Function and Regulation of myc-family bHLHZip Transcription Factors during the Animal and Plant Cell Cycle

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Abstract
The myc-family of proto-oncogenes (c-, N- and L-myc) encodes transcription factors of the basic region helix-loop-helix leucine zipper (bHLHZip) family and plays important roles in cell proliferation, metabolism, apoptosis and many other fundamental cellular processes. myc-family genes are frequently deregulated in cancer, often correlating with poor prognosis. c-Myc is estimated to regulate 15% of all genes.

This thesis focuses firstly on the mechanisms behind posttranslational negative regulation of Myc activities induced by growth inhibitory signals, secondly on the regulation of transcription of genes involved in G1/S transition during cell cycle by Myc, and thirdly on characterization of myc-related bHLH genes in Arabidopsis.

Our results show that the cytokine interferon (IFN)-γ induces ubiquitylation and degradation of Myc-family proteins in N-myc-amplified neuroblastoma cells and in Myc-transformed monocytic cells and induces expression of the Myc antagonist Mad1. IFN-γ-induced turnover of Myc involves reduced phosphorylation of Ser-62 through inhibition of cyclin E/ cyclin-dependent kinase 2 (Cdk2), which was identified as a Ser-62 kinase. The mechanism behind this inhibition was shown to be IFN-γ-induced expression of the Cdk-inhibitor p27Kip1. p27Kip1 was further demonstrated to target the Myc/cyclin E/Cdk2-complex directly at Myc target promoters, resulting in transcriptional repression of Myc target genes. This study also shows that during cell cycle progression, Myc associates with S-phase genes already in early G1 but cannot activate those genes until in late G1/S. This correlated with binding of the E3 ubiquitin ligase and cofactor Skp2 to these promoters. We propose that Skp2 and cyclin E/Cdk2 cooperate in this regulation. Taken together, this thesis suggests that Myc intimately interact with cyclinE/Cdk2, p27Kip1, and Skp2 in the control of the cell cycle genes, which might provide potential new strategies for cancer therapy.

Our study in the plant Arabidopsis thaliana (A) characterized 84 myc-related At bHLH genes. Expression profiling of these genes in proliferative tissues showed that 15 of the bHLH genes were expressed in a cell cycle regulated manner, suggesting that they may play a role in cell division in plants.

Keywords: Myc, Mad1, interferon-γ, cyclin E/Cdk2, p27Kip1, Skp2, transcription, cell cycle, bHLH, Arabidopsis thaliana
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献给我的家人和我的祖国

To my family and my homeland

为什么我的眼中常含泪水，是因为我对这土地爱得深沉

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


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Abbreviations

AAA  ATPases associated with various cellular activities
ALT  alternative lengthening of telomeres
ATPases  adenosine triphosphatase
bHLHZip  basic helix-loop-helix leucine zipper
BLE  basal level enhancer
CAK  Cdk-activating kinase
CDK  cyclin dependant kinase
CKI  Cdk inhibitor protein
CoA  coactivator
CoR  corepressor
DR  death receptor
DSBs  DNA double-strand break
GS  growth signal
GSK  glycogen synthase kinase
GTF  general transcription factors
HAT  histone acetyltransferase
HDAC  histone deacetyltransferase
Inr  initiator
MAP  mitogen activated protein
MAPK  mitogen-activated protein kinase
MB  Myc box
NDR  nucleosome-depleted region
NF-κB  nuclear factor-κB
NLS  nuclear localisation sequence
ORF  open reading frame
PI3K  phosphatidylinositol 3-kinase
pRb  retinoblastoma protein
PTM  post-translation modification
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5-acetyltransferase</td>
</tr>
<tr>
<td>SID</td>
<td>Sin3 interaction domain</td>
</tr>
<tr>
<td>STAT</td>
<td>the signal transducers and activators of transcription protein</td>
</tr>
<tr>
<td>TAD</td>
<td>transcription activation domain</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRAP</td>
<td>TR-associated protein</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRRAP</td>
<td>Transactivation/transformation Associated Protein</td>
</tr>
<tr>
<td>UPS</td>
<td>ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growthfactor</td>
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1 Background

1.1 Cancer

Cancer is a complex disease involving dynamic changes in the genome (for reviews see (Hanahan and Weinberg, 2000)). Tumorigenesis is a multi-step procession during which the genome of the cancer cells suffers disruption from point mutation to changes in chromosome complement (for review see (Kinzler and Vogelstein, 1996)). Cells in the tumour proceed via a process similar to Darwinian evolution, that the cells with a serials of genetic change acquire one or another growth advantage, driving the formation of cancer (for reviews see (Hanahan and Weinberg, 2000)).

There are three types of genes involved in tumorigenesis: oncogenes, tumour-suppressor genes, and stability genes (caretaker) (Vogelstein and Kinzler, 2004). Mutations on only one allele of oncogenes are able to promote the gene activity, and are sufficient for cells to gain growth advantage, but which is not enough to cause cancer. Tumor-suppressor genes and stability genes form strong barriers for malignant tumorigenesis in human cells. Unlike oncogenes, tumor-suppressor genes usually need to be mutated on both alleles for function lost. The deregulation of both oncogenes and tumour-suppressor genes works to promote cell proliferation, decrease cell apoptosis, and inhibition of cell arrest, hence increasing total number of tumour cells. Mutated stability genes don’t contribute to cancer cell number directly; instead, they decrease genome stability through the impaired function of DNA damage repair mechanisms, thereby creating opportunities for cell transformation. Two mutations are required for cultured rodent cells to acquire tumorigenic competence, while at least three are required for human cells (Hahn et al., 1999). Deregulation of cyclin E
causes genetic instability in both rodent and human cells (Spruck et al., 1999, Minella et al., 2002), which may due to impaired mini-chromosome maintenance proteins (MCM), a key component of the pre-replication complex (pre_RC) and may be involved in the formation of replication forks and in the recruitment of other DNA replication related proteins, loading on replication origins. In addition to gene mutation, apoptosis may also lead to genomic instability when DNA in dead cells being incorporated into neighboring cells by phagocytosis (Holmgren et al., 1999).

An instable genome renders cell opportunities of going through the six alterations for tumorigenesis, which are self-sufficiency in growth signal, insensitivity to anti-growth signals and block of differentiation, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

![Outline of cellular processes affected by genetic alterations in cancer cells.](image)

Figure 1: Outline of cellular processes affected by genetic alterations in cancer cells.
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1.1.1 Self-sufficiency in growth signals

Normal cells need to receive mitogenic growth signals (GS) from their environment via trans-membrane receptors for proliferation. Oncogenes act by mimicking normal growth signals in one way or another so that the cells are able to proliferate independent of extra cellular stimuli.

There are several ways to gain growth signal autonomy. One is the process called autocrine stimulation, through which the cancer cells
synthesize GS of their own and then create a positive feedback signal loop. In some other cancer cells, surface receptors transducing GS are overexpressed so as to generate cells hyperresponsive to growth stimuli (Slamon et al. 1987). Another possible way is the capability of cancer cells to switch the types of extracellular matrix receptors (integrins) they express, favouring those that transmit pro-growth signals (Lukashev and Werb, 1998; Giancotti and Ruoslahti, 1999). The most complex mechanisms of acquired GS autonomy derive from the mutation of genes encoding proteins that transduce and process signals emitted by ligand-activated GS receptors and integrins in cytoplasm. One example is the Ras-proteins. Ras relies on its intrinsic GTPase activity to terminate signalling by hydrolyzing GTP to GDP. This process is catalysed by GTPase-activating proteins (GAP). Mutations in ras are found in 30% of human cancers, resulting in impaired Ras GTPase activity and resistance to GAPs. The active Ras-GTP activates the Raf kinase and then induces mitogen-activated protein kinases (MAPKs). Downstream targets of Ras-MAPK pathway phosphorylate H3 at Ser 10 and Ser 28 as well as a variety of transcription factors, which leads to chromatin structure destabilization and promotes expression of genes such like c-myc, c-jun, and c-fos (Dunn et al., 2005, Ito, 2007, Diaz-Flores and Shannon, 2007).

1.1.2 Insensitivity to anti-growth signals

In normal cells, anti-proliferative signals function to maintain cellular quiescence and tissue homeostasis. These signals drive cells out of active proliferating cycle into quiescence (G0) or into postmitotic states associated with differentiation straits. Insensitivity to anti-growth signals is an acquired genetic alteration of cancer cells.

Much of the anti-proliferative signals circuitry is associated with the cell cycle, especially the components involved in G1/S phase transition. Most, if not all, of the anti-proliferative signals are funnelled through the cell cycle regulator and tumour suppressor retinoblastoma protein (pRb). When in a hypophosphorylated state, pRb blocks proliferation by sequestering and altering the functions of E2F transcription factors that control the expression of genes essential for G1/S transition (Weinberg, 1995). This mechanism will be specified in the next chapter. pRb is mutated in many cancer cells, which causes continuously active of E2F, resulting in S phase entry even when in absence of mitogen stimuli. Loss of the tumour suppressor p53 is another common phenomenon in tumourgenesis that occurs in more than 50% of human cancers (Hollstein et al., 1996; Vousden and Prives, 2005). p53 is well known as the “guardian of the genome”, which is activated by
various types of cellular stress, including DNA damage and oncogenic stress. p53 stops the proliferation of precancerous cells by inducing cell cycle arrest or apoptosis, resulting from its integration of life or death signals such as oncogene activation, DNA damage, mitotic impairment or oxidative stress (Harris and Levine, 2005). Loss of p53 dramatically disrupts cells differentiation and promotes cancer formation in several oncogene-driven mouse tumour models. Even loss of one p53 allele will have similar effect in 20% of all mice (for review see (Stiewe, 2007)). Moreover, the level of p53 proteins has significant influence on cell fate. Low p53 level favours growth arrest whereas high p53 level triggers apoptosis (Laptenko and Prives, 2006)

In addition to avoidance of anti-proliferative signals, lack or block cellular differentiation is another hallmark of cancer (for review see Tenen, 2003). This is particularly evident in hematopoetic tumours. Tumour cells use various strategies to avoid differentiation; one of them is deregulation and translocation of specific transcription factors that control the process of cells from self-renewing stage to mature differentiated stage. Beside, overexpression of oncogenes can also block differentiation, e.g c-myc. It is known that c-myc can induce a shift from differentiation to proliferation when overexpressed (for review see (Grandori et al., 2000)). Depletion of c-myc inhibits cell proliferation, resulting in G0/G1 growth arrest in normal cells, while more variable arrest patterns (G0/G1, S, or G2/M) in different tumour cell lines (Wang et al., 2007). However, there is also evidence showing that Myc promotes differentiation in B lymphocyte (Habib et al., 2008). This will be discussed in more details in the later chapter about Myc.

1.1.3 Evasion of apoptosis
The expansion of tumour cell population not only relies on the cell proliferation but also the cell death. Evasion of apoptosis, programmed cell death, is another important capability acquired by cancer cells. Apoptosis is an energy–dependent process characterized by several morphological features namely nuclear condensation and fragmentation, membrane blebbing and non-inflammatory phagocytosis of the cell fragments (Wyllie et al. 1980). There are two main pathways of apoptosis, the intrinsic and extrinsic pathway, although there is evidence for cross-talk between the two (for review see Meyer et al. 2006).

Intrinsic pathway of apoptosis can be triggered by DNA damage, oncogene activation, hypoxia, or limited growth signal. Intrinsic pathway signals converge on the mitochondria where decision of survival or apoptosis
of the cell will be made by balance between pro- and anti- apoptotic molecules. The BCL2 family of proteins is the main player of this process. This family can be divided into anti-apoptotic group (BCL2 and BCL-XL) and pro-apoptotic group (BAX, BAK, BID, PUMA, NOXA and BIM). Balance of the two groups will decide the fate of cells (Gross et al. 1999). When pro-apoptotic signals win, cytochrom C is released from mitochondria. It binds to Apaf-1 and activates caspase-9, which in turn cleaves and then activates downstream caspase-3 and caspase-7 (for review see Lavrik 2005). Caspases are a family of proteases that cleave substrates at those consensus sites containing asparagine residues. The caspase cascade can also be initiated by extrinsic pathway of apoptosis, also known as the death receptor pathway. The death receptors (CD95/Fas, TNFR and DR5) response to TNF-related apoptosis-inducing ligand (TRAIL). Upon death ligand simulation, the receptors oligomerise, bringing intracellular death domains (DD) together so they can interact with adaptor molecules such as FADD and TRADD (Danial 2004). Adaptor proteins contain both DD and death effector domain (DED), which in turn interacts with the DED domain of caspase-8, triggering its cleavage and activation. Activated caspase-8, like caspase-9, activates downstream caspases such as caspase-3 and caspase-7.

Resistance to apoptosis can be achieved through various strategies in cancer cell. Enhancement of survival molecules is one of the possible mechanisms. Loss of the pro-apoptotic tumour suppressor p53 function is the most common way. It senses DNA damage signals and leads cells to growth arrest, where the damage can be repaired, or into apoptosis (Vousden, 2000). Apoptosis is mediated by p53 through both transcription-dependent and –independent methods (Schuler and Green, 2005). p53 can not only activate transcription of apoptosis inducing genes like BAX and BIM, but also can be translocated from nucleus to function at mitochondria through binding directly to BCL2/BCL-XL and BAK (for review see Meyer et al. 2006). P63 and p73, two relatives and evolutionary predecessors of p53, are thought to be required for p53 to transactivate pro-apoptotic genes. (Flores et al., 2002, 2005).

1.1.4 Limitless replicative potential
The three acquired capabilities of cancer cells mentioned above all dedicate to uncouple the cell growth from its environment though disruption of cell-to-cell signalling. However, they are not enough to generate the huge mass of cells required for malignant tumour constitution. All mammalian cells
carry an intrinsic program independent of the cell-to-cell signalling pathway that limits cell multi-replication. Break of the mortality barrier is necessary for cancer formation.

Cells in culture have limited replicative potential (for review see Hayflick, 1997). Cells having gone through a certain amount of cell doublings (50 for human cell cultures) will stop growing. During each cell cycle, telomeres, which are composed of several thousand repeat of a short 6 bp element at the ends of chromosomes, will suffer a loss of 50 bp telomeric DNA. The continuous erosion of telomeres will eventually lead to complete loss of its ability to protect the ends of chromosomes, resulting in end-to-end fusion of chromosomes and massive cell death.

Telomeres maintenance is the strategy used by almost all types of cancer cells to gain limitless proliferative potential. There are two methods of telomeres maintenance. One is upregulation of telomerase, an enzyme adding hexanucleotide repeats onto the telomeric DNA, which is used by 85–90% cancer cells (Bryan and Cech, 1999). The other method is called alternative lengthening of telomeres (ALT), where the maintenance is achieved through recombination based inter-chromosomal exchange of sequence information (Bryan et al., 1995; Ceth, 2004).

1.1.5 Sustained angiogenesis, Tissue invasion and metastasis

Nutrients and oxygen supplies are crucial for cell survival and functions. All cells in tissue can live only within a small distance (10µm) from capillary blood vessel. The rapid formation of blood vessel, a process named angiogenetic switch, is required for large size neoplasias. New blood vessels may either sprout from preexisting mature ones or form freshly by recruiting bone-marrow-derived endothelia progenitor cells (for review see Bertolini et al., 2006; Rafii and Lyden, 2008). Further, evidence shows that endothelial progenitor cells control the angiogenic switch in mouse (Gao et al., 2008). Angiogenesis can be achieved through over-expression of angiogenesis inducers such as VEGF and suppression of angiogenesis inhibitors through transcription and proteases regulations (for review see Hanahan and Weinberg, 2000).

Sooner or later the cancer development will achieve the stage known as metastasis, where the primary cancer cells invade adjacent tissues and made colonies at distant sites. Metastasis is responsible for 90% of human cancer death (Sporn, 1996). Metastasis initiates with dissemination, which is the
process that invasive tumour cells enter the bloodstream through vasculature. Intravasation is also enhanced by an epithelial-to-mesenchymal transition that endows carcinoma cells with embryonic plasticity and added motility (Thiery, 2002, Huber et al., 2005). The efficiency of dissemination increases with tumour size. Intrinsic functions for evading of detachment-triggered cell death and extrinsic cooperation such as platelet aggregates are required for tumour cells to survive stresses during disseminating through bloodstream (Nierodzik and Karpatkin, 2006). Metastatic cells enter the parenchyma of a target organ by breaching the capillaries in which they are embedded. In the entry, they need cooperation of organic specific components of the tumour microenvironments for successful colonization (for review see (Nguyen and Massagué, 2007)). As each organ presents a highly specialized microenvironment, distinct sets of functions are required for colonizing different tissues (Gupta and Massagué, 2006).

1.2 The eukaryotic cell cycle

Cells reproduce through a serial of events known as the cell cycle, which includes duplication of the genome and cell division. The cell cycle is divided into four phases, G1 phase (G for gap), S phase (S for synthesis of DNA), M phase (M for mitosis), and G2 phase. In M phase, the replicated chromosomes are divided to form two new nuclei and the cell is then split in two by cytoplasmic division. Most cells require much more time for growth and organelle duplication, for this reason, two extra gap phases are inserted in cell cycles, the G1 and G2 phase. The two gaps also provide time for cells to monitor the internal and external environment signals to ensure that conditions are favourable and preparation are complete before the cells go into S phase and mitosis. Checkpoint in G1 phase is reported both in yeast and mammalian cells, where the cells decide to proceed or exit the cell cycle upon integration of anti- and pro-growth signals (for review see (Kastan and Bartek, 2004)). Once the cells pass the checkpoint, cell cycle will continue even if the signals that stimulate cell growth and division are removed. Checkpoint in G2 phase prevents cells from entering mitosis with damaged DNA, providing an opportunity for DNA repair and stopping the proliferation of damaged cells. The critical target of the G2 checkpoint is the mitosis-promoting activity of the cyclinB/Cdk1 kinase (Katsuhiro, 2004, Kastan and Bartek, 2004). Cells can also go into quiescence, the G0 phase, in either G1 or G2 phase, when internal and external signals are unfavourable, staying for days or even years before resuming proliferation. The G1, S, and G2 phase together are called interphase in which the cells
spend the majority of its time and perform the majority of its cellular functions. Some human cells divide every 24 hours; in this case interphase takes about 22~23 hours, with 1 or 2 hours for mitosis, which depends on the variety and size of the cells.

Figure 2: A simplified overview of the mammalian cell cycle. Printed with permission of Anna Dimber.

1.2.1 Cyclins, cyclin-dependent kinases and their inhibitors
There are two types of proteins that constitute the major players in the regulation of the cell cycle, the cyclins and cyclin-dependent kinases (Cdns). There are 9 Cdns (referred as Cdk1-9) and at least 16 cyclins found in animal (Johnson and Walker, 1999) whereas there are 7 Cdns (CdkA-G) and 7 cyclins (A, B, C, D, H, P and T) in plants (for review see (Francis, 2007)). Cyclins in different organisms might not be phylogenetically related, like in animal and yeast, but they still carry out similar functions, e.g. the activation of G1/S transcription by phosphorylating the transcription repressors Whi5 (yeast) or Rb (animal and plant).
Cdks are a group of serine/threonine kinases and each Cdk associates with a cyclin. The association is mediated via the cyclin box, a sequence of 100 amino acids conserved in all cyclins. Cdk activities in eukaryotic cells are very low in G1, and rise progressively in mid-late G1, S, and peak and rapidly decrease during mitosis (Morgan, 1987). Full activation of the cyclin/Cdk complex occurs when a separate kinase, the Cdk-activating kinase (CAK), a complex of cyclin H and Cdk7, phosphorylates an amino acid near the entrance of the Cdk active site, then allows the complex to phosphorylate and activate certain set of substrate proteins. Different groups of cyclins and Cdks are linked to different cell cycle phase. Mammalian G1 phase has three D type cyclins and two E type cyclins, which interact to Cdk4/6 or Cdk2 respectively. Various combinations of D type cyclins are expressed in different cell lines whereas cyclin E are expressed ubiquitously. The cyclins associated to S phase are A and E type, both binding with Cdk2. In G2/M phase, cyclin A and cyclin B, together with Cdk1, control cell cycle process (Arellano and Moreno, 1997, Nigg, 1995).

The different cyclins and Cdks are biochemically similar. Their apparent differences in behaviour seem to reflect more of the spatial and temporal expression than of the specificity of substrates (for review see Murray, 2004)). For example, Cyclin B, which is normally excluded from the nucleus during interphase, can induce DNA replication when fused with nuclear localization sequence from cyclin E and thereby enter the nucleus (Strausfeld et al, 1996), suggesting that it can recognize the same substrates as cyclin E/Cdk2. Further, evidences show that none of the G1 cyclin/Cdks is unique enough to impair cell cycle progress in mouse when being disrupted, and members in some classes, like D type cyclins, can compensate each other (for review see (Sherr and Roberts, 2004)). It is also possible that even though cyclin/Cdks are controlling cell cycle, there are still some other kinases that can be involved in this process (Murray, 2004). To sum up, the multiplicity of cyclins reflects redundancy and divergence that send different family members to different places at different time, which assures biological functions in different parts of the cells to be regulated separately. Although substrate specificity may not be the defining trait of different cyclin/Cdks, it does exist at certain level. The differences in substrate recognition between different cyclins associated to the same Cdk or between Cdk1 and Cdk2 is subtle (up to 5−fold), which is both small enough to help explain the survival capability of cells suffering multiple depletion of cyclins and Cdks and big enough to explain why such genetically disrupted cells often confront somewhat abnormalities during cell cycle.
The activity of a cyclin/Cdk complex can be inhibited by phosphorylation at two residues in the Cdk active site by the protein kinase Wee1, and the inhibitory process can be reversed by dephosphorylation of same sites by a phosphatase Cdc25. The other way of cyclin/Cdk complexes inactivation is through Cdk inhibitor proteins (CKIs), Ink4 and Kip/Cip families, p21Cip1, p27Kip1, and p57Kip2. p27Kip1 is a tumour suppressor that is highly expressed in quiescent cells and low in tumour cells. An important difference between p27Kip1 and other tumour suppressor proteins, like p53 or p16, is that the down-regulation of p27Kip1 in cancers is caused by accelerated degradation of the p27Kip1 protein instead of mutations of the p27Kip1 gene or epigenetic silencing (Slingerland and Pagano, 2000). Regulation of p27Kip1 is mediated by two independent phosphorylation sites on C-terminal of the protein. Phosphorylation on Thr187 by cyclinE/Cdk2 promotes its degradation whereas phosphorylation on Thr198 by AKT leads to its stabilization (Kossatz et al., 2006). In G0 phase, p27Kip1 exists in monomeric form. Phosphorylation on Thr198 is needed to prevent proteolysis of the cyclin unbound p27Kip1. When cells entering G1, Thr198-phosphorylated p27Kip1 prefers to bind cyclinD/Cdk4/6 complex. Loss of Thr198-phosphorylation shifts the binding preference from cyclinD/Cdk4/6 to cyclinE/Cdk2. p27Kip1 is also known to help cyclinD/Cdk4/6 activation when binding to the complex.

