observed effects of SCF^{Skp2} are indirect through another E3 ligase. Such an E3 ligase would not only require SCF^{Skp2} activity but also direct interaction between Skp2 and c-Myc, which we find rather unlikely. Nevertheless, our studies have revealed a potential mechanism by which c-Myc turnover and c-Myc function is controlled which contributes to the overall understanding of c-Myc.

Control of G1/S transition and translational initiation by repressing p21 and cooperating with Skp2 – a working hypothesis for c-Myc function (papers I-III)

In this thesis, several aspects of c-Myc function have been studied. Paper I addressed the ability of c-Myc to repress expression of the CKI p21Cip1 which is important for the regulation of G1/S transition. Paper II established a link between post-translational modifications of c-Myc by phosphorylation and ubiquitylation as a prerequisite for proteasome-mediated degradation. Paper III both elaborated on the subject of degradation by identifying an E3 ligase that promotes c-Myc degradation and further connected ubiquitylation to transcriptional activity. Our findings also show that Skp2 E3 ligase activity promotes c-Myc induced S-phase

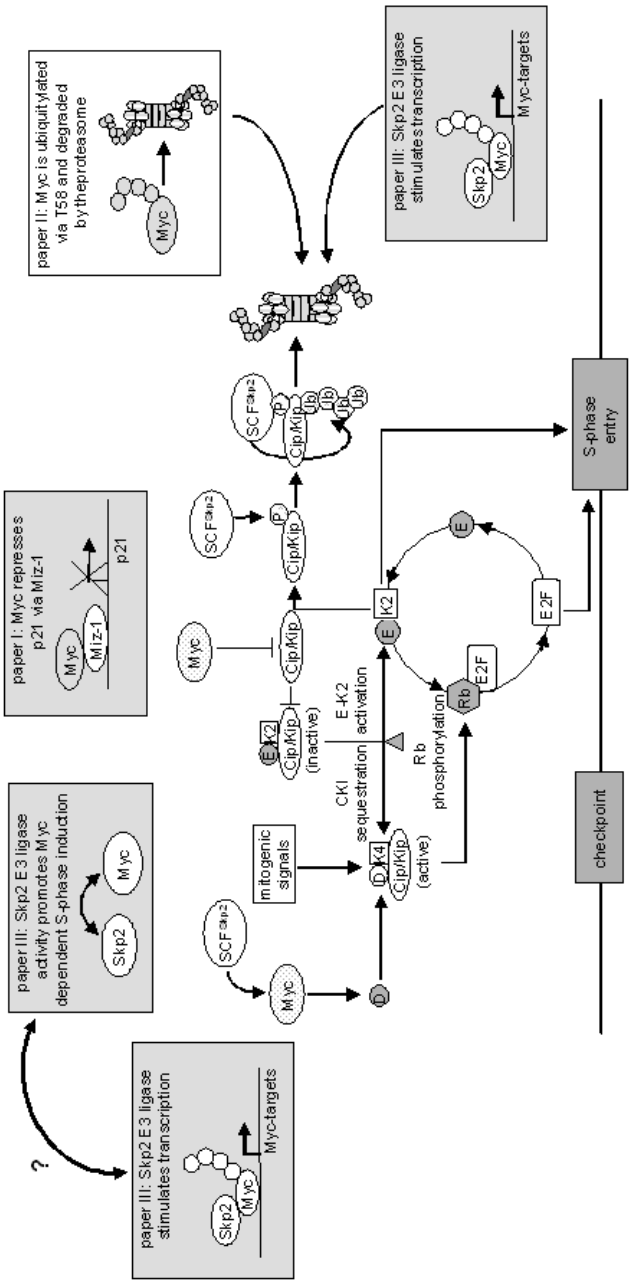


Figure 12: Hypothetical model of Skp2/Myc cooperation at the G1/S transition. See text for details. D: cyclin D. E: cyclin E, K2: cdk2, K4: cdk4

entry. How this is achieved is not entirely clear but I propose that it involves Skp2 transcriptional coactivator function to stimulate c-Myc ability to induce

transcription of genes necessary for cell cycle transition in the following way (see Fig. 12). The first step at G1/S transition is the inhibition of CKIs. This is achieved partly by p15Ink4b and p21Cip1 repression by c-Myc (Gartel et al., 2001, Seoane et al., 2001, Staller et al., 2001, Herold et al., 2002, Seoane et al., 2002). It is unclear at present whether Skp2 possibly also contributes to this function of c-Myc. Further c-Myc and Skp2 seem to cooperate to induce degradation of p27Kip1 . Transcriptional upregulation of cyclin D2 by c-Myc is dependent on SCF^{Skp2} and leads to upregulation of cdk4 and 6 activity and the sequestering of p27Kip1 by cyclin D2. As a result of p27 sequestering, cyclin E/cdk2 complexes are activated and phosphorylate p27 and pRb. These phosphorylations lead to SCF^{Skp2} mediated degradation of p27Kip1, the release of E2F from pRb and other cdk2 dependent events. The release of E2F results in the transcription of genes essential for G1/S transition. At the onset of S-phase, c-Myc and Skp2 associate directly and both transcriptional activity and proteasome-mediated degradation of c-Myc is mediated via SCF^{Skp2}. Finally both c-Myc and Skp2 are degraded at the end of S-phase, c-Myc by SCF^{Skp2} E3 ligase and Skp2 by an autocatalytic mechanism. This model remains highly speculative and much more work remains to be done to elucidate the precise mechanisms and networks by which c-Myc carries out its function.

Conclusions

1. c-Myc represses transcription from the p21 promoter in a Miz-1 dependent fashion. c-Myc/Miz1 function as growth/differentiation switch at the promoter.

2. c-Myc is ubiquitylated and degraded by the proteasome.

3. Thr58 seems to play a role in the ubiquitylation and proteasome-mediated degradation process.

4. SCF^{Skp2} is participating in c-Myc ubiquitylation and degradation in a Thr58-independent manner.

5. Skp2 is a cofactor for c-Myc function in cell cycle regulation and transcriptional regulation.

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