In plant, the two major cyclin-dependent kinases are CDKA and CDKB. CDKA regulates the transition from the G1 to S, which associates with cyclin D (CYCD) and cyclin A (CYCA) in G1 and S phase, respectively. CDKB regulates G2 to M phases of the cell cycle. It associates with G2/M cyclins CYCA and cyclin B (CYCB). The CDK inhibitors (CKIs) are called Kip-related proteins, KRP (or ICK). They are related to animal p27kip1 (for review see (De Veylder et al., 2007)).

1.2.2 The ubiquitin and proteasome system (UPS) and degradation of G1 phase cyclins

Proteins are ubiquitylated through three enzymes, known respectively as E1, E2, and E3. Ubiquitin, a small 8kDa protein, is activated by the ubiquitin-activating enzyme E1 in an ATP-dependent manner. The activated ubiquitin is transferred to ubiquitin-conjugating enzyme E2. Finally, the conjugated ubiquitin is covalently attached to a substrate protein by the ubiquitin ligase E3 to make a polyubiquitin chain via UbK48, leading to degradation of the substrate protein through proteasome. While there is only
one E1 enzyme and several E2 enzymes, there are numerous (up to 1000 members) E3 enzymes that determine the specificity of substrates recognition. The big E3 ligase family can be divided into four classes based on the structure specificity: HECT-type, RING-finger-type, U-box-type, and PHD-finger-type. The largest E3 ligases family Ring-finger family is composed of single and multisubunit E3 ligases. The single unit E3 ligases have substrate recognition element and Ring-finger in one single protein. For example, the p53 E3 ligase MDM2 belongs to this group. Multisubunit E3 ligases contain a ring-finger protein, a member of cullin family, an adaptor, and a substrate recognition protein. The subfamilies are SCF, VCB and APC complexes shown in Fig4. The 26S proteasome comprises a 20S core component and two 19S regulatory complexes. The 20S core is conserved from yeast to humans; it contains four α- and β rings surrounding a barrel shaped cavity. The two inner β rings form a central chamber that carries out proteolytic function. The 19S complex contains 18 distinct proteins; including six highly related ATPases of the AAA family. Ubiquitylated proteins are recognized by the ATPase subunit S6’ (spt6), unfolded and channelled via a central passage into the degradation chamber, cut into peptides, finally released through the entry channel (Lam et al, Finley, 2000, Hutschenreiter et al., 2004).

Figure 3: The ubiquitin and proteasome system.
The oscillation of cyclins during the cell cycle process is the result of orchestrated cyclin synthesis and proteolysis. Clearance of cyclins gives the cells enough time for growth and integration of intrinsic and extrinsic signals before there are enough cyclins accumulated to drive cells into another round of replication and division. All cyclins are destroyed through UPS pathway. Two types of E3 ligase are involved in cell cycle regulation, SCF complex and anaphase-promoting complex (APC). The name of SCF is derived from the three earliest identified components: Skp1 (adaptor protein), Cullin1 (scaffold protein), and F-box proteins (substrate recognition protein). The other components are the Ring-finger protein Rbx1 and Rub1/Nedd8. SCF complex functions from late G1 to early M phase, whereas APC actives from mid-mitosis to the end of G1 phase. Two F-box proteins, Skp2 and Fbw7, are thought to involve in G1/S phase regulation (for review see (Nakayama and Nakayama, 2005, 2006)).

Skp2 was originally discovered as protein associated with the cyclinA/Cdk2 complex, and was named as p45. It is since then well established as a growth promoter and oncoprotein. Reported substrates of Skp2 include p27<sup>kip1</sup>, p21<sup>cip1</sup>, p53, p130, cyclin A, cyclin D1, free cyclin E, E2F1, ORC1, CDT1, CDK9, MYC, B-MYB, SMAD4, RAG2, UBP43, FOXO1 and papilloma virus E7 (for review see (Nakayama and Nakayama, 2005)). Cyclin D is phosphorylated and exported from nucleus to cytoplasm through glycogen synthase kinase 3-β (GSK-3β), a kinase inhibited by Ras/Pi3K/AKT pathway (Diehl et al. 1998, Alt et al. 2000), and recognized by F-box Skp2 in SCF complex, leading to its degradation. Skp2 also mediates degradation of free monomeric non-phosphorylated cyclin E (Clurman et al., 1996, Nakayama et al., 2000, Koepp et al., Strohmaier et al., 2001, Won et al., 1996).
Fbw7/hCdc4 is frequently mutated in tumours and is considered to be a tumour suppressor gene (Calhoun et al., 2003, Rajagopalan et al., 2004). In cell culture models, loss of Fbw7 leads to genomic instability (Rajagopalan et al., 2004). Cyclin E is phosphorylated at multiple sites by GSK-3β (Thr380) and is autophosphorylated by Cdk2 (Thr380), producing the phosphodegron sequence recognized by Fbw7/hCdc4 (Clurman et al., 1996; Welcker et al., 2003; Won and Reed, 1996). The involvement of GSK-3β in cyclin E stability suggests that cyclin E, just like cyclin D1, can be directly influenced by at least one mitogen-dependent kinase-signaling cascade (Ras/PI3K/AKT). A double phosphorylation at Thr380/Ser384 creates high-affinity binding of cyclin E to Fbw7 whereas a single phosphorylation at Thr62 makes a low-affinity binding (Hao et al., 2007). Recent observations suggest that phosphorylated cyclin E, together with Pim1, binds to one of two isoforms of Fbw7, Fbw7α, potentiating prolyl isomerase activity of Pim1 to isomerize the trans bond between pralines 381 and 382 of cyclin E. The isomerized cyclin E can be efficiently ubiquitylated by another Fbw7 isoform, Fbw7γ (van Drogen et al., 2006). In experiments performed by Tsunematsu and Mao, expression of cyclin E was unaffected in Fbw7–/– embryos (Tsunematsu et al., 2004), and concentration of cyclin E increased only in the placenta (Tetzlaff et al., 2004), which suggested that Fbw7 regulates cyclin E degradation in a tissue specific manner. The other reported E3 ligase for cyclin E is Cul3-based E3 ligase. Cul3 exclusively targets monomeric cyclin E (Clurman et al., 1996, Singer et al, 1999). The N terminus of Cul3 binds to one of many BTB domain-containing proteins, a family of proteins that have been shown to contain both the adaptor and substrate recognition moiety in a single polypeptide (Geyer et al., Xu et al., 2003). Mechanisms of Cul3 degradation pathway is still not very clear, but it is known that phosphorylation on Thr380 of cyclin E is not required here. Decreased levels of Cul3 increases both cyclin E protein levels and the number of cells in S phase. Cul3-based E3 ligase is essential to maintain quiescence in mammalian cells (McEvoy et al., 2007).

1.2.3 G1/S transition regulation

In early embryos, DNA synthesis starts directly after mitosis. In post-embryonic cells, a gap G1 is inserted into the two phases. Yeast and animal have long G1 phase, where, as mentioned above, the cells decide to proceed into S phase or enter quiescent stage G0 depends on the availability of nutrients and growth factors. As a result, deregulation of G1/S transition is universal in cancers. Prohibition of fast entry into replication is achieved
through Cdk inhibitors (CKIs) and S-phase negative regulator proteins (e.g. Rb family). A major role of G1 cyclins/Cdk complexes seems to be to disable cell cycle inhibitory proteins, after which the mitogenic signals can be delivered to the core cell cycle machinery to assure smooth process of cell proliferation and division (Sherr and Roberts 1995, 1999)

1.2.4 The retinoblastoma protein pRb

pRb family proteins play a crucial role in the cell cycle, especially DNA replication, by controlling the expression of E2F-dependent genes that are necessary for S phase entry, and important for CKI degradation, like cyclin E and Skp2. Hypophosphorylated pRb binds promoters of such genes by forming a complex with E2F. It actively represses the activity of these promoters, at least in part by recruiting chromatin modulating factors such as histone deacetyltransferase 1 (HDAC1) (Luo et al., 1998), SWI/SNF type of chromatin remodeling factors (Harbour and Dean, 2000; De Luca et al., 1997), polycomb group proteins (Dahiya et al., 2001) or histone methyltransferases (Nielsen et al., 2001) that act on the surrounding nucleosome structure to create functionally inactive chromatin. pRb proteins can also block G1/S transition through E2F- independent mechanisms. They inhibit cyclinE/Cdk2 kinase activity either by binding directly to this complex or by increasing p27kip1 expression and stabilizing p27kip1 proteins. The latter can be achieved through several mechanisms. First, pRb binds to Skp2, thus interfering with the Skp2-p27kip1 interaction and thereby preventing p27kip1 ubiquitylation (for review see (Giacinti and Giordano, 2006)). Second, pRb acts as a scaffold for Skp2 and its E3 ligase APC/Ccdc1, facilitating Skp2 degradation, resulting in stabilization of p27kip1 (Binne et al. 2007). pRb phosphorylation is regulated by mitogenic signals and is exerted by the cyclinD/Cdk4/6 complex in the early and mid-G1, and cyclinE/Cdk2 in late G1. pRb can also be regulated at both the transcriptional and protein levels by the transcription factor ICBP90 (inverted CCAAT box binding protein of 90 kDa), resulting in S phase entry (Hopnefer, 2000).

The pRb/E2F pathway is also conserved in plants. The E2F transcription factor that controls expression of S-phase specific genes associates with the retinoblastoma protein-related corepressor RBR in G1 to keep these genes silenced. In late G1, CYCD/CDKA phosphorylates RBR, resulting in release of E2F and activation of S-phase genes (for review see (De Veylder et al., 2007)).
1.2.5 Role of the G1 cyclins in the G1-S transition

The D-type cyclins (D1, D2, and D3) activation is induced by mitogenic stimuli and is closely regulated at many levels, including gene transcription, cyclin D translation and stabilization, assembly of D cyclins with their Cdk partners (Cdk4 and Cdk6), and import of the holoenzymes into the nucleus, where they ultimately phosphorylate their substrates (Sherr and Roberts 1999) through Ras/Raf/MAP kinase signal transduction cascade pathway (Diehl et al. 1998, Alt et al. 2000). Each type of cyclin D could receive different upstream signals. Cell proliferation, which controlled by different mitogens in various cell types, is regulated via expression of different D-type cyclin gene. cyclin D1 availability is controlled by a balance between the Ras/Raf/MAPK (cyclin D1 synthesis) and Ras/PI3K/AKT (cyclin D1 stability) mitogenic pathways (for review see (Malumbres and Barbacid, 2001, Massagué, 2004)). Once activated, cyclinD/Cdk4/6 complex can promote cell division through phosphorylation of substrate proteins such as pRb and Smad3. The main inhibitors of cyclinD/Cdk4/6 complexes are the Ink4 family (p16Ink4A, p15 Ink4B, p18Ink4C, and p19Ink4D) that can be induced by growth inhibitor such as TGF-β. They bind to the Cdk4/6 kinases and prevent their association with cyclin Ds.

The primary role of cyclin D is to activate cyclinE/Cdk2 in mid/late G1 phase. In association with Cdk4/6, cyclin Ds initiate phosphorylation of pRb family protein (pRb, p107, and p130), thus helping to release transcriptional factor E2F from its suppression. The freed E2F will activate a group of genes required for S phase entry (Trimarchi and Lees 2002), among these are genes encoding cyclin Es. CyclinD/Cdk4/6 complexes can also activate cyclin E by sequestering Cip/Kip proteins, hence titrating these inhibitors away from cyclinE/Cdk2 complexes. Binding of Cip/Kip proteins to cyclinD1/Cdk4 stabilizes the complex and facilitates its nuclear import, without inhibiting cyclin D associated kinase activity (Blain et al., LaBaer et al., 1997, Cheng et al., 1999). Cyclin Es, acting with Cdk2s, target and hyperphosphorylate pRb family proteins that are already partially phosphorylated by CyclinD/Cdk4/6 complexes. Only after this latter modification pRb renders inactive and thus unable to occlude the transcription-activating domains on the E2F and to block cell cycle advance. Overexpression of cyclin E can greatly accelerate G1/S transition in cultured mammalian cells (Resnitzky et al. 1994). Ectopic cyclin E expression causes decreased mitogen requirement and increased cell size. All this data suggest that cyclinE/Cdk2 is crucial in G1/S transition. But reports from 2003 challenged this concept when they showed that neither elimination of cyclin
E by targeting both genes encoding cyclin E1 and E2 nor disruption Cdk2 vitally impaired continuous cell cycle in either embryonic or post-embryonic cells (Geng et al., Ortega et al., 2003). Although these reports suggest that cyclin E is dispensable for cell division, and there is redundant activity that substitutes its loss, data accumulated during many years indicate that it plays a fundamental role in G1 in wild-type cells. cyclin E is regulated at both transcriptional and post-transcriptional levels. It forms a positive feedback loop with pRb and E2F, which makes the inactivation of pRb independent of mitogens once cyclinE/Cdk2 becomes active. cyclin E is regulated by other transcriptional factors in other circumstances, e.g. in pancreatic and hepatic cells it is target of LRH-1 (Botrugno et al., 2004). The cyclin E mRNA is also regulated by alternative splicing, but the relevant functions of its different isoforms are not clear. cyclinE/Cdk2 complex carries on most of its biological function through substrate phosphorylation. Its substrates include proteins involved in G1/S transition regulation (such as pRb and p27^{kip1}), histone transcription (p220/NPAT) and acetylation (CBP/p300), and centrosome regulation (nucleophosmin B23 and CP110) (for review see (Möröy and Geisen, 2004, Hwang and Clurman, 2005)). cyclin E also plays an important role in DNA synthesis and functions synergistically with CDC6 to facilitate MCM (2-7) loading on replication origins (Coverly et al., 2002). Surprisingly, cyclin E mutant that fails to activate Cdk2 is able to interact with the critical replication licensing protein CDT1 and with MCM proteins, thus promoting the MCM loading, as good as wild type cyclin E (Geng et al., 2007). This result implies that the replication licensing function of cyclin E is independent of Cdk2.

When cells enter S phase, cyclin E is degraded and Cdk2 then associates with cyclin A. In late S phase, CylinA/Cdk1 and CylinB/Cdk1 complexes are necessary for S phase completion and M phase entry. To exit mitosis, cyclin B destruction is required.

1.2.6 The Skp2 autoinduction loop at the G1/S transition

Ectopic expression of Skp2 allows cells to enter S phase in absence of mitogens (Sutterluty et al., 1999). The recent discovery that Skp2 is a transcriptional target of E2F (Zhang and Wang, 2006) gave rise to a positive feedback loop termed as the Skp2 autoinduction loop (Yung et al. 2007), which unifies many cell cycle regulators implicated in G1 restriction point control, such as Skp2, p27^{kip1}, cyclinE/Cdk2 and pRb.
p27<sup>Kip1</sup> deficiency almost completely rescues the aberrations obverted in Skp2-/- cells, suggesting that p27<sup>Kip1</sup> is the primary downstream effector of Skp2 (Nakayama et al., 2004). Direct physical contact between Cyclin E but not Cdk2 and p27<sup>Kip1</sup> is required for p27<sup>Kip1</sup> recruitment to SCF<sub>Skp2</sub>. Arg306 of Skp2 is required for recognition and ubiquitylation of phosphorylated p27<sup>Kip1</sup> (Ungermannova et al., 2005). p27<sup>Kip1</sup> requires the accessory protein Cks1 for its recognition and ubiquitylation by the SCF<sub>Skp2</sub> ligase (Ganoth et al., 2001; Spruck et al., 2001). Cks1 binds to the leucine-rich repeat (LRR) domain and C-terminal tail of Skp2. p27<sup>Kip1</sup> binds to the phosphate binding site on Cks1 though its phosphorylated Thr187 side chain, whereas binds to both Cks1 and Skp2 through another side chain of an invariant Glu185 (Hao et al., 2005).

Skp2 triggers p27 degradation, which leads to activation of cyclinE/Cdk2, resulting in pRb phosphorylation, release of E2F, and further E2F-dependent Skp2 expression. While the overall flow through the loop is directional, two steps are bi-directional. One is that cyclinE/Cdk2 inactivates pRb while pRb sequesters E2F to transcription. The other is that p27<sup>Kip1</sup> inhibits cyclinE/Cdk2 activity while cyclinE/Cdk2 down-regulates p27<sup>Kip1</sup> level through phosphorylation of its T187A. The Skp2 autoinduction loop doesn’t have any intrinsic on/off mechanisms. Its regulation is dependent on external mitogens and anti-mitogens. External mitogens turn on the loop through induction of cyclin D, but its turnover is not restricted to decreased levels of cyclin D or inhibition of Cdk4/6. Knock-in of p27<sup>T187A</sup> mutant that cannot be degraded by Skp2 is also able to block the loop (Yung et al., 2007). Blockade of the skp2 autoinduction loop strongly inhibits but not completely eliminates cells procession to S phase, which suggests that the loop may not be the only way for restriction point control. One possible option is that E2F can bind to cyclin D1 promoter and acts as another feedback loop which induces cyclin D1, finally resulting in pRb phosphorylation and further E2F release (for review see (Assioan and Yung, 2008)). Ubiquitylation and proteolysis of p27<sup>Kip1</sup> also occur at early G1 phase, a process independent of Skp2. p27<sup>Kip1</sup> phosphorylated on Ser10 binds to CRM, a carrier protein for nuclear export, being translocated to the cytoplasm (Ishida et al., 2002) where it is degraded by Kip1 ubiquitylation-promoting complex (KPC). KPC2 stabilizes KPC1, recruits polyubiquitylated p27<sup>Kip1</sup> and sends it to proteasome for degradation (Kamura et al., 2004, Kotoshiba et al., 2005, Hara et al., 2005).
1.3 Overview of transcriptional regulation

Transcription is the process where a DNA sequence is enzymatically copied by RNA polymerase to produce a complementary RNA nucleotide sequence. Proofreading mechanisms of transcription are fewer and less effective than that of DNA replication, therefore, transcription has a lower copying fidelity than DNA replication. The template strand of DNA is transcribed by RNA polymerase. The product, primary transcript RNA, has 5’→3’ direction and it is identical to the coding (non-template) strand of the DNA. After three main RNA processing steps, 5’ capping, 3’ polyadenylation, and RNA splicing, the mature mRNA is exported from nucleus to cytoplasm where the translation takes place.

Gene expression is tightly controlled in cells, which is carried out through a serial of events by transcription activator and suppressor, polymerase I, II, and III, chromatin modifiers, and other general transcription factors (GTF). Polymerase I (Pol I) transcribes ribosomal RNA, while Polymerase III (Pol III) transcribes tRNA and various small RNA. Polymerase II (Pol II) transcribes protein-encoding genes.

1.3.1 Initiation of transcription

Transcription initiation is the first step of gene expression. Formation of the preinitiation complex (PIC) at the right time and at the right promoter is a prerequisite of mRNA synthesis. The process is governed through regulations affecting both the multi-subunit transcription factor complex and
DNA template. In prokaryotes, transcription initiation is executed by RNA polymerase (RNAP) and initiation specific subunit, σ factor, though it is much more complicating in eukaryotes. I will focus on transcription initiation by RNA polymerase II (Pol II) in eucaryotic cells in the following description.

Figure 6: Overview of the transcription apparatus assembling on the promoter. Printed with permission from Robert Tjian.

Transcription directed by RNA polymerase II needs several elements: cis-acting elements, trans-acting elements, and the basal transcription machinery. The cis-acting elements include the core promoter, proximal promoter elements/promoter-proximal basal level enhancers, and gene-specific signal-responsive upstream promoters/distal enhancers. The core promoter often contains a TATA box (TATA), usually located 25 nucleotides upstream from the transcription start site, the initiator (Inr) and the downstream promoter element (DPE), which directs transcription by RNA polymerase II. Proximal enhancer elements, including BLE, GCbox, and CCAAT box, are usually bound by transcription factors such as Sp1 that enhance transcription. The gene-specific signal-responsive upstream promoters interact with different types of DNA-binding transcription factors that can be either activators or suppressors of transcription (for review see (Lemon 29).
and Tjian, 2000)). The trans-acting elements are composed of various transcription activators and associated complexes that act in a gene-specific manner. Binding of transcription activators or activation of pre-bound activator usually is the initial step of RNA polymerase II directed transcription, followed by recruitment of the preinitiation complex (PIC) along with chromatin remodeling protein complexes. DNA-binding transcription activators contain at least two important domains, the DNA binding domain and the transactivation domain (TAD). They can be classified into different groups upon the DNA binding motif, such as helix-turn-helix motif, zinc finger motif, leucine zipper motif, and helix-loop-helix (HLH) motif. Many of the activators binding sites are located either in DNA regions not associated with histones such as linker regions or nucleosome-depleted regions (NDR), leading to direct access of the activators to the promoters, or in regions having lowest nucleosome occupancy, leading to outcompeting histones by the activators to create open chromatin and binding. Some genes are controlled by global suppressors that create a repressive chromatin environment. Transcription of these genes relies on release of the suppressor instead of binding of activator on promoters. There are also the third types of genes having “open” proximal promoter maintained by architectural factors or DNA sequence element, which makes them constitutively active, thus can be transcribed without activators (for review see Morse, 2007). This kind of activator-independent transcription is referred as basal transcription.

The basal transcription machinery is the core player in transcription, which includes RNA polymerase II and the preinitiation complex (PIC) made of six general transcription factors referred as TFIIA, TFII B, TFIID, TFII E, TFIIF and TFIIH. Assembly of the basal transcription machinery to the promoters is a multi-step process. The first step is that TFIID recognizes and binds to promoter. TFIID is a multicomplex containing the TATA-binding protein (TBP) and 12-15 TBP associated factors (TAFs), being responsible for core promoter recognition, coactivator function, catalysis of protein modification, and targeting to specially acetylated nucleosomes. TAFs recognize core promoter through binding to Inr (TAF2) and DPE (TAF6). They also provide coactivator interfaces for enhancer-binding transcription factors. TAFs show tissue specific promoter selection, for example, TAF4b is expressed in highly differentiated human B cell line and TAF1 and TAF10 are required for cell cycle progression (for review see Hochheimer and Tjian, 2003)). TAF1 has intrinsic histone acetyltransferase (HAT) activity (Mizzen et al., 1996). Interestingly, TAF1 can recognize and
bind to diacetylated H3 and H4 N-terminal trails, which suggests that TFIID can initiate PIC binding even at nucleosome occupied promoters (Jacobson et al., 2000). Upon TFIID binding, the DNA is distorted and thus serves as a physical landmark for the location of an active promoter in the midst of a very large genome. It also brings together DNA sequences on both side of the binding to allow subsequent protein assembly steps, such as the adjacent binding of TFIIB. The N-terminal of TFIIB, named as the B finger, occupies almost the same location of the RNA/DNA hybrid in a transcribing complex. RNA/DNA hybrid containing more than 5 RNA residues has to compete with TFIIB for space in the Pol II saddle. Pol II escapes from promoter for elongation upon RNA victory, otherwise, the transcription initiation is aborted. After TFIIB brings the DNA to a point on polymerase surface then locates the transcription start point close to polymerase active center, TFIIE enters PIC and recruits TFIIH that uses ATP to spry apart the DNA double helix, resulting in formation of a transient bubble. TFIIF interacts with the non-template strand and upstream duplex DNA to trap the bubble, allowing the transcription initiation on template DNA (for review see (Kornberg, 2007)). TFIIH can also phosphorylate Pol II at the C-terminal domain (CTD), changing its conformation so that Pol II is released from the PIC to start transcription elongation.

It has been suggested that transcription is a dynamic and stochastic process rather than static one. Highly mobile transcription factors and chromatin-associated proteins bind to and are released from promoters transiently through a stochastic mechanism known as “hit and run” without any productive result. Transcriptionally productive complexes, which have low mobility, are rarely formed on promoters. The transcription activity happens only when the specific required factors are recruited at appropriate time points (Métivier et al., 2006).

1.3.2 Coactivators and cosuppressors

Transcription activities need not only DNA-binding transcription factors but also additional proteins called co-regulators (coactivators and cosuppressors). Animal cells utilize from 1000 to 10000 transcription regulators. Diverse combinational interaction between DNA-binding transcription factors and co-regulators gives high order complexity to the basal transcription apparatus with limited number of components. Further, cellular signal pathway control of transcription that assures right gene expression under certain cellular environment, is mediated through modification of co-regulators.
Studying the interacting between gene regulators and coactivators / cosupressors is essential to understand transcriptional regulation in eukaryotes.

Coactivators can be divided into two groups: proteins that interact with or are components of the basal transcription machinery, and proteins that modify or remodel the chromatin structure (Näär et al., 2001, Featherstone, 2002). The first group contains the TBP-associated factors (TAFs) and the mediator complex. Mediator, which is conserved in many organisms from yeast to humans, is a big protein complex with more than 20 subunits. It contacts both activators and Pol II to transduce transcriptional information from enhancers to core promoters (Kornberg, 2005, Malik and Roeder, 2005). It is worth noting that mediator not only acts positively but also negatively for transcriptional regulation (Kornberg, 2007). Human mediator was first identified through a stable intracellular thyroid hormone-dependent association with thyroid hormone receptor (TR) (Fondell et al., 1996). Further, TRAP/mediator complex binding of TR through TRAP220/MED1 subunit is necessary for TR-dependent transcription by Pol II (Fondell et al., 1996, Zhu et al., 1997, Yuan et al., 1998). Different activators target specific mediator subunits, for example, Elk-1 and E1A interact with Sur2/MED23 subunit, whereas p53 interacts with TRAP80/MED17 subunits (for review see (Roeder, 2005)). The chromatin-remodeling or -modifying proteins contain two subgroups; one subgroup is involved in histone modification for facilitating the access of activators to DNA, such as Gcn5 (subunit of yeast SAGA complex), PCAF (the mammalian SAGA-like complex), CBP (CREB-binding protein), and p300. The other subgroup is ATP-dependent chromatin remodeling complexes, such as SWI/SNF complex in yeast or their mammalian counterpart BRG1 or BRM (Featherstone, 2002, Spiegelman and Heinrich, 2004). Some of these proteins will be discussed later.

Cosuppressors behave opposite to coactivators. Unlike coactivators that recruit HAT activity, cosuppressors often recruit HDACs (histone deacetyltransferase), resulting in a compacted chromatin that inhibits protein access to promoters; whereas coactivators stabilize PIC, cosuppressors make inhibitory contacts to interfere transcription initiation. Examples of those cosuppressors are the SMRT (silencing mediator of retinoid and thyroid hormone receptor) and N-CoR (nuclear hormone receptor-corepressor) (Privalsky, 2004). One should also bear in mind that since the same modification, e.g. H3K4 methylation, can cause either positive or negative
regulation of transcription in different contexts, identity of a given protein may change between cosuppressor and coactivator under different circumstances.

1.3.3 Chromatin regulations

Chromatin structure of packing DNA into nucleosomes is a significant barrier that has to be overcome for transcription. The nucleosome core contains 147 bp of DNA wrapped 1.65 turns around the histone octamer made of two copies of each H2a, H2B, H3, and H4. The 14 contact points between histones and DNA make it one of the most stable protein-DNA complexes. The dynamics of chromatin structure are closely regulated through multiple mechanisms including histone modifications and chromatin remodelling. These histone modifications include methylation of arginine (R); acetylation, methylation, ubiquitylation, DP-ribosylation, and SUMOylation of lysines (K); and phosphorylation of serines and threonines. Modifications related to active transcription are referred as euchromatin modifications, whereas modifications associated with inactive regions are called heterochromatin modifications (for review see (Li et al., 2007)). Acetylation usually seems to be activating whereas SUMOylation is repressing. Methylation and ubiquitylation carry out variable functions according to sites and context. These modifications are not exclusive from each other; instead, crosstalks happen among them. One modification can sent completely different messages depending on the other histone modifications present.

1.3.4 Histone acetylation

Histone acetylation is traditionally seen as the mark of active transcription. Unlike other histone modifications, it can happen at multiple lysine sites. Histone acetyltransferases (HATs) catalyse the transfer of an acetyl group from acetyle-CoA to lysine residues on the N-terminal tails of the histones (Yang et al., 2004). Two main superfamilies of HAT have been characterized: the GNAT and the MYST families. In mammals, the most well known GNAT (Gcn-related N acetyltransferase) proteins are GCN5 and PCAF (p300/CREB-binding protein-associated factor). GCN5 is involved in acetylation of histone H3 and, to lesser extent, H4. Its N-terminal possesses chromatin substrates recognition function, whereas its C-terminal interacts with the DNA-dependent kinase holoenzyme, which inhibits GCN5 HAT activity through phosphorylation. PCAF functions as HAT and as a coactivator in several processes: myogenesis, nuclear-reporter–mediated activation, and growth-factor-signalled activation. It can also
acetylate non-histone transcription factor such as p53, TFIIE, and TFIIF. P300/CBP is another family of HATs, which acetylate the N-terminal tails of all four core histones. Like PCAF, substrates of P300/CBP include proteins other than histones, such as p53 and HIF2. Other HATs include Hat1, Hat2 and Elp3. Hat1 is shown to be involved in DNA double strand break repair. Elp3 is able to acetylate all four histones. The MYST is named after its founding members MOZ, Ybf2/Sas3, Sas2, and Tip60. Other members include yeast Esa1, drosophila MOF, human HBO1 and MORF. Tip60 participates in specific gene transcriptional activities through local histone acetylation, particularly H4 acetylation. Being similar to p53, it also responses to DNA damage. MOZ and MORF are found transformed in some types of leukaemia. HBO1 may play a role in DNA replication since it interacts with replication factors MCM2 and ORC1. Most HATs exist as multisubunit complexes in vivo. The complexes are usually more active than their respective catalytic subunits alone. In contrast to HATs, HDACs remove acetyl groups from histones, usually creating a repressive chromatin environment for transcription. Like HATs, HDACs cannot bind directly to DNA; recognition of different deacetylation sites mainly depends on the cofactors they form a complex with. (for review see (Verdone et al., 2005, 2006)).

HATs and HDACs target histone through two mechanisms, one is that HATs and HDACs are recruited to histones by proteins binding to specific DNA sequences. The other mechanism depends on prior histone modification serving as the recognitive mark (Clayton et al., 2006), such as methylation at H3K4 site (Barratt et al., 1994, Clayton et al., 2000). Most of acetylation happens at the flexible and highly basic N-terminal tail of histone. However, a lysine (K56) within the core domain of H3 has also been found acetylated (Xu et al., 2005). Several mechanisms of how histone acetylation affects transcription have been brought up. First, the acetylations on lysine residues neutralize charge on histones, thus decreases affinity between histones and negatively charged DNA backbone, which leads to increased nucleosome mobility. On this respect, biological functions of histone acetylation rely on the number of lysines modified. Second, addition of an acetyl group to histone creates a new binding surface for transacting factors possessing a protein module dubbed as bromodomain (for review see (Verdone et al., 2005, 2006)). There are more than 30 bromodomain-containing proteins in human. They selective recognize acetylated histones, for example, Brd2 prefers interaction with H4K12, binds less well to H2B, and does not interact with H3 and H2A; TAF1 recognizes H3 and H4, to
less level H2B, but not H2A; PCAF interacts with H3 and H4, but not H2A and H2B (Kanno et al., 2004). Third, some acetylations directly affect higher-order chromatin structure; acetylation of H4K16 inhibits formation of compacted 30nm fibers since the H4 N-terminal is crucial for contact between nucleosomes (Shogren-Knaak et al., 2006). New insight into histone acetylation suggests it a dynamic process involving rapid acetylation turnover on lysine residues (Clayton et al., 2006). Experiments showed that blocking acetylation turnover using HDAC inhibitor inhibits transcription of some genes (Hazzalin, and Mahadevan, 2005), which might imply histone acetylation and deacetylation affect transcription as continuous circuit and loss of any link will impair machinery of gene expression.

1.3.5 Histone methylation

Compared to acetyltransferases, histone methyltransferases are much more specific. They usually methylate one single lysine on a single site (Bannister and Kouzarides, 2005). Methylations don’t change electrical state of histones. Lysine methylations provide binding of proteins containing chromodomain, tudordomain, or PHD domain. Dogmatically, three methylation sites are implicated in activating transcription: H3K4, H3K36, and H3K79, whereas another three methylation sites are implicated in repressing transcription: H3K9, H3K27, and H4K20 (for review see (Kouzarides, 2007)). This dogma has been challenged in the last several years.

H3K4 is methylated by the Set1 complex in three patterns, mono- (enriched at 3’ end of the gene), di- (in the middle), and tri-methylation (at 5’ end) (Pokholok et al., 2005). Monomethylation occurs at a basal level without association of Pol II. Set1 associated with elongating Pol II at the transcription start region and converts monomethyl to dimethyl and trimethyl (Li et al., 2007). H3K4me is able to bind to various proteins carrying different biological functions. For instance, as mentioned above, H3K4 methylation binds chromodomain-containing protein Chd1, thus recruiting HAT GCN5 for histone acetylation. Besides these positive-acting transcription effector recruitment activities, H3K4me3 is also found associated with negative-acting effector recruitment. In response to DNA damage, H3K4me3 recruits the Sin3-Hdac1 HDAC through binding of the PHD domain of one of the subunits Ing2 (Shi et al., 2006). One explanation about why so many complexes bind to one single PTM (post-translation modification) was proposed by Berger (Berger, 2007). She suggested that positive acting complexes (such as GCN5, MLL, and NuA3) might be
recruited during initiation and elongation, followed by recruitment of negative-acting complexes (JMJD and Sin3-Hdac) during attenuation of transcription. Methylation of H3K9 is implicated in gene silencing by recruiting HP1 (heterochromatin-like protein 1) to the promoters to form pericentromeric heterochromatin (Bannister, Lachner et al., 2001). However, findings recently indicate that H3K9me3 and the γ isoform of HP1 are enriched in the coding region of active genes, and actually increase when transcription is induced (Vakoc et al., 2005, Brinkman, 2006), a phenomenon resembling H3K36. Similarly, methylation of DNA, usually occurring at promoters and genes to repress transcription, is reported to occur frequently across ORFs of many actively transcribed genes. Taken together, H3K9 and DNA methylation may have a similar role as methylated H3K36 in transcription, that is, to reestablish the nucleosome structures over the transcribed DNA region after Pol II passage (Berger, 2007).

So far two classes of demethylases have been identified: the LSD1/BHC10 class and the jumonji class. LSD1 demethylates H3K4me1 and H3K4me2 to repress transcription. However, it demethylates H3K9 to activate transcription when presenting in a complex with the androgen receptor. Jumonji class demethylases remove H3K4me1, H3K4me3, H3K9me2, H3K9me3, H3K36me2, and H3K36me3 (for review see (Berger, Kouzarides, 2007)). The selectivity of demethylases for mono-, di-, and tri-methylation assures a larger functional control of lysine modification (Shi and Whetstine, 2007).

1.3.6 Chromatin remodelling
The histone modifications described above regulate nucleosome dynamics through transient histone dissociation resulting from the electrical contact changes between histones and the DNA backbone. There is another type of dynamic nucleosome regulation concerning histone displacement. Unlike the former changes, which happen on a millisecond time scale, the latter occurs on a time scale of minutes in yeast (Dion et al., 2007). It has been reported that during efficient transcription, 80% of all core histones are removed from transcribed regions of yeast genes. Maximal removal of core histones happens only at high Pol II density, that is, one molecule per 100-150 bp (Kristjuhan and Svejstrup, Schwabish and Struhl, 2004.). With lower Pol II density, partial displacement of histones occurs only with H2A/H2B (Nacheva et al., 1989). This observation also coincides with the nucleosome structure where H3/H4 are located centrally in the nucleosome particle and in
tight contact with DNA (Luger, 2006) whereas H2A/H2B flank the H3/H4 tetramer and bind to DNA less tightly. Numerous proteins are involved in this histone displacement and chromatin remodelling process, including actively transcribing Pol II, histone chaperons, and chromatin-remodeling complexes. For instance, the protein complex FACT (facilitates chromatin transcription), a histone chaperon specially interacting with all core histones, assists pol II to remove H2A/H2B from nucleosomes. Besides this, together with other histone chaperons such as Spt6, Spt2 and Hir proteins, it helps to rebind the H2A/H2B dimer to the nucleosomes in transcribed DNA region after Pol II passage (for review see (Kulaeva et al., 2007)). In transcription of the yeast PHO5 gene, nucleosomes cannot be reassembled in the absence of Spt6, which leads to constitutively active of PHO5 promoter even after removal of transcriptional activators (Pho2 and Pho4) from the promoter (Adkins and Tyler, 2006). ATP-dependent chromatin-remodeling complexes include SWI/SNF, ISWI, RSC, and Mi-2/NuRD. They change location or confirmation of nucleosomes. This activity requires the intrinsic ATPase activity of the common subunit SWI2/SNF2 (for review see (Naar et al., 2001)). Experiments show that recruitment of SNF2 human homolog BRG1 to the IFN-β promoter catalyzes removal of nucleosome 25 bp downstream from the TATA box, thus facilitating binding of general TF (Lomvardas and Thanos, 2001).

1.4 Transcriptional activity of modification by ubiquitylation

Ubiquitylation was long regarded solely as mark for proteasome degradation, but accumulating evidence indicates also nonproteolytic function of Ub. All seven lysine residues in Ub can be used to form isopeptide bonds with substrate proteins or another ubiquitin molecule. Different ubiquitin linkage patterns may serve different or, in some case, still-to-be-defined biological functions. For example, monoubiquitylation has been reported to be involved in endocytosis, transcription, and DNA damage response. Multiple monoubiquitylations are used in endocytosis. Whereas K48 polyubiquitylation leads to proteasomal degradation, K63 polyubiquitylation results in degradation-free modification of protein, which, for instance, is involved in cytokine signalling cascades. K29 polyubiquitylation seems to label proteins for lysosomal degradation (for review see (Crosetto et al., 2006, Herrmann et al., Mukhopadhyay and Riezman, 2007)). The effects of ubiquitylation on transcription will be described from two perspectives as below: ubiquitylation on chromatin and ubiquitylation on transcription factor proteins.
1.4.1 Histone ubiquitylation

The most studied histone ubiquitylation occur on H2A and H2B. There are reports about ubiquitylation of other histones in mammalian cells recently: H1 ubiquitylation by TAF1, H3/H4 ubiquitylation by Cul4-DDB-Roc complex (Pham and Sauer, 2000, Wang et al., 2006). H2B ubiquitylation generally occurs in euchromatin and is indicative of active gene transcription. An ubiquitin moiety is conjugated to lysine 123 (lysine 120 in human) at the C-terminal of H2B, mediated by the E2 conjugating enzyme Rad6/Bre1. Rad6 has a C-terminal cysteine containing catalytic site involved in ubiquitin conjugation and a N-terminal site binding to three different E3 ligases, Rad18, Ubr1, and Bre1, which target different substrate. In the case of histone ubiquitylation, Rad6 binds to Bre1, RNF20/40 and UbcH6 are human homologues of Bre1 and Rad6 respectively. Rad1/Bre1 is recruited to upstream promoter elements by binding to DNA sequence-specific activators. Following phosphorylation of the CTD domain of Pol II by TFIIH subunit kin28, BUR kinase complex associates with Pol II and assists recruitment of the elongation factor PAF that bridges the interaction between Rad6/Bre1 and Pol II. This interaction may bring Rad6/Bre1 into proximity of BUR, which activates the complex by phosphorylating Rad6 on serine 120. Ubiquitylation of H2B regulates subsequent methylation of H3 at lysine 4 and 79. It also promotes removal of H2A/H2B dimer from nucleosomes by the histone chaperon FACT (for review see (Osley, 2006, laribee et al., 2007)). Another consequence of monoubiquitylation of H2B is recruitment of the 19S regulatory subunit of proteasome, which serves as a transcription regulator binding to the coding and 3' end of genes. Chaperon activity of the 19S proteasome is required for H3K4me and H3K79me regulation. The mechanism behind this regulation is still unclear (Ezhkova and Tansey, 2004, Lee et al., 2005, Collins and Tansey, 2006).

Two ubiquitin proteases are found in yeast, Ubp8 and Ubp10. Ubp8 is a subunit of SAGA histone acetyltranferase complex, and it deubiquitylates H2BK123 in a SAGA dependent manner. Deletion of Ubp8 has similar effects on gene expression as H2BK123R mutant, which suggests both ubiquitylation and deubiquitylation are important for transcription (Henry et al., 2003). Ubp10 usually targets H2B in transcriptionally silenced region, like heterochromatin, to keep low level of H2B monoubiquitylation and H3K4me/H3K79me, which may help spreading of silencing factors such as Sir proteins that prefer to bind unmodified histones (for review see (Osley, 2006, Kouzarides, 2007, Laribee et al., 2007)).
H2A ubiquitylation occurs on 10% of histone H2A in mammalian cells but it is not conserved in yeast. H2A is ubiquitylated at lysine 119 by PcG PRC1L (Polycomb-repressive complex-1-like) family regulators and is usually associated with transcription repression. PcG PRC1L complex are factors required for homeotic gene silencing in flies and vertebrates. Human PRC1L have three RING domain subunits, Ring1B, Ring1A, and Bmi1. Ring1B possesses E3 ligase activity towards H2A and Ring1A, which is promoted by Bmi1 (Wang et al., 2004, Cao et al., 2005). Two PcG complexes are responsible for homeotic gene silencing. First, PRC2 with histone methylation activity is recruited to polycomb response elements (PRE) in promoter, and then methylates H3K37, leading to binding of PRC1 to the promoter and, finally, H2A ubiquitylation (Osley, 2006). The contribution of H2A ubiquitylation to gene silencing is still to be investigated. A JAMM/MPN + domain containing H2A deubiquitinase complex named as 2A-DUB or KIAA1915/MYSM1 was defined recently (Zhu et al., 2007). 2A-DUB is found to bind to the p300/CBP-associated factor (p/CAF). In addition, 2A-DUB favours H2A monoubiquitylation in hyperacetylated nucleosomes. These two sets of data suggest a potential coordination of histone acetylation and deubiquitylation (Zhu et al., 2007). Experiments also show that H2AK119R mutant dramatically decreased number of association of linker histone H1 (Zhu et al., 2007), which is consistent with the observation that H2Aub1 enhances H1 association to reconstituted nucleosomes (Jason et al., 2005). Further, reduced H2A monoubiquitylation was shown to increase H1 phosphorylation that is known to promote dissociation of H1 from nucleosomes (de Napoles et al., 2004, Wang et al., 2004). Put together, H2A monoubiquitylation, might be promoted by histone acetylation, enhanced H1 phosphorylation and then disassociation from nucleosomes (Zhu et al., 2007).

1.4.2 Role of UPS in gene expression

The ubiquitin-proteasome system (UPS) regulates gene transcription in several ways (for review see (Muratani and Tansey, 2003)). First, it controls localization of some transcription activators. For example, NF-κB interacts with the inhibitor IκB in cytoplasm. IκB is phosphorylated, ubiquitylated and then degraded upon inflammatory signals, leading to release of NF-κB from its sequestration. The free NF-κB enters the nucleus where it activates transcription (Palombella et al., 2004). Second, UPS can selectively target coactivators thereby regulating the activator activity. The ubiquitin ligase RLIM recognizes both transcription factor LIM and its coactivator CLIM, and specially targets CLIM for degradation (Ostendoff et al., 2002), which
allowing the recruitment of the alternative coactivator, resulting in reprogramming of the transcription process. The third and most common way of UPS to regulate transcription is through controlling activator protein level. It has been noticed that transcriptional potency of some activators is positively correlated with their turnover rate (unstable when active). For instance, proteasome inhibitors that stabilize the estrogen receptor (ER) block estrogen-receptor-mediated transcription. In addition, several steroid-receptor-interacting coactivators, such as Sug1/Trip1, Rsp5/Rpf1, E6-AP and Ubc9, have been identified as components of the UPS (for review see (Von Mikecz, 2006)). Furthermore, it has been demonstrated that degrons, a specific sequence of amino acids that directs the starting place of degradation, in activator largely overlap with certain type of transcription activation domain (TAD) (Salghetti et al., 2000), and components of transcriptional machinery recruited by TAD can promote destruction of activators (Chi et al., 2001, Nelson et al., 2003). Transcription activator can be divided into three classes according to their ubiquitin-dependent proteolysis (for review see (Lipford and Deshaies, 2003)). Degradation of class A activator is completely uncoupled from transcription. Transcription efficiency of class A activator relies on their concentration in the cell. Samples of this class include β-catenin, p53, and HIF-1α. β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β), followed by its ubiquitylation and degradation. Upon Wnt signalling, GSK-3β is inactivated and turnover of β-catenin is shut off, leading to rapid induction of target genes (Yost et al., 1996, Aberle et al., 1997, Hart et al., 1999). Class B activators are degraded only when bound to DNA, and the rate of degradation determines the window of time available to stimulate transcription. The activator binds to DNA and recruits GTF, leading to recruitment of both Pol II and E3 ligase. The activator will be able to start transcription if Pol II comes faster to the promoter than E3, otherwise the activator is degraded and transcription fails. Same as class A activators, activity of class B activators increases when shutting off their turnover, resulting in increase of their concentration. In contrast, degradation of class C activators is necessary for transcription induced by them, so obstruction of their turnover inhibits transcription. The mechanism might be that degradation of the activator disrupts the PIC complex and helps the release of Pol II for elongation, or that clearance of spent PIC and resemblance of a new one is required for each round of transcription, the "single-shot" mechanism (Thomas and Tyers, 2000, Lipford and Deshaies, 2003). Examples of this class are Gcn4 and Myc. Yeast transcription factor Gcn4 binds to promoter, and recruits Pol II. Srb10, a subunit of the Pol II complex, together with
another cyclin-dependent kinases (CDKs) Pho85, phosphorylates Gcn4, leading to its ubiquitylation by SCF$^{Cdc4}$ and proteolysis (Chi et al., 2001). Indeed, this degradation stimulates Gcn4 induced transcription. Moreover, SCF$^{Cdc4}$ mutant and ubiquitin mutant that lack proteolysis function impair transcription of Gcn4 target genes (Lipford et al., 2005).

Apart from the UPS degradation, the proteasome also regulates transcription in a proteolysis-independent manner. Five ATPases of the 19S complex have been linked to transcription either biochemically or genetically. In yeast, the ATPases assist recruitment the SAGA acetyltransferase complex to the Gal1-Gal10 promoter and stimulate interaction between SAGA and DNA-binding transcription activators (Lee et al., 2005). In the study of yeast CDC20 transcription, 19S lid and base components as well as 20S core complex of proteasome are detected at the CDC20 promoter. Mutations in a 19S base subunit cause a modest decrease of CDC20 transcript whereas mutations in 20S subunits have no effect (Morris et al., 2003).

1.5 The transcription factor Myc

1.5.1 The bHLH transcription factors in animals and plants

The basic region/helix-loop-helix (bHLH) proteins constitute a large family of DNA-binding transcription factors with important regulatory roles in the lives of eukaryotic cells. The basic region binds to so called E-box DNA recognition sequences (CANNTG) in regulatory regions of target genes, whereas the HLH domain functions to form hetero- or homo-dimers. The bHLH family can be divided into six groups, A to F, according to E-box binding, conservation of residues in the other parts of the motif, and the presence or absence of additional domains (Atchley and Fitch, 1997, Ledent, 2002). The A-group proteins, which are only found in animals, bind to the E-box with sequence CAGCTG, while proteins of the B-group, which is the most ancient one found in all eukaryotic kingdoms, bind to E-box with sequence CACGTG, which is called G-box in plant. The B-group can be sub-divided into two groups, one containing a leucine zipper, the other without this motif. The leucine zipper motif is involved in homo- and heterodimerization and is characterized by leucines or other hydrophobic residues at every 7th position. Group C proteins have a PAS domain and bind to ACGTG or GCGTG DNA sequences. Hypoxia-inducible factor (HIF) belongs to this group. Group D lacks a basic domain and hence does...
not bind DNA. Group E, containing an orange domain and a WRPW peptide, binds preferentially to N-box sequences CACGCG or CACGAG. Group F has an additional COE domain that is involved in dimerization and DNA binding (for review see (Jones, 2004)).

The B-group bHLH genes can be found in all eukaryotic organisms, including plants. In *Arabidopsis thaliana* it constitutes the second largest transcription factor family, comprising 162 members (Heim et al 2003, Bailey et al 2003, Toledo-Ortiz et al., 2003), only after the biggest MYB superfamily with 190 members (Riechmann et al., 2000). The bHLH genes in plants and animals are significantly diverged in evolution beyond the conserved DNA-binding domain (Buck and Atchley, 2003). The handful of plant bHLH genes studied so far are involved in anthocyanin biosynthesis, phytochrome signaling, globulin expression, fruit dehiscence, and carpel and epidermal development. In contrast to the indispensability of their animal counterpart in development, plant bHLH genes are often redundant and deletion of individual member has limited phenotypic effects. Interestingly, many plant bHLH proteins seem to form networks in similar way as animal bHLH genes. For instance, two related phytochrome-interacting factors (PIFs) in *Arabidopsis*, PIF3 and PIF4, can form both homodimers and heterodimers and all three dimers can bind to the G-box DNA sequence motif CACGTG for subsequent transcriptional regulation (Toledo-Ortiz et al., 2003), which resembles the way of Myc/Max/Mad proteins in animals (see below).

1.5.2 Myc/Max/Mad network

The animal Myc family, which plays an important role in cell proliferation, metabolism, apoptosis and cancer development, is the prototype of the bHLHZip group. In this group are also found the Myc heterodimerization partner Max and the Mad/Mnt that are alternative heterodimerization partners for Max and antagonizers of Myc. With Max in the center, these three types of proteins define the so called Myc/Max/Mad network.
The Myc family has several members, including c-Myc, N-Myc, and L-Myc. The myc oncogene was first found in an avian retrovirus and was named v-myc, which stands for myelocytomatosis, the disease caused by it (SheINESS and Bishop, 1979, SheINESS et al., 1978). Shortly after, a v-myc homologue was isolated in chicken and named c-myc (Vennstrom et al., 1982). c-myc is evolutionally conserved in all vertebrates and some non-vertebrates such as sea star Asteria vulgaris (Walker et al., 1992) and Drosophila (Gallant et al., 1996). Subsequently, two other member of myc family, the N- and L-myc were identified as amplified or highly expressed genes in the childhood tumour neuroblastoma (Kohl et al., Schwab et al., 1983) and in small lung cancer (Nau et al., 1985).

Myc comprises an N-terminal TAD domain and a C-terminal bHLHZip domain. There are two evolutionally conserved sequences named as Myc box 1 and 2 (MB1 and 2) in the TAD domain that are involved in the recruitment of various cofactors. MB1 also harbours several closely located serine and threonine residues that mediate Myc degradation as well as the apoptotic and oncogenic activities of Myc (Sears, 2004, Adhikary and Eilers, 2005, Hemann et al., 2005). Surprisingly, MB 1 is necessary for cell transformation but not for transcription (Kato et al., 1990, Oster et al., 2003, Herbst et al., 2005). Myc without MB 2 is defective for cell transformation, apoptosis, differentiation, and largely impaired in transcription activity (Stone et al., 1987, Freytag et al., 1990, Evan et al., 1992, Cowling et al., 2006). The precise role of MB2 in Myc function is still unclear and it was reported to be dispensable for Myc-driven cell proliferation (Cowling and Cole, 2007a). Recently, two other Myc boxes, MB 3 and MB 4, were identified, and both seem to be required for cellular transformation. In some assay, deletion of MB 3 was reported to promote Myc-induced apoptosis while deletion of MB 4 promoted G2 arrest, and both deletions effected Myc transactivation much less than MB 2 deletion. MB 4 also seems necessary for apoptosis. MB 1, 3, 4, however, are not required for cell proliferation (Xiao et al., 1998, Herbst et al., 2005, Cowling et al., 2006)). The Myc protein further contains a nuclear localization site (NLS) that is important for nuclear import and a central acidic domain with several phosphorylation sites that might be important for some unknown aspects of
its function. The C-terminal bHLHZip domain of the Myc protein is required to heterodimerize with the bHLHZip protein Max (Luscher and Larsson, 1999). Heterodimerization with Max is crucial for Myc DNA binding to the CACGTG E-box and execution of all Myc functions. There is no evidence of Myc homodimers in cells. Max is a ubiquitously expressed phosphoprotein with several isoforms resulting from differential splicing. The most commonly expressed 21 and 22 kDa isoforms have differential DNA binding and transcriptional activities (for review see (Hurlin and Huang, 2006)). Unlike Myc, Max can form homodimers that bind to the E-box DNA, and the binding can be inhibited by phosphorylation on its N-terminal serine residues (Kato et al., 1992, Berberich and Cole, 1992, Koskinen et al., 1994). Max also binds to other proteins that antagonize Myc activities. These proteins include Mnt (Hurlin et al., 1997), Mad1-4 (Mad1, Mxi1, Mad3, and Mad4) (Ayer et al., Zervos, et al., 1993, Hurlin et al., 1995) and Mga (Hurlin et al., 1999). Mnt, Mad1-4, and Mga are all transcriptional repressors. They interact with Sin3A and Sin3B corepressors through the N-terminal Sin3-interaction domain (SID) (Ayer et al., Schreiber-Agus et al., 1995), which in turn recruits a number of different proteins, including HDAC1 and HDAC2 (for review see (Ayer, 1999)). The Max/Myc dimers are usually formed in proliferating cells whereas Max/Mad in resting or differentiated cells (Ayer and Eisenman, 1993). Max/Myc and Max/Mad heterodimers are found essentially mirroring the expression of Myc and Mad proteins respectively. Myc also interacts with a number of additional proteins through either C- or N-termini. Examples of the C-terminal interacting proteins 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., 1998). Examples of the N-terminal interacting proteins are Skp2 (von der Lehr et al, Kim et al., 2003), p107 (Beijersbergen et al., Gu et al., 1994), Bin1 (Sakamuro et al., 1996), TRRAP (McMahon et al., 1998), and TBP (Hateboer et al., 1993). Some interactions related to this thesis will be described later.

1.5.3 Myc in transcription

Myc binds very ubiquitously throughout the genome. There are tens of thousands of genomic Myc-binding sites, encompassing up to 15% of all genes (Orian et al., Fernandez et al., 2003, Cawley et al., Patel et al., 2004, Bieda et al., 2006). These binding sites are often located in the regions close to the CpG islands (Fernandez et al., 2003), the mark for DNA methylation resistance, and are rich of H3K4/K79 methylation and H3 acetylation.
The fact that the number of binding sites exceeds the number of Myc molecules implies that Myc binds to these sites temporarily and regulates transcription on a hit-and-run manner (Adhikary and Eilers, 2005). In spite of the important roles that Myc plays in cell biology, the induction of transcription by Myc is quite weak. Indeed, Myc activates the majority of target genes only by two-fold (Patel et al., 2004). Besides its role in RNA Pol II transcription, c-Myc associates with E-boxes in ribosomal DNA regulatory region and activates RNA Pol I transcription (Arabi, et al., 2005). Further, Myc also binds to Pol III-transcribed genes and induces Pol III activity (Gomez-Roman et al., 2003).

An important role of Myc seems to be recruitment of coactivators for transcription; TRRAP (Transactivation/transformation Associated Protein) is one of the most studied among them. TRRAP is a large protein (3830 amino acids) with limited homology to the phosphatidyl-inositol-3-kinase (PI3K) that binds Myc through the conserved domain MB2. TRRAP was shown to be a core subunit of GCN and Tip60 HAT complexes (McMahon et al., 1998). In accordance with this, Myc has been demonstrated to recruit HAT activity (McMahon et al., 1998, Liu et al., 2003). The Tip60 complex acetylates H2A/H4 histones. In vivo, H4 acetylation is more associated with Myc target gene activation than H3 acetylation (Frank et al., 2001). The other TRRAP complex binding to Myc is a complex containing the Swi/Snf-related p400 protein. The consequence of this binding is unclear since p400 is devoid of HAT activity (Fuchs et al., 2001). Other HATs interacting with Myc are the CREB-binding protein (CBP) and p300. They are recruited by Myc to promoters and enhance Myc-induced transcription. The binding site of CBP/p300 to Myc is still controversial. One report claimed that they bind through the C-terminal domain of Myc whereas the other mapped binding site to the N-terminal 100 amino acids (Vervoorts et al., 2003, Faiola et al., 2005). Recruitment of the HATs mediated by TRRAP suggests that Myc plays a role in chromatin structure regulation. Indeed, about half of Myc-bound sites were intergenic (>10 kb away from transcriptional start sites) (Bieda et al, 2006), implying a potential non-transcriptional function for Myc on chromatin. Moreover, GCN5 is identified not only a coactivator but also a target gene of Myc (Knoepfle et al., 2006), which makes a positive feedback loop between Myc-induced transcription and Myc-induced chromatin regulation. Myc also binds to two ATPase domain-containing proteins found in Tip60 and several other chromatin remodeling complexes independent of TRRAP; these are Tip48 and Tip49 (Wood et al., 2000). A point mutation in Tip49 that abolishes its
ATPase activity inhibits Myc-induced transformation but not proliferation, and enhances Myc-induced apoptosis (Wood et al., 2000, Dugan et al., 2002). Some Myc target genes are reported to be transcribed independent of increased histone acetylation but still dependent on TRRAP, such as *Tert*. Some are independent of both, such as *Cad*, *Cdk4*, and *Hsp60* (Eberhardy et al., 2000, Nikiforov et al., 2002). Interestingly, Pol II was observed being stalled at the *Cad* promoter in the absence of Myc. It was also found that two components of Pol II CTD kinase P-TEFβ, Cdk9 and cyclin T1, interact with c-Myc through the TAD domain. Myc may therefore activate *Cad* transcription by stimulating promoter clearance, perhaps via recruitment of P-TEFβ (Eberhardy and Farnham, 2000).

Another coactivator binding Myc through MB2 is Skp2. Skp2 is also an ubiquitin E3 ligase of Myc. Binding of Skp2 leads to Myc ubiquitylation and degradation as well as enhanced Myc target gene expression. Further, Myc-driven transcription was shown to be Skp2 dependent. Moreover, Myc-dependent association of Skp2, ubiquitylated proteins, and subunit of the proteasome to c-Myc target gene cyclin 2D was demonstrated in vivo (von der Lehr et al., Kim et al., 2003). Another coactivator linking Myc ubiquitylation with Myc-induced transactivation is the E3 ligase HectH9 (also called ARF-BP1, ARF-binding protein 1). HectH9 promotes the formation of lysine 63-linked polyubiquitin chain at six lysine residues in the C-terminal of Myc in vivo and in vitro. The K63-linked chains do not lead to proteasomal degradation of Myc, but seem to be required for transcription of Myc target genes, recruitment of the CBP/p300 coactivator, and Myc-induced cell proliferation (Adhikary et al., 2005). The binding of Skp2 to Myc is competed by the forkhead-associated (FHA) domain-containing protein SNIP1. SNIP1 was shown as an inhibitor of both TGF-β and NF-κB signaling pathway. It blocks the interaction of Smad4 and p65/Rel A, respectively, with CBP and p300 (Kim et al., 2000, 2001, Fujii et al., 2006). SNIP1 is also a coactivator of Myc that functions to both enhance Myc transcriptional activity and inhibit the UPS-mediated Myc degradation. The N-terminal of SNIP1, which can interact with the CBP/p300 complex, is required for this coactivator function. Interestingly, both Skp2 and SNIP1 are oncogenes but they seem to antagonize each other for Myc degradation or stabilization. They are differentially expressed during cell cycle: SNIP1/c-Myc complexes predominate in G1, whereas Skp2/c-Myc complexes predominate in late G1/S (von der Lehr et al., 2003, Fujii et al., 2006), which may suggest that Myc regulates different sets
of genes incorporation with different coactivators at different phases of cell cycle (Larsson, 2006).

In addition to its role as a transcriptional activator, Myc is also able to repress transcription for instance of the cell cycle arrest genes, p15<sup>ink4a</sup>, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> (Gartel and Shchors, 2003), when interacting with Miz1. Miz1 binds to initiator (Inr) like elements in the promoters of these genes and upregulates their expression. Myc suppresses this transcription activating function of Miz1 by interacting with it through the C-terminal HLH domain (Herold et al., 2002, Peukert et al., 1997, Seoane et al., 2001, Staller et al., 2001, Wanzel et al., 2003, Wu et al., 2003). Recently, Miz1 was shown to inhibit ubiquitylation of Myc by HectH9, perhaps by competing with the binding of c-Myc for this E3 ligase, thereby antagonizing the ubiquitylation -dependent transcription activity of Myc (Adhikary et al., 2005). Another protein that negatively affects Myc transactivation activity is the tumour suppressor alternative reading frame protein ARF/p19<sup>Arf</sup>. ARF is known to activate p53 by suppressing either Mdm2-mediated or HectH9–mediated p53 ubiquitylation, resulting in inhibition of Myc induced tumorigenesis (Sherr et al., 2001, Chen et al., 2005, Honda and Yasuda, Tao and Levine, Weber et al., 2003). ARF was recently found on Myc dependent promoters via direct binding to Myc, and it inhibits transactivation of some Myc target genes such as eIF4E, nucleolin, hTERT, Cdk4 and Cul1, but does not affect Myc-induced repression of GADD45 and p15<sup>ink4a</sup> (Datta et al., Qi et al., 2004). This function of ARF is independent of p53 expression.

As mentioned above, Max/Mad represses transcription by recruiting HDACs. Knockdown of Mad leads to hyperproliferation of cells or restoration of growth in myc−/− cells. Mad deficient cells, like Myc overexpressed cells, can be transformed by the Ras oncogene. Moreover, some Myc target genes can be induced by Mad knockdown, which provides a strikingly different view of Myc-mediated gene regulation: Myc may not activate gene transcription but disrupt Mad-mediated gene repression (for review see (Cowling and Cole, 2006)).

1.5.4 Myc stabilization and degradation
Myc protein has very short half-life, about 20-30 minutes. Myc stabilization and degradation is regulated via post‐translational modifications. Several phosphorylation sites have been detected in the N-terminal of Myc. Two sites in the MB1 domain of Myc have been investigated intensively; these
are Threonine58 and Serine62. They are also hotspots for mutation in v-Myc and c-Myc proteins from human Burkitt’s lymphoma (Bhatia et al., 1993, Smith-Sørensen et al., 1996). T58 and S62 are followed by proline residues in c-Myc, which makes them potential targets of proline-directed kinases, such as GSK3, MAP kinases and Cdks.

Three kinases are known to phosphorylate Myc on S62; these are extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and Cdk1 for N-Myc (for review see (Hann, 2006)). The ERK-mediated S62 phosphorylation can be induced by oxidative stress and mitogenic stimuli (Benassi et al., Vogel et al., 2006, Sears, 2004, Wang et al., 2006b). Upon mitogenic stimulation, the Ras-Raf signalling pathway activates MAPK/ERK kinase 1 or 2 (MEK1/2) dependent on types of stimuli and duration of stimulation, which in turn induces ERK, followed by S62 phosphorylation. JNK is activated through another Ras-dependent pathway, the PI3K-Rho-ROCK pathway. Ras sequentially activates PI3K, RhoA/C and Rho kinase (ROCK). Inhibition of ROCK prevents c-Myc phosphorylation (Watnick et al., 2003). ROCK induces MAPK/ERK kinase kinase-1, resulting in JNK activation (Zhang et al., 2005). All three isoforms of JNK are able to phosphorylate S62 on Myc (Noguchi et al, 1999); JNK1 is more involved in stress-induced apoptosis whereas JNK2 in more involved in cell cycle regulation (MacCorkle and Tan, 2005). Besides S62, JNK can also phosphorylate another site on Myc N-terminus, S71 (Noguchi et al., 1999). The third S62 kinase is Cdk1. In vitro, Cdk1 can phosphorylate c-Myc at S62 (Lutterbach and Hann, 1994). In complex with Cyclin A or B1, Cdk1 phosphorylates N-Myc S62 in G2/M phase of the cell cycle (Sjostrom et al., 2005). S62 phosphorylation stabilizes the Myc protein (Sear et al., 2000). A prior phosphorylation of S62 is required by phosphorylation of T58 (Lutterbach and Hann, 1994) carried out by GSK3 (Gregory et al., 2003). GSK3β is inhibited by PI3K/Akt pathway, through which PI3K induces the Akt kinase that phosphorylates and inactivates GSK3β (Liang and Slingerland, 2003).

Myc is degraded through the UPS (Flinn et al., Gross-Mesilaty et al., 1998, Salghetti et al., 1999). Three E3 ligases, SCF^{Skp2}, SCF^{Fbw7/Cdc4}, and HectH9, have been shown to ubiquitylate Myc. HectH9 conjugates K63-linked ubiquitin chain on its C-terminal domain of Myc, which does not lead to proteolysis. Skp2 and Fbw7/Cdc4 interact with the N-terminal domain of Myc. Ubiquitylation by SCF^{Skp2} leads to both degradation of Myc and activation of Myc target genes whereas ubiquitylation by SCF^{Fbw7/Cdc4}
seems to lead to mere degradation. Skp2 binds to Myc through two regions, MB2 and amino acid 379-418, then ubiquitylates Myc (Kim et al., Von der Lehr, 2003) efficiently on the amino acid 129-147 where one lysine K144 is located (Kim et al., 2003). There is no evidence of dependence on phosphorylation for Skp2-mediated ubiquitylation of Myc. In contrast, Fbw7/Cdc4-mediated ubiquitylation and degradation of Myc is dependent on T58 phosphorylation (Welcker et al., 2004). Sears and colleagues suggested that ubiquitylation and degradation of Myc is dependent on T58 phosphorylation followed by S62 dephosphorylation (Sears et al., 2000). Consistent to this model, inhibition of protein phosphatase PP2A that dephosphorylates S62 but not T58 significantly inhibits c-Myc proteolysis. Further, Pin1 prolyl isomerase was suggested to promote c-Myc proteolysis via catalyzing the isomerization of proline residues to enhance S62 dephosphorylation by PP2A (Yeh et al., 2004). Recently, the deubiquitylating enzyme, Usp28, was shown to target c-Myc (Popov et al., 2007). The Fbw7 isoform Fbw7α was suggested to bridge the interaction between Myc and Usp28, after which the latter deubiquitylated Myc, leading to its stabilization. The Fbw7γ isoform, on the other hand, does not have this bridging function and can therefore ubiquitylate and degrade Myc (Popov et al., 2007).

1.5.5 Biological function of Myc

Myc effects cell cycle progression

Myc starts to appear at the early G1 phase during cell cycle and targets various key cell cycle genes for G1/S checkpoint passage. c-Myc is sufficient to overcome the G1/S checkpoint and induce cells into S phase in absence of mitogenic stimuli (Eilers, 1999). Moreover, Rat 1a myc-/- cells exhibit a much longer doubling time (50 hours) than wild type cells (20 hours) with prolonged G1 and G2 phases while c-Myc deficient MEFs exhibit an even slower doubling time (100 hours) compared to wild type MEFs (20 hours) (de Alboran et al., 2001, Mateyak et al., 1997). CHIP assays show that c-Myc binds to E-box in promoters of cyclin D1, cyclin D2, Cdk4, and cyclin B1 (Bouchard et al., 2001, Fernandez et al., 2003, Herneking et al., 2000, Menssen and Herneking, 2002). Also, the phosphatase Cdc25 that removes inhibitory phosphorylation of Cdk2 is also identified as Myc target gene (Galaktionov et al., 1996). In addition, Myc directly represses transcription of Cdk inhibitor genes such as p15\textsuperscript{ink4b}, p21\textsuperscript{cip1}, and p27\textsuperscript{kip1} (Claassen and Hann, 1999, Gartel et al., 2001). Transcription of the Cks1 component of the SCF\textsuperscript{Skp2} complex, the E3 ligase for p27\textsuperscript{kip1} ubiquitylation
and degradation, is induced by Myc (Keller et al., 2007). All the Myc functions mentioned above lead to activation of the cyclin E/Cdk2 kinase complex, which in turn phosphorylates pRb, resulting in E2F activation. Besides, Myc is able to activate the expression of E2F1, E2F2, and E2F3 genes independent of Cdk kinase activities. Induction of E2F2 and E2F3 by Myc could be direct, since a series of Myc binding sites are essential for induction of these two genes (Sears et al., 1997, 1999, Leone et al., 1997, Adams et al., 2000). Interestingly, E2F2 and E2F3 are essential for Myc to induce S phase entry (Leone et al., 2001). Another central player of cell proliferation, the pRb protein, has been shown to be inhibited by the HLH protein Id2 that is a positively transactivated target gene of c-Myc (Lasorella et al., 2000).

**Myc in cancer**

Co-transfection of Ras and myc oncogenes into REFs leads to formation of transformed foci containing tumorigenic cells (Land et al., 1983). Myc-family genes are deregulated in a broad range of cancer such as Burkitt lymphoma, neuroblastoma, breast cancer, colon, cervical, small-cell lung carcinomas, osteosarcomas, glioblastomas, melanoma and myeloid leukaemias (Dang, Nesbit et al., Schlagbauer–Wadl et al., 1999, Henriksson and Luscher, 1996). As described above, Myc stability is regulated by Ras oncogene. Besides, some Myc target genes involving in cell proliferation are controlled by Ras through regulation of FOXO family of transcriptional factors that repress expression of these genes by binding to their promoters thus inhibiting formation of PIC (Bouchard et al., 2004). Similar to GSK3, FOXO factors can be phosphorylated by AKT, resulting in their nuclear export. c-Myc is also induced by the Wnt signaling pathway that activates the downstream TCF/β-catenin transcriptional factor complex on the c-Myc promoter (He et al., 1998). A recent report demonstrated that c-Myc can activate the Wnt pathway (for review see (Cowling and Cole, 2007)). Interestingly, two of the most expressed genes by c-Myc in mammary epithelial cells are the Wnt pathway inhibitors DKK1 and SFRP1 (Cowling et al., 2007).

In contrast to its transformation capability, Myc can also induce apoptosis through two independent pathways. One is the p19Arf pathway through which Myc stabilizes p53. p19Arf is directly regulated by E2F that is a downstream factor of Myc (for review see Adhikary and Eilers, 2006). Second, Myc promotes release of cytochrome c independent of Arf/p53 (Juin et al., 1999). It is reported that mutation at S71 of c-Myc results in low
apoptotic potential and low level of cytochrome c release (Chang et al., 2000), suggesting a role of S71 phosphorylation in Myc-induced apoptosis. Experiments also showed that c-Myc expression is essential for activation of the pro-apoptotic protein BAX (Juin et al., 2002). During apoptosis, various BH3-Only proteins induce BAX dimerization and insertion into outer mitochondrial membrane, resulting in release of cytochrome c through creation of alteration of membrane pores (for review see (Pelengaris et al., 2002)).

**Myc in cell growth and differentiation**

Cell division requires not only DNA replication but also doubling of proteins and cellular organelles. The first correlation between Myc and the cell growth was the discovery that the rate-limiting translation factors eIF4E and eIF2α are c-Myc target genes (Coller et al., 2000, Jones et al., 1996). Diminished expression of Drosophila dMyc results in smaller but normally developed flies whereas overexpression of dMyc leads to larger cells without significant change in cell dividing rate (Johnston et al., 1999). In particularly, dMyc overexpression increases both the amount of pre-rRNA and nucleolar size, resulting in increased ribosome activity (Grewal et al., 2005). dMyc transcriptionally regulates ribosome biogenesis, therefore enhances protein translation within cells (for review see (de la Cova and Johnston, 2006)). Evidences of Myc in growth control also found in mammalian cells: c-Myc overexpression in B lymphocytes of transgenic mice results in cell growth without cell division (Iritani and Eisenman, Schuhmacher et al., 1999). c-Myc was reported to associate with ribosomal DNA and activate transcription of rRNA genes by RNA polymerase I (Arabi, et al., Grandori, et al., 2005).

In addition to promoting cell proliferation Myc often inhibits cell differentiation. It has been shown that Myc is down-regulated during differentiation and, constitutive overexpression of Myc blocks differentiation in various cell types, including neuroblastoma, myeloid cells, erythroleukemia cells and teratocarcinoma cells (Coppola and Cole, 1986, Freytag, 1988, Gonda and Metcalf, 1984, Lachman and Skoultchi, 1984, Larson et al, 1988, Thiele et al., 1985). This inhibitory function of Myc can be achieved by the repression of certain genes important for differentiation; for example, Myc represses C/EBPα that induces differentiation in 3T3-L1 adipocytes (Mink et al., 1996). The promyelocytic leukemia protein PML4 can induce differentiation by destabilizing Myc when binding to it, which followed by reactivation of Myc repressed genes (Buschbeck et al., 2007).
However, there are exceptions. Habib and colleagues showed that Myc is necessary to stimulate both proliferation and differentiation in primary B cells together with amplified calcium signaling (Habib et al., 2007).

Members of the Mad family, antagonists of Myc, are often expressed in differentiating or quiescent cells. Cells in the mad1-/- and mxi1-/- mice display an enhanced proliferative capacity (Foley and Eisenman, 1999). Increase of both mRNA and proteins level of Mad1 and Mxi1 are detected after induced differentiation in hematopoietic cells (Ayer and Eisenman, Zervos et al., 1993, Larsson et al., 1994). Increased expression of Mad1 proteins leads to a shift from Myc/Max complex to Max/Mad complex on promoter binding of target genes, such as hTERT and cyclin D2 (Ayer and Eisenman, 1993, Hurlin et al., 1995, Larsson et al., 1997, Bouchard et al., Xu et al., 2001). Further, MAD1 and p27Kip1 were reported to cooperate to promote terminal differentiation of granulocytes and to inhibit Myc expression and cyclin E-Cdk2 activity (McArthur et al., 2002). However, just like for Myc, there may be exceptions since Mad 1 inhibits cell growth and proliferation but does not seem to promote differentiation in human U-937 monoblasts (Hultquist et al., 2004).

1.6 Signaling pathways in the cell

The cell and its extracellular environment communicate via a variety of signaling pathways that mediate the cellular response up to environmental changes. There are at least seven major signalling pathways that control cell fate decision in animals: Wnt, TGF-β, nuclear receptor, Jak/STAT, hedgehog (Hh), receptor tyrosine kinase (RTK), and Notch (Gerhart, 1999). They regulate target genes expression in response to the binding of a signalling ligand to the pathway’s receptor. Three principles underlie the signalling pathway regulation. First, “activator insufficiency”: signal-regulated transcription factor alone is not able to strongly activate gene expression, which assures that any given signalling pathway is not sufficient to activate transcription of all its target genes. Second, “cooperative activation”: robust expression of target genes needs combination of signal-regulated factors and cell-or tissue-specific local activators. Third, “default repression”: signalling pathway target genes are by default repressed in absence of signalling (Barolo and Posakony, 2002). Some signalling pathway related to this thesis will be discussed as followed.
Interferons (IFNs) are proteins that belong to the large class of glycoproteins known as cytokines. They are produced by immune system cells in response to the presence of double-stranded RNA, a key indicator of viral infection. Interferons have potent antiviral, growth inhibitory, and immunomodulatory functions. Human interferons can be divided into three classes according to the receptors they bind to. Type I interferons, including IFN-α, IFN-β and IFN-ω, bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains (Liu, 2005). Type II interferon has only one member, IFN-γ, binds to interferon-gamma receptor (IFNGR) made up of two subunits IFNGR1 and IFNGR2 (Bach et al., Pestka et al., 1997). Type I interferons are present in most cells whereas IFN-γ is mainly secreted by T helper type I (Th1) lymphocytes and natural killer (NK) cells. Type III interferons consist of three IFN-λ molecules referred as IFN-λ1 (IL29), IFN-λ2 (IL28A) and IFN-λ3 (IL28B) (Vilcek, 2003). They signal through a receptor complex comprising IL10R2 and IFNLR1 (Bartlett et al., 2005).

IFN-γ primarily signals through the JAK-STAT pathway. The players in this pathway include the Janus kinase family (JAK 1-3 and TYK2) and the signal transducers and activators of transcription proteins, STATs (STAT 1-6, including STAT5a and STAT5b). Biologically active IFN-γ is a noncovalent homodimer that binds to the IFNGR1 receptor. Upon ligand binding, JAK2 is autophosphorylated and then activated, and it in turn transphosphorylates JAK1. Activated JAK1 phosphorylates tyrosine residues Y440 on each chain of IFNGR1, resulting the formation of a docking site for the SH2 domain of STAT1. After being recruited to the receptor, STAT1 is phosphorylated on the Y701 residue, perhaps by JAK2. Phosphorylated STAT1 then dissociates from the receptor and enter the nucleus to activate or repress IFN-γ target genes, which are mostly transcription factors (for review see (Schroder et al., 2004)). The first wave of IFN-γ target gene expression occurs with 15-30 minutes after IFN-γ treatment (Kerr and Stark, 1991).

The other IFN-γ induced pathway is the PI3K pathway. Activation of PI3K leads to production of phosphatidylinositol (3,4,5) P3 or the PIP3 second messenger. PIP3 recruits PH-containing proteins like PDK and AKT to the membrane. IFN-γ induced activation of PI3K and AKT is essential for phosphorylation of the serine residue S727 on STAT1 that is required for its maximal transcriptional activity towards target genes (Decker and
Growth inhibition is a common effect of IFN-γ on cells. IFN-γ usually arrests cell cycle at the G1/S phase, where they may exit cell cycle and enter into G0. IFN-γ inhibits proliferation mainly by inducing CKIs like p21cip1 and p27kip1 (Chin et al., 1996, Mandal et al., 1998, Takami et al., 2002). It also represses c-Myc expression by either suppressing pRb phosphorylation or promoting Mad expression (Dey et al., 1999, Raveh et al., 1996). Anti- or pro-apoptotic function of IFN-γ is dependent on the level of activate IFNGR receptors. Highly activated IFNGR receptors lead to high expression of IFN-regulatory factor-1 (IRF-1) that is required for IFN-γ induced apoptosis (for review see (Schroder et al., 2004)). Recent report shows that 60% of all deregulated genes during c-myc and Ras induced transformation are reverted to the normal levels by IRF-1 (Kröger et al., 2007). The other IFN-γ induced proapoptotic genes include PKR, DAPs, cathepsin D, Fas, and TFN-α receptor (Schroder et al., 2004).

1.6.2 Retinoic acid (RA)

Retinoid refers to the naturally occurring compounds derived from vitamin A. Retinol is taken up from blood and bound to cellular retinol-binding protein (CRBP) in cytoplasm, hence metabolized by the retinol dehydrogenase (RoDH) to retinal. Retinal is then metabolized by retinaldehyde dehydrogenases (RALDHs). Retinoids are key regulators of differentiation, proliferation, and inflammation. RA has two isoforms, all-trans-RA and 9-cis-RA. They bind to nuclear transcription factors, the RA receptors RARs (RARα, β, and γ) and the retinoid X receptors RXRs (RXRa, β, and γ). RARs and RXRs act as heterodimers and recognize the RA-response elements (RAREs) on RA-responsive genes (for review see (Maden, 2002)). In absence of ligand, RAR/RXR represses gene expression by recruiting HDACs via corepressors (CoR) and Sin3 (Glass and Resenfeld, 2000). Upon ligand binding, a conformational change occurs in ligand binding domain, which resulting in dissociation of CoR and binding of coactivators (CoAs) from gene promoter. An association between vitamin A/ retinoic acid deficiency and carcinogenesis has been known since 1925 (Wolbach and Howe, 1925). RA induces cell cycle arrest and differentiation via degradation of Skp2 and stabilization of p27kip1 in several cancer cell lines (Borriello et al., 2006, Dow et al., 2001, Nakamura et al., 2003, Zancai et al., 2005).
2 Aims of this study

The general objective of this thesis is to gain more knowledge about the function and regulation of the transcription factor and onco protein Myc. Further understanding of how Myc is regulated and how Myc regulates its target genes during different cellular processes may potentially lead to the development of new therapeutic strategies for treatment of cancer.

The thesis can be divided into one animal and one plant part. The animal part is based on previous findings from our group, that growth inhibitory signal, like IFN-γ, can affect Myc function post-translationally. One of the aims of the thesis is to elucidate the mechanism(s) behind this anti-Myc activity. Another part of the thesis addresses the question how certain cell cycle-regulated transcription cofactors for Myc, such as the E3 ubiquitin ligase Skp2, which has been described previously in the group, promotes Myc-driven G1/S transition.

The basic mechanisms of cell cycle regulation in plants are similar to those in animals. In the plant part of this thesis, we are interested in finding out whether Myc-related bHLHZip proteins can be found in plants and whether they carry out similar functions as in animals.

2.1 Specific Aims

- To investigate whether combined RA+IFN-γ treatment affects the expression and/or activity of members of the Myc/Max/Mad network in N-myc-amplified neuroblastoma cells (paper I).
• To elucidate the mechanism(s) by which IFN-γ counteracts Myc activity, how this is linked to the IFN-γ-induced overall reduction in Myc phosphorylation and to identify the targeted phosphorylation sites (paper II).

• To shed light on the interplay between Myc, Skp2, p27Kip1 and cyclinE/Cdk2 in Myc-induced transcription of target genes during the G1/S cell cycle transition (paper II, III).

• To investigate whether Myc-related bHLH proteins can be found in plants and whether they carry out similar functions as in animals (paper IV).
3 Results and discussion

3.1 Combined IFN-γ and retinoic acid treatment induces differentiation and targets the N-Myc/Max/Mad1 network in N-myc-amplified neuroblastoma cells (paper I)

Neuroblastoma (NB) is one of the most common solid childhood tumours and originates from sympathetic neuroblasts of the peripheral nervous system. It occurs primarily in children under 5 years of age with a median age at diagnosis of 18 months, and accounts for 7-10% of cancers and 15% of childhood cancer deaths. NB tumours usually locate to the adrenal gland and the sympathetic ganglia of the abdomen, neck, thorax, and/or pelvis and can spread to regional lymph nodes, bone and bone marrow (for review see (Brodeur, 2003)). Neuroblastoma tumours can be divided into several different types: benign tumours, lower stage malign tumours, highly aggressive/often untreatable tumours, and tumours differentiating spontaneously despite of their metastatic spread (Brodeur et al., 1993). N-myc amplification, ploidy, deletions or loss of heterozygosity of chromosome 1p, and gains at chromosome 17q have been established as diagnostic and prognostic markers for neuroblastomas. N-myc amplification occurs in 25% of all neuroblastomas and in 40% of advanced stage cases, strongly correlating with advanced disease, drug resistance, and poor outcome (Brodeur, 2003, Cohn and Tweddle, 2004, Schwab, 2004). The ectopic expression of N-myc in non-amplified neuroblastoma cell lines results in enhanced proliferative potential whereas neuroblastoma cell lines that are transfected with N-myc antisense (AS) DNA oligos exhibit growth inhibition and a more differentiated phenotype (Cohn and Tweddle, 2004, Strieder and Lutz, 2002). Further, targeted expression of an N-myc transgene to
neural crest cells has been reported to cause neuroblastoma-like tumours in transgenic mice (Weiss et al., 1997).

Retinoic acid (RA) is known to induce differentiation to some extent in N-myc amplified neuroblastoma cell lines, often accompanied by decreased expression of N-Myc (Schwab, 2004, Strieder and Lutz, 2002). We and others have shown that IFN-γ strongly enhances RA induced differentiation and growth inhibition of N-myc amplified human neuroblastoma cells in vitro (Cornaglia-Ferraris et al., 1992, Guzhova et al., 2001, Wada et al., 1997, Wuarin et al., 1991). In order to investigate how combined RA+IFN-γ treatment affects expression and/or activity of members of the Myc/Max/Mad network, the N-myc- amplified human neuroblastoma cell lines LA-N-5 and IMR-32 were treated with RA, IFN-γ, or their combination, after which differentiation assays, immunoprecipitation (IP), Western blot, Northern blot, RT-qPCR, and chromatin immunoprecipitation (CHIP) assays were performed.

In the differentiation assays, we found that IFN-γ strongly enhanced RA-induced differentiation of these cell lines, which is consistent with previous reports. The protein level of N-Myc was substantially decreased after combined RA+ IFN-γ treatment in both cell lines whereas RA alone only modestly reduced N-Myc levels in LA-N-5 cells. In contrast to the previous finding that RA+ IFN-γ treatment decreases N-Myc mRNA levels to almost 30% in L-A-N-5 cells (Guzhova et al., 2001), the N-Myc mRNA levels were only slightly reduced in IMR-32 cells in response to RA+ IFN-γ treatment, which suggests that RA+ IFN-γ downregulates N-Myc protein levels at a posttranslational level in these cells. Another interesting observation was that RA+ IFN-γ treatment increased the expression of Mad1 in both IMR-32 and LA-N-5 cells, whereas the effects of either agent alone varied between the cell lines. Expression of Mad1 mRNA followed the same pattern as Mad1 protein levels in IMR-32 cells, implying that Mad1 is regulated transcriptionally upon RA+ IFN-γ treatment. Neither protein nor mRNA levels of Max changed evidently after the different treatments. Taken together, RA+ IFN-γ affects the N-Myc/Max/Mad network by decreasing N-myc mRNA (LA-N-5 cells), decreasing N-Myc protein levels and increasing Mad1 mRNA.

The amount of Max/N-Myc heterodimers was also shown to decrease significantly to 15% compared to untreated cells in IMR-32 cells after RA+ IFN-γ treatment. This could be partly due to increased N-Myc degradation.
(see below) and possibly also due to other posttranslational regulation affecting the formation and/or stabilization of Max/N-Myc heterodimers (Bahram et al., 1999). We also wanted to investigate how the occupancy of N-Myc, Max, and Mad1 on their target genes was affected by the RA+ IFN-γ treatment. CHIP experiments were performed and the association of the relevant proteins was investigated on the promoter of the N-Myc target gene ODC. We found that after RA+ IFN-γ treatment, binding of N-Myc to the ODC promoter decreased to ~40% compared to untreated cells whereas binding of Mad1 increased ~2.5-fold. RA alone also induced the same phenomenon, but to lesser extent. The association of Max with the ODC promoter did not change significantly irrespective of treatment. In summary, RA+ IFN-γ treatment of neuroblastoma cells leads to a shift from N-Myc/Max towards Mad1/Max complex occupancy on the ODC promoter. RA- or RA+ IFN-γ treatment also resulted in a reduction of acetylation of histone H4 in the same region of this promoter. This suggests that RA and RA+ IFN-γ induce a repressive chromatin state by decreasing recruitment of histone acetyltransferases through N-Myc, and/or increasing recruitment of histone deacetylases through Mad1. Finally, we investigated the effects of the different treatments on the transcription of N-Myc target genes by using RT-qPCR. The combined RA+ IFN-γ treatment strongly reduced transcription of the ODC and hTERT target genes to around ~20% of control whereas both RA and IFN-γ treatments alone reduced mRNA levels to lesser extent.

In conclusion, IFN-γ+RA induces differentiation accompanied with decreased N-Myc expression and increased Mad1 expression in neuroblastoma cells, leading to a shift from N-Myc/Max to Mad1/Max complexes on target gene promoters, resulting in reduced transcription of N-Myc target genes.

3.2 IFN-γ increases degradation and ubiquitylation of Myc in a Ser-62- dependent manner (paper I, II)

The U-937 differentiation model was established in 1976 from a patient with generalized histiocytic lymphoma (Sundstrom and Nilsson, 1976). U-937 cells are arrested at a monoblastic stage under normal conditions. They can be induced into differentiation by using 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 agents leads to G0/G1 arrest accompanied
with decreased c-Myc mRNA levels and increased expression of Mad1 (Ayer and Eisenman, 1993, Larsson et al., 1994). We previously reported that human monocytic U-937 cells constitutively expressing the v-Myc protein of the avian OK10 retrovirus, which carries a stabilizing mutation at Thr-58, are unable to undergo TPA-induced differentiation. However, the TPA-induced differentiation and G1 arrest is restored upon IFN-γ treatment (Oberg et al., 1991). We also found that IFN-γ destabilizes the Myc/Max complex, which correlates with reduced phosphorylation of Myc (Bahram et al., 1999). The aim of paper I and II was to further investigate the mechanisms behind the IFN-γ-induced inhibition of Myc functions.

First, we investigated the effects of IFN-γ treatment on c- and v-Myc protein stability in v-Myc-transformed U-937-myc6 cells. IFN-γ+TPA decreased the half-life of both c- and v-Myc from approximately 30 to 20, and 150 to 50 minutes respectively. Correspondingly, IFN-γ+RA, but neither RA nor IFN-γ alone, increased the rate of N-Myc protein degradation from a half-life of approximately 40 minutes to 15-20 minutes in IMR-32 neuroblastoma cells (Paper I). Also, the fluorescence intensity of c-Myc/Max BiFC (Bimolecular Fluorescence Complementation) fusion pairs was reduced after IFN-γ treatment, which indicated a decrease in c-Myc protein levels since Max expression is not affected by this treatment (Bahram et al., 1999). A similar reduction was observed with the c-Myc-GFP protein. IFN-γ treatment also increased the c-Myc turnover rate in other cell lines, including Colo-320 carcinoma cells with amplified c-myc and human 2fTGH fibrosarcoma cells. A cycloheximide (CHX) chase experiment performed with 2fTGH cells treated with IFN-γ+TPA showed that Myc turnover increased already within 4 hours and was further accelerated after 8 hours of treatment. TPA alone did not affect c- or v-Myc protein stability.

The IFN-γ induced increase in Myc degradation seems to occur via the UPS since evidence showed that increased ubiquitylation of c-Myc happened synchronously with increased turnover in 2fTGH cells. IFN-γ induced Myc degradation was sensitive to proteasome inhibition in U-937 cells, and IFN-γ stimulation increased the signal intensity of c-Myc/Ub BiFC, reflecting increased c-Myc ubiquitylation. Interestingly, degradation of Myc was not affected by IFN-γ in the 2fTGH sublines U3A and U4A that lack STAT1 and JAK1 respectively, suggesting that both STAT1 and JAK1 are required for IFN-γ induced Myc turnover. There was no evidence
from Myc-GFP fusion and BiFC experiments that Myc or Myc/Max was redistributed within the cell upon IFN-γ treatment prior to degradation.

As mentioned previously, the stability of Myc is regulated through phosphorylation at Ser-62 and Thr-58. Antibodies against phospho-T58/S62 were used to investigate how IFN-γ affects the phosphorylation state of Ser-62 of Myc. U-937-myc6 cells were first treated with IFN-γ for 24 hours followed by proteasome inhibition for 2 hours prior to harvest in order to avoid differences in total Myc levels resulting from different sensitivity to Fbw7/Cdc4-mediated degradation. The result showed an IFN-γ-induced reduction of v-Myc Ser-62 phosphorylation. To investigate whether the IFN-γ-induced increase in Myc turnover is dependent on Ser-62, we transfected Myc-T58A and Myc-S62A plasmids, respectively, into 2fTGH cells, followed by blocking protein synthesis with CHX. IFN-γ treatment increased degradation of Myc-T58A but not of Myc-S62A, suggesting that this degradation is Ser-62 dependent.

3.3 Cyclin E/Cdk2 is a new Ser-62 kinase for Myc (paper II)

Three kinases, Erk, Jnk, and Cdk1 (for N-Myc), are known to phosphorylate Myc on Ser-62. Erk functions via the MAPK/ERK pathway whereas Jnk functions via the PI3K-Rho-ROCK pathway (for review see Hann, 2006). To examine which kinases are involved in IFN-γ-induced Ser-62 phosphorylation, U2OS cells transfected with c-Myc-T58A (to ensure the exclusive study of Ser-62 phosphorylation) were treated with inhibitors of these three kinases and of other relevant factors, such as Mek1, p38 MARK, GSK3, PI3K, and ROCK, for 4 hours. The proteasome inhibitor MG115 was also added to the cells 2 hours prior to harvest in order to avoid possible effects of the kinase inhibitors on the Myc-T58A turnover. Roscovitine, a Cdk2/Cdk1 inhibitor, reduced Ser-62 phosphorylation most efficiently whereas Kenpaullone, which inhibits Cdk1 more efficiently than Cdk2, was not as effective as roscovitine, suggesting that Cdk2 is the kinase for IFN-γ-induced Ser-62 phosphorylation of Myc. Cdk1 may phosphorylate Myc in mitotic cells (Sjostrom et al., 2005) that are only a small fraction of all cells under our experimental conditions, and thus Cdk1-specific effects might be undetectable. Indeed, bacterially expressed GST-Myc was a substrate of cyclin E/Cdk2 and to a lesser extent of cyclin A/Cdk2 when Ser-62 was present. Further, phosphorylation of Ser-62 and Thr-58/Ser-62 was readily detected in U-937 cells arrested in S phase by treatment with the DNA polymerase inhibitor aphidicolin. These
phosphorylations can be strongly attenuated with roscovitine treatment. Consistently, both IFN-γ alone and IFN-γ+TPA in combination inhibited Cdk2 kinase activity. Ser-62 phosphorylation of both c- and v-Myc was reduced after 4 hours and more significantly after 8 hours of roscovitine treatment. Interestingly, mimicking IFN-γ, roscovitine increased the turnover of both c- and v-Myc in U937-myc6 cells, which further implicates that Cdk2-dependent phosphorylation of Ser-62 might act to antagonize IFN-γ induced Myc turnover.

3.4 p27Kip1 reduces phosphorylation of Myc on Ser-62 and increases turnover of Myc in a Ser-62 dependent manner (paper II)

p27Kip1 is a well-known inhibitor of the Ckd2 kinase, we therefore next wanted to investigate what potential role p27Kip1 plays in IFN-γ induced inhibition of Cdk2 activity on Myc. p27Kip1 protein levels were shown to increase in U-937-myc6 cells after 2 hours treatment with IFN-γ+TPA and thereafter continued to rise gradually, correlating with a decrease in Cdk2 activity. At the same time, the p27Kip1:Cdk2 complex formation was increased while the stability of Cdk2 kept unchanged. Cells overexpressing or lacking p27Kip1 were used together with wt c-Myc, Myc-T58A, Myc-S62A, or Myc-T58A/S62A constructs in order to further investigate the role of p27Kip1 in the phosphorylation and IFN-γ-induced turnover of Myc. The results demonstrated that p27Kip1 reduced the phosphorylation and increased the turnover of both the wt and the Myc-T58A mutant, but not of the Myc-S62A mutant. Furthermore, IFN-γ-induced Myc degradation seems to be dependent on p27Kip1 since Myc stability was unaltered in p27-/- MEF cells after IFN-γ treatment.

SCF^Fbw7/Cdc4 and SCF^Skp2 are two E3 ligases acting on Myc. Are they involved in the IFN-γ induced Myc degradation? To answer this question, we transfected p27Kip1 or control vector into HCT116 Fbw7/Cdc4-/- cells and investigated the turnover rate of Myc. Myc proteins were stabilized in Fbw7/Cdc4-/- cells as expected. However, the stabilization was eliminated by ectopic expression of p27Kip1. To study if p27Kip1-induced Myc degradation required Skp2, cells were transfected with the dominant negative Skp2ΔF mutant, which resulting in stabilization of Myc proteins. However, p27Kip1 coexpression increased Myc turnover irrespective of the presence of Skp2ΔF, suggesting that Skp2 does not participate in p27Kip1-induced Myc degradation.
3.5 Cyclin E/Cdk2 and p27Kip1 interact with Myc at target promoters and regulate Myc-driven transcription (paper II, III)

Since cyclin E/Cdk2 phosphorylates c-Myc we were interested to see whether this might occur at Myc target promoters. An interaction between Myc and cyclin E was detected by coimmunoprecipitation (coIP) in U937-myc6 cells. The relatively low signal indicated a weak or transient interaction. The presence of Myc, cyclin E, and Cdk2 on Myc target gene promoters was investigated by CHIP. Myc bound to the E-box region of the cyclin D2 promoter as expected. Cyclin E and Cdk2 were also shown to bind on the same region. Further, re-ChIP showed that Myc directly associated with Cdk2 at the cyclin D2 promoter (paper III).

We also examined whether interactions between Myc and p27Kip1 could be detected in IFN-γ treated U-937 cells by coIP. Weak but measurable amounts of Myc were specifically coIP-ed with p27Kip1 antibodies. The interaction between Myc and p27Kip1 could be visualized in cells treated with IFN-γ, IFN-γ+TPA, and to a lesser extent in TPA-treated cells but was hardly detectable in untreated cells. CHIP experiments demonstrated that p27Kip1 indeed associated specifically with the E-box region of the cyclin D2 promoter. Interestingly, the presence of p27Kip1 at this promoter increased after IFN-γ+TPA treatment. Moreover, a re-ChIP experiment was performed, showing that Myc indeed forms complexes with p27Kip1 at this promoter.

Consistent with this, a reduction in Ser-62 phosphorylation of Myc also occurred on Myc target gene promoters. By using Q-ChIP, we demonstrated that the occupancy of both total and phosphorylated Myc decreased substantially at the cyclin D2 promoter in response to IFN-γ treatment, as well as the acetylation of histone H4. It is unclear whether the reduced presence of Myc is due to its turnover at the promoter or degradation independent of its binding to DNA.

We next examined how IFN-γ treatment affects Myc-driven transcription using luciferase reporter gene constructs. IFN-γ and IFN-γ+TPA treatment strongly repressed the reporter activity. Moreover, the mRNA expression of two endogenous Myc-activated genes, hTERT and ODC, were significantly decreased upon IFN-γ+TPA treatment whereas expression of the Myc-repressed gene p21Cip1 was strongly enhanced. Finally, a Myc-driven promoter/luciferase reporter together with Myc and/or
cyclinE/Cdk2 was transfected into U2OS cells to investigate the effect of cyclinE/Cdk2 overexpression on Myc-induced transcription. Cotransfection with cyclinE/Cdk2 strongly increased Myc-induced transcription.

Put together, these data suggest that cyclinE/Cdk2 phosphorylates Myc at Ser-62 on target gene promoters. The stabilization of Myc prolongs its binding to the target gene promoters and thus enhances the transcription of these genes. IFN-γ represses cyclinE/Cdk2 kinase activity by inducing p27^Kip1, which is transiently recruited to Myc target gene promoters and inhibits the activity of Myc-bound cyclin E/Cdk2, resulting in reduced Myc phosphorylation and loss of Myc (and interacting proteins) from chromatin.

3.6 c-Myc activates late G1/S phase genes in cooperation with Skp2 (paper III)

To study the impact of Skp2 on the regulation of Myc-induced transcription of target genes during the cell cycle, the P493-6 human B lymphoid cell line carrying a conditional tetracycline (Tc)-regulatable c-myc construct was used. Tc turns off c-myc after which the cells go into quiescence. After removal of Tc, the myc gene is activated and cells re-enter cell cycle synchronously (Schuhmacher et al., 1999). In agreement with previous publications we found that Myc protein levels were strongly increased in these cells within 4 hours after Tc removal. Expression of cyclin A increased after approximately 20 hours, indicating progression of cells into S phase, whereas Skp2 protein was started to be expressed around 12 hours and kept increasing until 32 hours, in consistence with the previous finding that Skp2 expression starts in mid/late G1 and reaches its maximum in S and G2 phases of the cell cycle (for review see Nakayama and Nakayama, 2005). Correspondingly, the levels of both Myc and Skp2 mRNA increased after 4 and 12 hours, respectively. mRNA levels of Myc target genes, including RFC4, ornithine decarboxylase (ODC) and cyclin D2, were also investigated with RT-qPCR at the same time intervals. The results showed that ODC mRNA increased immediately after c-Myc was activated in early G1. Interestingly, the transcription of RFC4 did not start until around 12 hours after induction and was maximal at 18 hours, resembling the expression pattern of Skp2.

Myc is known to interact with Skp2 from late G1 in the nucleus (von der Lehr et al., 2003, Fujii et al., 2006), and Skp2 is recruited by Myc to target promoters, which not only ubiquitylates and degrades Myc but also enhances Myc induced transcription and S phase entry. (Kim et al.2003, von
Here we raised the question whether Skp2 could play a role in the induction of Myc target genes at late G1 phase. CHIP experiments performed with P493-6 cells showed that Myc bound strongly to E-box-containing promoter regions of both the ODC and RFC4 genes already at 6 hours after Myc induction, although the expression of RFC4 was not activated until several hours later. In contrast, ODC mRNA was induced with the same kinetics as the Myc binding to its promoter. Interestingly, Skp2 only bound to the RFC4 promoter but not to the ODC promoter. This occurred only at the later time points, thus correlating with the Skp2 expression pattern.

Taken together, we suggest that Myc binds to its target gene promoters soon after its activation and may induce transcription of different genes by cooperating with different coactivators. Some target genes such as ODC are transcribed immediately, perhaps in correlation with the action of the coactivator TRRAP. Other genes, like RFC4, are kept silent until certain cofactor, in this case possibly Skp2, is recruited at late G1 phase. However, it remains to be shown that Skp2 plays an active role in turning on this promoter.

3.7 Intimate connections between Myc, cyclinE/Cdk2, p27Kip1, and Skp2 might provide potential new strategies for cancer therapy (paper I, II, III)

The results mentioned above imply several possibilities of how Skp2 and cyclin E/Cdk2 are involved in the regulation of Myc driven transcription during G1/S transition. Cyclin E/Cdk2 binds to Myc target gene promoters during cell cycle progression, thus enhancing Myc-induced transcription. The mechanism by which cyclin E/Cdk2 can carry out a coactivator function for Myc is unclear. One possibility is that cyclinE/Cdk2-mediated phosphorylation of Ser62 stabilizes Myc, thereby increasing its time of association with chromatin. In addition, cyclin E/Cdk2 has been reported to repress the Myc antagonist Mad1 (Rottmann et al., 2005). It is possible that upon anti-proliferative signals such as IFN-γ, repression of cyclin E/Cdk2 on Mad1 is released, resulting in a shift from Myc/Max to Mad1/Max complexes on Myc target gene promoters. cyclin E/Cdk2 is also able to phosphorylate other components in the Myc transcriptional complex such as the histone acetyltransferase cofactor CBP (Ait-Si-Ali et al., 1998, Vervoorts et al., 2003). The kinase activity of cyclin E/Cdk2 might be repressed by
p27\textsuperscript{kip1} in response to anti-proliferative signals such as IFN-\(\gamma\), resulting in inhibition of Ser-62 phosphorylation.

This study also implies a possibility that Myc binds to the promoter of a subgroup of target gene, but cannot induce transcription until specific cofactors such as Skp2 are recruited to the promoter at late G1 phase. Recently, Skp2 has been found to antagonize the binding of p53 to p300, thus suppressing p300-mediated acetylation of p53 and the transactivation capability of p53 (Kitagawa et al., 2008). This brings up a potential pathway in which Skp2 may function in similar way to remove the repression on Myc, thereby enhancing Myc induced transcription. We also show that p27\textsuperscript{kip1}, which is known as a substrate of Skp2-mediated degradation, binds to promoters in complex with Myc and might inhibit cyclinE/Cdk2 kinase activity towards Myc at the promoter. One interesting hypothesis is that Skp2 ubiquitylates and degrades p27\textsuperscript{kip1} at the promoter, thereby releasing its inhibition of cyclin E/Cdk2, which in turn would assist the cyclin E/Cdk2-mediated coactivator function on Myc induced transcription. However, this remains to be investigated.

Both RA and IFN-\(\gamma\) have been used as single agents for clinical neuroblastoma therapy (Matthay et al., 1999, Yang et al., 2003). The effects of RA+IFN-\(\gamma\) on the N-Myc/Max/Mad network demonstrated with our work suggest a new therapeutic strategy of “differentiation therapy” for neuroblastomas with amplified N-myc. In this respect, it is encouraging that even transient inactivation of c-myc in c-Myc-driven tumours is sufficient to induce permanent tumour regression (for review see (Pelengaris et al., 2002)). While Myc and cyclin E are well established oncoproteins, a role of p27\textsuperscript{kip1} in tumor formation has been postulated but has only become apparent recently (Sicinski et al., 2007). Thus the Myc/Cyclin E/Cdk2/p27\textsuperscript{kip1} axis may also provide a target for intervention. Indeed, many cytokines or other agents that stimulate p27\textsuperscript{kip1} expression (Blain et al., 2003) as well as specific inhibitors of Cdk2/Cdk1 (Goga et al., 2007; Shapiro, 2006) are already in clinical use or under development, and could in the light of our new findings be of relevance for combined multi-drug therapies for patients with aggressive Myc–driven tumours.

### 3.8 Several Arabidopsis B-group bHLH genes show expression pattern correlated to cell cycle regulation

The plant Arabidopsis bHLH group, containing 162 genes (Heim et al 2003, Bailey et al 2003, Toledo-Ortiz et al., 2003), is the second largest transcription factor family in Arabidopsis thaliana. Most of the plant bHLH
genes belong to the B group and are involved in a number of different processes, including regulating trichome and root hair initiation (Bernhardt et al., 2003; Payne et al., 2000; Zhang et al., 2003), carpel development (Heisler et al., 2001), regulating fruit opening (Liljegren et al., 2004; Rajani and Sundaresan, 2001), regulation of light signalling (for reviews see (Duek and Fankhauser, 2005; Monte et al., 2007), regulation of stress and hormone signalling. The basic mechanisms of cell cycle regulation in plants are similar to those in animals (for review see (De Veylder et al., 2007)), but the understanding about how transcription factors regulate the cell cycle in plants is very limited. Many regulators of the cell cycle that are controlled by Myc in animal are fairly conserved in plants, such as cyclins, Cdk, CKI, Rb/E2F. Therefore in this paper we investigated if Myc-related bHLH proteins could be found in plants and if they would carry out similar functions.

First we tried to identify myc-like B-group genes in Arabidopsis thaliana (At). The bHLH domain of Myc, Max, and Mad are highly conserved in different species, and can be used for screening the genome of Arabidopsis. The bHLHZip domains of a number of animal Myc, Max and Mad proteins were used to screen At databases utilizing the Psi- and Phi-BLAST programs. We identified 84 B-group At bHLH sequences that characterized by a characteristic H-E-R “fingerprint”. This “fingerprint” is made of H at position five, E at position eight, and R at position thirteen out of the 13 amino acids in the basic region, and functions crucially for recognition of the CACGTG E-box sequence.

A neighbour-joining phylogenetic tree was constructed with amino acid sequences of the bHLHZip domains of these At genes using MEGA 4.0. Accordingly, these At genes were divided into 15 phylogenetic subfamilies with high predictive values (bootstrap support of 50 or greater) and 8 orphan genes that could not be grouped into any of these subfamilies. This classification is overlapping but not entirely identical what was proposed in previous reports (Heim et al 2003, Bailey et al 2003, Toledo-Ortiz et al., 2003). All 15 subfamilies were evolutionary separated from the myc/max/mad genes and all other animal bHLH genes.

The sequence similarity between animal myc/max/mad genes and these At genes exits the highest in the bHLH domain. But many of these At genes also seem to contain, at least partly, a leucine zipper (Zip). However, no significant similarity was found outside the bHLHZip domain. No particular
At B-group bHLH subfamily was identified to be much more myc, max or mad-like than the other, although all of them had many features in common. Here we argue that the At B-group bHLH family genes are all more or less “myc-like”.

Since the expression of the animal myc genes are cell cycle regulated, to investigate whether these At bHLH genes might function in a similar way as the animal myc genes, we next examined the expression patterns of the At bHLH genes using several cell cycle systems. These included shoot apex, flower, leaf, silique/seed and root development systems (Birnbaum et al., 2003, Jacqmard et al., 2003; Mordhorst et al., 1998; Zhang et al., 2005) as well as auxin-induced early lateral root initiation (Himanen et al., 2004, Vanneste et al., 2005) and sucrose-induced suspension culture systems (Menges and Murray, 2002).

Four cell cycle regulatory genes were used as reference genes, including the G1 cyclins cyclin D3;3 and cyclin D3;1, the transcription factor E2Fa, and the Cdk inhibitors KRP1 and/or KRP2. The E2Fa and the G1 cyclins genes are highly expressed in proliferative tissues, whereas the opposite of KRP1 (Menges and Murray, 2002). 15 At bHLH genes showed expression profiles correlating with proliferative status in shoot apex, flowers, leaves and/or siliques/seeds.

Among these, three genes, including At1g10120, At1gf68810 and At3g25710, were coregulated with cyclin D3;3, cyclin D3;1 and E2Fa in all four tissues. 5 genes, including At1g26260, SPATULA, EGL3, At3g61950, and At5g53210, were coregulated with these cell cycle genes in three out of four tissues; while others had similar expression patterns as the cyclins in some tissues, but resembled more the expression of KRP1 in other tissues. Some of the genes were differentially expressed between tissues or during the development of a certain tissue, but in a way that seemed unrelated to the cell cycle.

Similar bioinformatic study was done also in published microarray expression profiling data from Arabidopsis root (Beemster et al., 2005; Birnbaum et al., 2003). The three root developmental stages 1, 2, and 3 represent active cell proliferation, cell longitudinal expansion, and cell maturation, respectively. As expected, highest expression of cyclin D3;1 and cyclin D3;3 and lowest expression of KRP1 and KRP2 was found in stage 1 and vice versa in the stage 3. Nine of the At genes showed expression
patterns that were similar to the cyclins, including At3g25710 that were amongst the genes that were coregulated with cyclins in all of the previous tissues, and At1g26260, SPATULA, EGL3 and At3g61950 that were coregulated in three out of four of the previous tissues. Two of the genes showed an expression pattern that was similar to KRP 1 and 2.

We next looked at the expression of these At genes in an auxin-induced early lateral root initiation system (Himanen et al., 2004). In this system pericycle cells in root are synchronized by using 1-N-naphthylphthalamic acid (NPA), followed by lateral root initiation and cell cycle progression induced by auxin. The published microarray expression data from Vanneste et al. (Vanneste et al., 2005) were used in this study. The dominant auxin signalling mutant solitary root (slr1) that is defective in induction of key cell cycle components, including cyclin D3;1 (Vanneste et al., 2005), was used as negative control. Interestingly, gene At1g26260, which was coregulated with cyclin D3 in four out of the five tissues studied above, was strongly induced after 6 hrs of auxin treatment in wt plants but not in the slr1 mutant, suggesting that it plays a role in auxin-signalling and further implicates its role in cell cycle regulation.

After the bioinformatic study mentioned above, RT-qPCR was used to investigate the mRNA expression kinetics of these selected At genes in sucrose-induced Arabidopsis suspension cell cultures. In this system, the cells are synchronized in G0 by sucrose starvation, followed with cell cycle re-entry induced by re-addition of sucrose (Menges and Murray, 2002). The expression patterns of these At bHLH genes fell into three different categories: cyclin D3-like genes, immediate early transiently expressed genes, and KRP1/2-like genes. Three out of the four cyclin D3-like genes, At1g10120, At5g50915 and ALCATRAZ, were coregulated with cyclin D3 genes in 4-5 different systems. There were three immediate early transiently expressed genes, At1g26260, SPATULA and At3g61950. They were all coregulated with the cyclins in 5-6 other systems. Finally, we have three KRP1/2-like genes, PIF3, EGL3 and At1g68810. PIF3 has shown KRP-like expression patterns in two other systems, whereas EGL3 and At1g68810 have rather been expressed in a cyclin-like pattern is most other systems.

In summary, our study characterized several Arabidopsis B-group bHLH genes that showed expression pattern correlated to cyclin D or KRP, respectively, which may offer a platform for future study about functions of these genes in plant cell cycle regulation.
4 Conclusion

Combined IFN-γ and retinoic acid treatment induces differentiation and targets the N-Myc/Max/Mad1 network in N-myc-amplified neuroblastoma cells.

IFN-γ increases degradation and ubiquitylation of Myc in a Ser-62-dependent manner.

Cyclin E/Cdk2 is a new Ser-62 kinase for Myc.

\(p27^{kip1}\) reduces phosphorylation of Myc on Ser-62 and increases turnover of Myc in a Ser-62 dependent manner.

Cyclin E/Cdk2 and \(p27^{kip1}\) interact with Myc at target promoters and regulate Myc-driven transcription.

c-Myc activates late G1/S phase genes in cooperation with Skp2.

Intimate connections between Myc, cyclinE/Cdk2, \(p27^{kip1}\), and Skp2 might provide potential new strategies for cancer therapy.

Several \textit{Arabidopsis} B-group bHLH genes show expression pattern correlated to cell cycle regulation.
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Combined IFN-γ and retinoic acid treatment targets the N-Myc/Max/Mad1 network resulting in repression of N-Myc target genes in MYCN-amplified neuroblastoma cells

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Abstract

The MYCN protooncogene is involved in the control of cell proliferation, differentiation, and survival of neuroblasts. Deregulation of MYCN by gene amplification contributes to neuroblastoma development and is strongly correlated to advanced disease and poor outcome, emphasizing the urge for new therapeutic strategies targeting MYCN function. The transcription factor N-Myc, encoded by MYCN, regulates numerous genes together with its partner Max, which also functions as a cofactor for the Mad/Mnt family of Myc antagonists/transcriptional repressors. We and others have previously reported that IFN-γ synergistically potentiates retinoic acid (RA)–induced sympathetic differentiation and growth inhibition in neuroblastoma cells. This study shows that combined treatment of MYCN-amplified neuroblastoma cells with RA + IFN-γ down-regulates N-Myc protein expression through increased protein turnover, up-regulates Mad1 mRNA and protein, and reduces N-Myc/Max heterodimerization. This results in a shift of occupancy at the ornithine decarboxylase N-Myc/Max1 target promoter in vivo from N-Myc/Max to Mad1/Max predominance, correlating with histone H4 deacetylation, indicative of a chromatin structure typical of a transcriptionally repressed state. This is further supported by data showing that RA + IFN-γ treatment strongly represses expression of N-Myc/Mad1 target genes ornithine decarboxylase and hTERT. Our results suggest that combined IFN-γ and RA signaling can form a basis for new therapeutic strategies targeting N-Myc function for patients with high-risk, MYCN-amplified neuroblastoma. [Mol Cancer Ther 2007;6(10):2634–41]

Introduction

Neuroblastoma originates from sympathetic neuroblasts of the peripheral nervous system and is one of the most common solid childhood tumors, accounting for 7% to 10% of the childhood cancers and around 15% of childhood cancer deaths (1). Twenty-two percent of neuroblastomas and ~40% of advanced stage cases exhibit amplification of the MYCN protooncogene, a condition that is strongly correlated with advanced disease, drug resistance, and poor outcome (1–3). Targeting expression of MYCN to neuroectodermal cells has been reported to cause neuroblastoma in transgenic mice, further suggesting a crucial role of deregulated MYCN in neuroblastoma development (4). Moreover, ectopic overexpression of MYCN in neuroblastoma cell lines lacking MYCN aberrations results in enhanced growth potential and tumorigenicity, whereas antisense MYCN experiments have the opposite effect (2, 5).

MYCN is a member of the MYC gene family, including MYC, MYCN, and MYCL. All MYC family genes stimulate cell growth and proliferation, counteract terminal cell differentiation, sensitize cells to apoptosis in a similar fashion, and are all involved in the development of various types of tumors when deregulated (for review, see refs. 6–8). The MYC family is a subfamily of the basic region/helix-loop-helix (bHLH)/leucine zipper class of transcription factors which bind E-box DNA recognition sequences in promoters of target genes by heterodimerization with the obligatory partner Max, thereby activating transcription (for review, see refs. 6–8). However, Max also forms heterodimers with the Mad/Mnt family of basic region/helix-loop-helix/leucine zipper proteins, including Mad1, Mad3, Mad4, and Mnt/Rox, as well as with Mga, thereby forming alternative E-box–binding complexes (6, 9–11). Mad/Mnt proteins repress c-Myc–induced transcription, cell proliferation, and transformation and are therefore considered to be antagonists of Myc. To repress transcription, Mad/Mnt proteins interact with the repressor protein Sin3, which is part of repressor...
complexes containing histone deacetylases (6, 9–11). c-Myc, on the other hand, activates transcription at least in part by recruiting histone acetyltransferases complexes to target promoters through interaction with the cofactor/adapter protein TRRAP (6–8). Modulation of chromatin structure is therefore one way for both the Myc and Mad/Mnt family proteins to regulate transcription. Recent reports suggest that c-Myc may control up to 10% to 15% of all genes in the genome, thereby affecting numerous fundamental cellular processes, including cell cycle, cell growth, protein synthesis, metabolism, apoptosis, senescence, differentiation, genomic stability, angiogenesis, adhesion, etc. (6–8, 12).

Based on the gene expression patterns during development and differentiation and the antagonistic functions of the Myc and Mad family proteins, one current hypothesis suggests that the Myc/Max/Mad network proteins constitute a molecular switch that determines cell fate by regulating an overlapping set of genes. This model is supported by evidence showing that the occupancy of E-box–containing regions of the CYCLIND2 and hTERT target gene promoters in vitro shifts from c-Myc/Max toward Mad1/Max predominance during hematopoietic differentiation, correlating with histone deacetylase recruitment, histone deacetylation, and repression of gene activity (13, 14). It is still unclear how large fraction of c-Myc target genes are coregulated by the Mad/Mnt family, but both families also seem to control unique sets of genes by interacting with alternative partners that bind non–E-box motifs, such as Mizi1, which is used by c-Myc for repression of certain genes (8, 12, 15, 16).

The poor outcome of patients with MYCN-amplified neuroblastoma despite intensive therapy has called for irreversible tumor cell differentiation and growth arrest, or alternatively apoptosis, in both cases resulting in strong repression of N-Myc target genes. These results suggest that combination therapy with IFN-γ could be of interest for treatment of patients with neuroblastoma harboring MYCN amplification.

Materials and Methods

Cell Cultures and Induction of Differentiation
The human neuroblastoma cell lines LA-N-5 and IMR-32 were cultured in RPMI 1640 and DMEM, respectively, supplemented with 10% FCS and antibiotics. Cells plated the day before at 1 × 10⁶ cells per 6-cm dish were induced to differentiate in medium containing 1 μmol/L all-trans RA (Sigma), 100 to 200 units/mL IFN-γ (generously provided by Dr. G.R. Adolf, Ernst-Boehringer Institute) or their combination. Cell number and viability were determined by trypsin blue exclusion. The morphology of the cells was evaluated under a phase contrast Zeiss microscope (Carl Zeiss), and photographs were taken through an Olympus phase contrast microscope. The percentage of morphologically differentiated cells was determined by counting at least 100 randomly chosen cells, wherein cells exhibiting neurites at ≥50 μm were scored as positive. Neurite outgrowth index (24) was defined as the product of the percentage of cells with neurites at ≥50 μm and the average total neurite length.

Immunoprecipitations and Western Blot Analysis

Cell labeling and immunoprecipitation were done as described previously (25). Briefly, cells were labeled for 50 min in 1.5 mL of methionine (Met)–free RPMI 1640 containing 0.2 μCi of ³⁵S-Met. For high and low stringency precipitations has been described (25). The samples were analyzed on 10% to 15% SDS-PAGE gels. For studies of protein turnover, cells were pulse labeled for 40 min as above, followed by chase in medium containing 1 mmol/L cold Met and 10 μg/mL cycloheximide. Western blot analysis was carried out as described (25). The antibodies used for immunoprecipitation were C-19 Mad1 antibodies (Santa Cruz Biotechnology), IG-7 pan-Myc antiserum (25), and immunoprecipitated with specific antibodies. An equal number of TCA-precipitable counts were used for each sample. The washing procedure for high and low stringency immunoprecipitations has been described (25). The samples were analyzed on 10% to 15% SDS-PAGE gels. For studies of protein turnover, cells were pulse labeled for 40 min as above, followed by chase in medium containing 1 mmol/L cold Met and 10 μg/mL cycloheximide. Western blot analysis was carried out as described (25). The antibodies used for immunoprecipitation were C-19 Mad1 antibodies (Santa Cruz Biotechnology), IG-7 pan-Myc antiserum (25), AB-1 N-Myc antibodies (Oncogene Science), and C-17 Max antibodies (Santa Cruz). Radioactive signals were quantitated with a Phosphor Imager (Bio-Rad).

Quantitative Reverse Transcription – PCR and Northern Blot Analysis
Total RNA (4 μg) was converted to cDNA with Revert-Aid M-MuLV reverse transcriptase (Fermenta). Omnitube

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dctheory (ODC)—specific and MYC—specific primers and probes for reverse transcription–PCR were designed by the software Primer Express (Perkin-Elmer Applied Biosystems). As reference genes, GAPDH, β-ACTIN, and hTERT were used. Primer sequences are provided upon request. Quantitative PCR reactions were done in triplicates, using the Tagman PCR Core Reagent kit (Applied Biosystems) with the ABI PRISM 7700 Sequence Detector (Perkin-Elmer).

Northern blot analysis was carried out as described (23). For MAD1, a 1.3-kb NotI/EcoRI fragment from the cDNA clone pVZ1mad (kindly provided by D. Ayer) was used, whereas for MAX, a 540-bp EcoRI fragment from the MAX cDNA clone pVZ1max (kindly provided by R. Eisenman) was used. The probes for MYCN and GAPDH have been described (23).

Chromatin Immunoprecipitation Assays
Cells were lysed and crosslinked with formaldehyde as described (13). Chromatin DNA was sonicated to an average size of 300 to 500 bp. Immunoprecipitations were carried out in radiolabeled immunoprecipitation assay buffer with the following antibodies: R20 (preimmune serum), anti-Max, anti–N-Myc, and anti-Mad1 from Santa Cruz and anti–acetyl histone H4 from Upstate. Immunoprecipitates were collected with protein G–sepharose beads, washed, and eluted in TE, 1% SDS. Purified DNA samples were analyzed by quantitative PCR as above using primers specific for an E-box–containing regulatory region of the ODC gene (7). As control, a non–E-box region of the Fasl was used. Quantitative PCR primer sequences are provided upon request.

Results

Combined RA+IFN-γ Treatment Reduces N-Myc and Induces Mad1 Expression Correlating with Robust Differentiation in MYCN-Amplified Neuroblastoma Cells
IFN-γ has been reported to potentiate RA-induced differentiation in MYCN-amplified neuroblastoma cell lines (20–23). To investigate whether such cooperative effects correlate with regulation of N-Myc and/or Mad1 protein expression, we used IMR-32 and LA-N-5 cells, both with very high copy numbers of MYCN (3) and very high N-Myc expression (Supplementary Fig. S1). Supplemental Table S1† shows that IFN-γ enhanced RA-induced differentiation measured as the percentage of morphologically differentiated cells with neurites as reported previously (23). Furthermore, in contrast to the effects of RA or each agent alone, complete growth arrest rather than only growth retardation was induced by their combination (data not shown; Supplementary Table S1,* ref. 23).

A substantial decrease of N-Myc protein expression, as determined by immunoprecipitation of 35S-Met–labeled cell lysates, was observed in both cell lines in response to the combination RA+IFN-γ, whereas RA alone led only to a modest reduction in LA-N-5 cells (Fig. 1A and B), as quantitated in Fig. 1D (top). This is in agreement with previous reports on MYCN mRNA expression in LA-N-5 cells (22, 23). In contrast, only a slight reduction in MYCN mRNA expression was observed in IMR-32 cells in response to RA+IFN-γ treatment (Fig. 1C and D; ref. 23).

RA and RA+IFN-γ treatment increased the expression of Mad1 in IMR-32 cells (Fig. 1B and D). In contrast, in LA-N-5 cells, RA+IFN-γ and IFN-γ alone increased Mad1 expression (Fig. 1A and D). This may suggest that RA-induced or IFN-γ–induced signals alone may regulate Mad1 expression differently in different neuroblastoma tumors. The reason for this difference is unclear but may be due to deregulation in tumor cells of one or the other of the diverse signaling pathways that activate Mad1 expression. The combination of RA+IFN-γ is therefore more likely to result in Mad1 induction than either treatment alone. The expression of MAD1 mRNA, analyzed in IMR-32 cells (Fig. 1C and D), followed the same pattern of expression, suggesting that Mad1 is regulated at the level of transcription in response to signaling, as reported previously in other cell types (9–11). We did not have access to antibodies of high enough quality for protein analysis of Mxi1, Mad3, Mad4, or Mnt. Northern blot analysis, however, did not reveal increased expression of the corresponding genes in response to treatment with RA, IFN-γ, or their combination (data not shown). No major changes in Max protein and mRNA expression were observed after the different treatments (Fig. 1). In summary, the cooperative effects of RA+IFN-γ on differentiation and growth arrest correlated with decreased N-Myc and increased Mad1 expression.

RA+IFN-γ Costimulation Induces Increased N-Myc Protein Turnover

Interestingly, Fig. 1 suggests that RA+IFN-γ down-regulates N-Myc protein expression posttranscriptionally, because the steady-state level of MYCN mRNA was only slightly affected in IMR-32 cells. We therefore continued to explore this phenomenon in IMR-32 cells. To investigate whether RA+IFN-γ affects the turnover rate of N-Myc, cells were pulse labeled with 35S-Met, followed by chase, in excess cold Met, after which remaining labeled N-Myc was assessed at various time points. Figure 2A shows that RA+IFN-γ, but not either treatment alone, increased the speed of N-Myc protein degradation from ~40 min to 15 to 20 min half-life, as quantitated in Fig. 2B.

This suggests that RA+IFN-γ affects the N-Myc/Mad1 network at three levels by decreasing MYCN mRNA (LA-N-5 cells), increasing MAD1 mRNA, and increasing the rate of N-Myc protein turnover.

IFN-γ Costimulation Results in Reduced N-Myc/Mad Complex Formation

Because N-Myc requires heterodimerization with Max for its activity, we next studied whether N-Myc/Max heterodimerization were altered in response to treatment in
IMR-32 cells. As shown in Fig. 3, RA+IFN-γ, and to lesser extents RA and IFN-γ alone, significantly reduced the concentration of N-Myc/Max heterodimers (15% of untreated cells; Fig. 3B) beyond the effect on total N-Myc expression (60% of untreated cells; Fig. 1B and D). These results are in agreement with earlier studies in monocytic cells, wherein IFN-γ treatment in combination with 12-O-tetradecanoylphorbol-13-acetate was shown to destabilize Myc/Max heterodimers (25). Unfortunately, we were not able to measure Mad1/Max complexes in the neuroblastoma cells because they were below the level of detection in this assay. The slight reduction in total Max protein levels after the treatments was found to be not significant (Fig. 3).

We conclude that RA+IFN-γ in particular decreased the level of N-Myc/Max complexes in the IMR-32 cells, in part via increased N-Myc turnover and possibly also via other posttranslational effects on N-Myc/Max complex formation and/or stability.
Shift from N-Myc/Max toward Mad1/Max Occupancy at the ODC Target Gene Promoter after RA+IFN-γ Costimulation

We next investigated N-Myc, Mad1, and Max occupancy of the ODC gene, a target for both N-Myc and Mad (26–28), in vivo by performing quantitative chromatin immunoprecipitation assays. As shown in Fig. 4, α-N-Myc and α-Max antibodies readily immunoprecipitated an E-box–containing fragment of the ODC promoter from unstimulated IMR-32 cells, whereas only a weak signal was obtained using α-Mad1 antibodies. Neither preimmune control antibodies nor any immunoprecipitation of the non–E-box, but MEF2-binding, region of the Fas ligand gene, used as a reference, gave signals above background. After RA+IFN-γ treatment, the N-Myc signal decreased to ~40% of untreated cells, whereas binding of Mad1 increased ~2.5-fold. RA alone also decreased and increased N-Myc and Mad1 associations with promoter, respectively, but to lesser extents. The association of Max did not change significantly after the treatments. RA and RA+IFN-γ–induced differentiation also resulted in a decreased acetylation of histone H4 in the same region of the promoter, indicative of alteration of the chromatin structure into a more repressed state during the process, a hallmark of decreased Myc and/or increased Mad activity at target promoters.

In conclusion, our results suggest that the ODC promoter occupancy shifted from N-Myc/Max predominance in untreated cells toward Mad1/Max predominance after RA+IFN-γ–induced and, to a lesser extent, RA-induced differentiation, resulting in a more condensed chromatin structure at the promoter.

The ODC and hTERT N-Myc/Mad1 Target Genes Are Repressed in Response to RA+IFN-γ Costimulation

We next investigated whether the treatments affect the expression of N-Myc/Mad1 target genes. mRNA expression of the ODC and hTERT target genes of N-Myc and Mad (14, 26–30) was therefore measured in IMR-32 cells by quantitative reverse transcription–PCR. As shown in Fig. 5, ODC and hTERT expression was reduced to various degrees in response to RA or IFN-γ alone. However, RA+IFN-γ in combination led to strong repression of ODC and hTERT expression to around 20% of control.

Taken together, these results suggest that the combined treatment with RA+IFN-γ regulates expression of N-Myc and Mad1, as well as N-Myc/Mad1 heterodimerization, resulting in a shift from N-Myc/Max toward Mad1/Max occupancy at N-Myc/Mad1 target promoters. This in turn results in altered chromatin structure and transcriptional repression.
Discussion

IFN-γ is reported to potentiate RA-induced growth retardation and differentiation in several MYCN-amplified neuroblastoma cell lines (20–23). We show here that this correlates with reduced and increased expression of N-Myc and Mad1, respectively. In IMR-32 cells, the drop in N-Myc protein in response to RA+IFN-γ is much more pronounced than the slight reduction in MYCN mRNA expression, and we show that this is the result of increased turnover of the N-Myc protein. Recently, we have made similar observations in v-myc-transformed U-937 monoblastic cells, wherein combined IFN-γ and 12-O-tetradecanoylphorbol-13-acetate treatment results in ubiquitin/proteasome-mediated degradation of v-Myc.7 Elucidating the mechanism by which combined IFN-γ treatments increase Myc turnover will require further investigation.

In contrast, Mad1 seems to be regulated at the mRNA level, as has been shown in other cell systems (9–11). In addition, RA+IFN-γ treatment and, to a lesser extent, RA and IFN-γ alone seemed to regulate N-Myc/Max heterodimerization, suggesting that these signals regulate N-Myc activity posttranslationally at different levels. This is also reminiscent of the situation in v-myc-transformed U-937 cells, wherein 12-O-tetradecanoylphorbol-13-acetate+IFN-γ treatment inhibits v-Myc DNA binding and heterodimerization with Max (25, 31). Our present results further show that N-Myc/Max complexes were exchanged by Mad1/Max complexes at the ODC target promoter in RA+IFN-γ-differentiated and, to a lesser extent, RA-differentiated cells. This correlated with deacetylation of histone H4 in the same region of the promoter, suggesting that RA and RA+IFN-γ induce a repressive chromatin state by increased recruitment of histone deacetylases and/or reduced recruitment of histone acetyltransferases through Mad1 and N-Myc, respectively. These results are in good agreement with previously reported exchanges of c-Myc/Max to Mad1/Max complexes on the CYCLIND2 and hTERT target promoters during differentiation of human myeloid HL60 cells (13, 14). Our present results further show that N-Myc/Mad1 target genes ODC and hTERT much more efficiently than either agent alone, thus demonstrating a functional consequence of the cooperative signaling. Our results suggest that both the down-regulation of N-Myc and the up-regulation of Mad1 in response to RA+IFN-γ treatment contribute to this shift in N-Myc/Max/Mad1 promoter occupancy and repression of promoter activity. Because RA alone accomplished H4 deacetylation at least as well as RA+IFN-γ, it is likely that also other aspects of Myc-regulated transcription are affected by the combined treatment.

Apart from the N-Myc/Max/Mad network, it is likely that RA and IFN-γ also affects other regulators that may contribute to the differentiation of MYCN-amplified neuroblastoma cells. For instance, the cyclin-dependent kinase inhibitor p21Cip1 is up-regulated by IFN-γ via the Janus-activated kinase–STAT pathway in various cells (32). In addition, both p21Cip1 and p27Kip1 have been shown to be up-regulated during RA treatment of neuroblastoma and
hematopoietic cells (33–36). Interestingly, Mad1 and p27Kip1 have been shown to cooperate in promoting granulocytic differentiation and in regulating self-renewal/differentiation of hematopoietic stem cells (37, 38). IFN-γ was recently shown to restore promyelocytic leukemia protein nuclear body formation in neuroblastoma cells (39). Because promyelocytic leukemia protein interacts with Sin3-containing corepressor complexes and is required for the transcriptional repressor function of Mad1 (40), promyelocytic leukemia protein may contribute to the observed repression of N-Myc/Mad1 target genes in response to IFN-γ costimulation once Mad1/Max complexes have bound to their target promoters. Furthermore, recent reports suggest that promyelocytic leukemia protein may also regulate c-Myc function (41, 42).

The concept of “differentiation therapy” for cancer has gained considerable interest in recent years. Today, RA is used successfully in therapy for patients with acute promyelocytic leukemia (43) and treatment of neuroblastosma patients with high-dose 13-cis-RA treatment after myeloablative therapy and autologous bone marrow transplantation have shown encouraging results (1, 19, 44). The synergistic effects of RA+IFN-γ for differentiation of MYCN-amplified neuroblastoma cells, shown in vitro (20, 22, 23) as well as in vivo by inhibition of tumor formation of LA-N-5 neuroblastoma cells injected in nude mice (21), are further substantiated here by the demonstration of direct effects on the N-Myc/Max/Mad1 network.

This suggests that RA+IFN-γ combination therapy may be of therapeutic value for treatment of patients with MYCN-amplified neuroblastomas. In this respect, it is encouraging that even transient inactivation of c-Myc in c-Myc–driven murine tumors is sufficient for permanent tumor regression (17). The fact that both RA and IFN-γ have been used already as single agents in clinical trials for neuroblastoma therapy (19, 45) and that their combination synergistically inhibits neuroblastoma tumor growth as xenografts in mice (21) should facilitate this approach. IFN-γ acts synergistically with a number of signal pathways, for instance, in the induction of apoptosis by Fas-L and TRAIL in neuroblastoma cells (46–49). It would be interesting to study if IFN-γ can cooperate also with other retinoids, such as fenretinide (4HPR), which, unlike at-RA and 13-cis-RA, induces apoptosis rather than differentiation and has also been used in clinical trials in neuroblastoma (44). Although the clinical use of RA and IFN-γ may also have limitations due to side effects and development of drug resistance, insights into the precise molecular mechanisms by which IFN-γ, RA, and other agents cooperatively regulate the N-Myc/Max/Mad1 network will be essential for the development of novel therapeutic strategies, including new drug design for treatment of patients with high-risk neuroblastoma with MYCN amplification.

Acknowledgments


References


Figure 5. Regulation of the N-Myc/Mad1 target genes ODC and hTERT during IFN-γ–enhanced differentiation of IMR-32 cells. The cells were treated with differentiation agents as indicated, and the ODC and hTERT mRNA expression levels were quantified using quantitative reverse transcription–PCR analysis. Results are presented as percentage of untreated cells and are based on at least three independent biological experiments. The mRNA expression values have been normalized to those of GAPDH, β-ACTIN, and β-TUBULIN. *P < 0.05, **P < 0.01.
Interferon-γ represses Myc function via p27Kip1 by inhibiting cyclin E/Cdk2-mediated phosphorylation of Ser62

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Running title: IFN-γ represses Myc function via p27

Abstract

The c-myc oncogene is tightly regulated at multiple levels, including ubiquitin/proteasome-mediated turnover of its gene product. We have shown previously that interferon (IFN-γ) stimulates monocytic differentiation by antagonizing Myc function through post-translational mechanisms. Here we show that IFN-γ signaling inhibits phosphorylation of Myc at Ser-62, resulting in increased ubiquitylation and proteasome-mediated turnover. The latter occurs independent of the phosphorylation site at Thr 58 and the two E3 ubiquitin ligases Fbw7/hCdc4 and Skp2 that previously have been implicated in Myc turnover. Our data suggest that cyclin E/cyclin-dependent kinase 2 (Cdk2) acts as a Ser-62 kinase both in vitro and in vivo, and that the inhibition of Ser-62 phosphorylation and increased
Myc turnover in response to IFN-γ signaling is mediated through induction of the Cdk inhibitor p27Kip1. We further show that IFN-γ treatment results in reduced association of total and in particular phosphorylated Myc at target promoters in cells, correlating with reduced histone H4 acetylation and repression of Myc target genes. In conclusion, our results suggest that p27Kip1 and c-Myc promotes ubiquitylation and degradation of each other in a mutually antagonistic manner in response to signaling. This finding may potentially be of therapeutic relevance for tumors with deregulated Myc-expression.

Introduction

The c-myc proto-oncogene encodes a transcription factor that regulates a large number of genes involved in numerous fundamental cellular processes, including cell cycle progression, cell growth, apoptosis and metabolism [1, 2]. In addition, c-Myc has recently been reported to participate in non-transcriptional regulation of DNA replication [3]. Deregulated expression of myc family genes has been implicated in the development of many types of human cancers. The basic region helix-loop-helix/leucine zipper (bHLHZip) motif in the C-terminus of c-Myc mediates dimerization with the bHLHZip protein Max, which enables it to bind specifically to E-box DNA elements in promoters of target genes [1, 2, 4]. The N-terminus contains a transactivation domain (TAD), which includes two highly conserved regions known as Myc boxes 1 and 2 (MBI and MBII). The TAD and the C-terminus mediate interactions with a number of cofactors important for transcription regulation by Myc, including TRRAP, TIP48/TIP49, Miz-1, the Mediator, Skp2, the APIS complex, CBP/p300, HectH9 and P-TEFb [1, 2].

Expression of c-Myc is tightly regulated at multiple levels including rapid protein turnover via the ubiquitin (Ub)-proteasome pathway. Two E3 Ub ligases mediating c-Myc turnover, SCFSkp2 [5, 6] and SCFFbw7/Cdc4 [7, 8], have been identified. A third E3-ligase, HectH9, is involved in non-proteolytic regulation of c-Myc [9]. Skp2- and HectH9-directed ubiquitylation of Myc occurs as an integrated coactivator function for Myc-regulated transcription [5, 6, 9]. The Fbw7/Cdc4 pathway on the other hand seems to work entirely as a negative regulator of Myc [7, 8]. Efficient turnover of Myc requires phosphorylation at Thr-58 in MBI, a residue that is mutated in a high percentage of human Burkitt’s lymphomas and in several avian retroviral (v)-myc genes and is recognized by Fbw7/Cdc4. Mutation of Thr-58 results in decreased ubiquitylation and stabilization of Myc. These mutants still promote cell proliferation but are deficient in apoptosis, resulting in enhanced transforming potential [10]. Phosphorylation of Thr-58, mediated by glycogen synthase kinase 3 (GSK-3), requires “priming” phosphorylation at Ser-62 (for reviews see [11, 12]. The latter has been suggested to be targeted by Erk and to stabilize Myc.
[13]. Efficient ubiquitylation by Fbw7/Cdc4 seems to require phospho-
Thr-58-dependent and Pin1-assisted removal of the Ser-62 phosphorylation
by PP2A [14], a process that was recently shown to be further regulated by
the deubiquitylating enzyme Usp28 [15] and the CIP2A regulator of PP2A
[16].

Extra- and intracellular signaling also contribute to this complex
regulation of c-Myc. Skp2-induced degradation is regulated during the cell
cycle [6], while the Fbw7/Cdc4 pathway is regulated for instance by Ras-
and PI3-kinase/Akt signaling [2]. We showed previously that the v-Myc
protein of the avian OK10 retrovirus, which like c-Myc in many human
Burkitt’s lymphomas carries a stabilizing mutation at Thr-58, blocked TPA-
induced differentiation in the human monocytic U-937 model system.
However, in combination with IFN-γ, TPA-induced differentiation and G1
arrest were restored (Figure 1A) [17]. We found that IFN-γ destabilized
Myc:Max complexes, which correlated with reduced overall Myc
phosphorylation [18]. The aim of the present study was to further elucidate
the mechanism(s) by which IFN-γ inhibits Myc function. We find that
IFN-γ induces expression of the Cdk-inhibitor p27Kip1 (here referred to as
p27), which was shown to control Myc activity and stability by reducing
Ser-62 phosphorylation. Further, we identified cyclin E/Cdk2 as a major
Ser-62 kinase and the target of p27 activity. Importantly, IFN-γ treatment
resulted in reduced Ser-62 phosphorylation of Myc at promoters, loss of
Myc from chromatin, and repression of Myc target genes. These findings
shed new light on the regulation and function of Myc, but also points out
new ways by which cyclin E/Cdk2 and p27 influence gene expression
during the cell cycle.

Results

IFN-γ increases degradation and ubiquitylation of Myc

Addressing possible mechanism(s) for IFN-γ-induced anti-Myc activity, we
first investigated v- and c-Myc protein stability. v-Myc runs slightly faster
than endogenous human c-Myc in SDS-PAGE gels as demonstrated in v-
Myc-expressing U937-myc6 cells and parental U-937-GTB cells (Figure
1B), in agreement with previous observations [18]. U-937-myc6 cells were
treated with or without IFN-γ+TPA for 24 hrs and pulse/chased with 35S-
Met (Figure 1C, D). In untreated control cells, endogenous c-Myc had an
expected short half-life of 30 minutes. v-Myc exhibited a half life of
approximately 160 minutes due to the Thr-58 mutation [19]. IFN-γ+TPA
treatment increased both c- and v-Myc turnover from approximately 30 to
20 and 150 to 50 minutes, respectively (Figure 1D, note the logarithmic
scale on the Y-axis). The non-linear slopes of the curves might reflect the
existence of different subpopulations of Myc proteins with different stability,
as reported [20]. TPA treatment alone did not alter c-Myc or v-Myc
stability (data not shown), while IFN-γ alone had similar effects on Myc turnover as the combination IFN-γ+TPA (data not shown, see below). IFN-γ+TPA increased c- and v-Myc turnover also in complex with Max (data not shown). IFN-γ enhanced c-Myc turnover also in Colo-320 colon carcinoma cells with amplified c-myc (Figure 1D, right panel and Supplementary Figure 1A) and human 2fTGH fibrosarcoma cells (Figure 1E, left panel and Supplementary Figure 1B). Thus IFN-γ increases the turnover rate of c-Myc in several cell types, and notably also of v-Myc with a stabilizing Thr-58 mutation.

Many of the biological effects of signaling via the IFN-γ receptor are mediated through activation of the JAK/STAT1 pathway [21]. Indeed IFN-γ treatment did not affect Myc turnover in the 2fTGH sublines U3A and U4A that lack STAT1 and JAK1, respectively, indicating that both STAT1 and JAK1 are required for IFN-γ-induced Myc degradation (Figure 1E and Supplementary Figure 1B). The increased turnover rate in 2fTGH cells was accompanied by increased c-Myc ubiquitylation (Figure 1F). IFN-γ-induced degradation of c- and v-Myc in U-937-myc6 cells occurs via the proteasome since it was sensitive to the proteasome inhibitor LLnL (Figure 1G).

No difference in the subcellular localization of a c-Myc-GFP fusion protein was observed in response to IFN-γ (Figure 2A). The same was true for c-Myc complexed with Max as determined by Bimolecular Fluorescence Complementation (BiFC) [22] (Figure 2B). The BiFC and Myc-GFP fluorescence intensity was reduced with similar kinetics after IFN-γ-stimulation, reflecting increased degradation (Figure 2A-C). Increased YFP fluorescence intensity of a c-Myc/Ub BiFC fusion pair, indicative of increased c-Myc ubiquitylation, was observed in IFN-γ-stimulated compared to non-stimulated cells after proteasome inhibition, (Figure 2D and E). The fluorescence seemed to be predominantly localized to the nucleoli, suggesting that this could be a site of rapid Myc turnover.

IFN-γ reduces phosphorylation at Ser-62 and induces degradation of Myc in a Ser-62-dependent manner

The stability of Myc is regulated by phosphorylation at Thr-58 and Ser-62 (Figure 3A). We used phospho-specific c-Myc antibodies to determine whether IFN-γ affected phosphorylation at these two sites. Figure 3B demonstrates that both the phospho-S62 and, to a somewhat lesser extent, the phospho-T58/S62 antibodies recognizes singly phosphorylated Ser-62 as determined by expression of wt c-Myc and a T58A mutant, while none of the antibodies recognized S62A and T58A/S62A mutants (Figure 3B). Since Thr-58 phosphorylation requires priming phosphorylation of Ser-62 (Figure 3A) the phospho-T58/S62 antibody will not recognize the S62A mutant even though it has intact Thr-58.
Immunoblotting of U-937 cell extracts with pan-Myc antibodies in the mini-gel system applied in Figure 3C shows that the v-Myc protein runs closer to c-Myc than in the longer gel system used in Figure 1B above. IFN-γ treatment of U-937-myc6 cells resulted in a reduction of v-Myc Ser-62 phosphorylation (since v-Myc carries a Thr-58 mutation) (Figure 3D). In these experiments cells were treated with the proteasome inhibitor MG115 prior to harvest to avoid differences in total Myc levels, e.g. due to different sensitivity to Fbw7/Cdc4-mediated degradation. Endogenous c-Myc levels were downregulated by these treatments due to transcriptional shut off of the c-myc gene [18].

To examine whether Ser-62 is essential for IFN-γ-induced Myc degradation, the turnover of T58A and S62A Myc mutants were studied by blocking de novo protein synthesis with cycloheximide (CHX) in 2FTGH cells. IFN-γ increased the rate of degradation of Myc-T58A but not of Myc-S62A (Figure 3E and Supplemental Figure 1C). Taken together these results suggest that IFN-γ treatment decreases Myc phosphorylation at Ser-62 and that IFN-γ-induced Myc degradation is Ser-62 dependent.

Cdk2 phosphorylates Myc at Ser-62 in vivo and in vitro

Reduced Myc Ser-62 phosphorylation in response to IFN-γ treatment could be the result of inhibition of a kinase and/or stimulation of a phosphatase. To identify relevant kinases, U2OS cells transfected with c-Myc-T58A (to ensure exclusive study of Ser-62 phosphorylation) were treated with kinase inhibitors. To avoid possible effects on the turnover of Myc-T58A, the cells were treated with the proteasome inhibitor MG115. The Cdk2/Cdk1 inhibitor roscovitine [23] most efficiently reduced Ser-62 phosphorylation (Figure 4A). Since kenpaullone, which inhibits Cdk1 more efficiently than Cdk2, and PD98059, an Erk inhibitor, were not as effective as roscovitine, we considered Cdk2 as a candidate kinase. Indeed bacterially expressed GST-Myc was a substrate of cyclin E/Cdk2 and to a lesser extent of cyclin A/Cdk2 when Ser-62 was present (Figure 4B). We concluded from these results that Cdk2 phosphorylates Myc at Ser-62 in vitro and that the Cdk2/Cdk1 inhibitor roscovitine inhibits Ser-62 phosphorylation in vivo.

To further investigate whether Cdk2 could be an in vivo Ser-62 kinase regulating c-Myc during the cell cycle, we arrested U-937 cells in early S-phase by aphidicolin (which inhibits DNA polymerases) and treated the cells with MG115. Phosphorylation of Ser-62 and Thr-58/Ser-62 was readily observed in these cells (Figure 4C). Importantly, phosphorylation was strongly reduced in response to roscovitine, suggesting that Cdk2 is a major Ser-62 kinase during early S-phase. In addition, roscovitine increased the rate of degradation of both c- and v-Myc in U937-myc6 cells (Figure 4D).
and Supplementary Figure 1D). Thus roscovitine mimics IFN-γ-induced degradation of c- and v-Myc, further implicating Cdk2-dependent phosphorylation of Ser-62 in Myc turnover. Our findings support a role of Ser-62 phosphorylation in Myc stabilization as suggested previously [13]. However this modification can also enhance turnover by stimulating Thr-58 phosphorylation, which might explain why IFN-γ and roscovitine destabilize Thr-58 mutants more efficiently than wt Myc (Figure 1D and 4D).

**IFN-γ controls Myc phosphorylation and stability by inducing p27Kip1**

We next investigated whether IFN-γ affects Cdk2 activity in U-937-myc6 cells. In response to IFN-γ+TPA, Cdk2 activity was strongly reduced within 4 hrs of treatment as determined by in vitro kinase assays (Figure 5A). In addition reduced phosphorylation of the retinoblastoma protein pRb, a cyclin E/Cdk2 substrate was observed (data not shown). IFN-γ+TPA and IFN-γ treatment both inhibited Cdk2 activity, while TPA alone had minor effects (data not shown). The Cdk2 inhibition correlated with increased p27Kip1 protein expression, which was elevated by 2 hrs of stimulation and thereafter continued to rise gradually (Figure 5B). This was accompanied by increased p27:Cdk2 complex formation, while the steady state levels of Cdk2 were unaltered (data not shown). Also the expression of p21Cip1 increased albeit transiently (Supplementary Figure 2A). In parallel Ser-62 phosphorylation of both c- and v-Myc was reduced by 4 hrs and more prominently by 8 hrs (Figure 5C). Myc turnover began to increase already within 4 hrs and was further accelerated by 8 hrs of IFN-γ+TPA treatment (Figure 5D and Supplementary Figure 1E). Myc degradation therefore correlated kinetically with Myc dephosphorylation, inactivation of Cdk2 and upregulation of CKIs in response to IFN-γ. To rule out that these IFN-γ effects were due to indirect effects on the cell cycle, the cell cycle distribution was analyzed in response to IFN-γ+TPA or roscovitine treatments (Supplementary Figure 2B). Although G1 accumulation was indeed observed at 24 hours after the treatments as previously reported [17] (Supplementary Figure 2B and data not shown), none of the treatments altered the cell cycle distribution at early time points, when the effects on Myc phosphorylation and stability were first observed. The IFN-γ-induced reduction of Myc-Ser-62 phosphorylation might also be explained by upregulation of the serine/threonine phosphatase PP2A [14]. However, no noticeable changes in PP2A activity occurred after IFN-γ+TPA treatment (Supplementary Figure 2C). In conclusion, these results suggested inactivation of Cdk2 by p27 as a key regulatory step in the reduced Myc-Ser-62 phosphorylation and the increased Myc degradation in response to IFN-γ treatment.
Ser-62-dependent Myc stability is regulated by p27 independent of SCFFbw7/Cdc4

The role of p27 in Myc phosphorylation and turnover was analyzed further by enforced expression of p27. Coexpression of p27 reduced phosphorylation of both wt Myc and Myc-T58A (Figure 6A). A T58/S62A double mutant was used as a negative control. Further, p27 reduced the levels of wt Myc and Myc-T58A, but not of a Myc-S62A mutant (Figure 6B). Thus p27 mimicked the effects of IFN-γ. Furthermore IFN-γ-induced degradation of c-Myc was p27-dependent since IFN-γ treatment did not affect the steady state levels of Myc in p27-/- MEF cells (Figure 6C).

Next we addressed whether the SCFFbw7/Cdc4 E3 Ub ligase complex, which previously has been shown to operate via MB1, was involved in p27-induced degradation of c-Myc. As expected, c-Myc was stabilized in Fbw7/Cdc4-/- HCT116 cells (Figure 6D and Supplementary Figure 1F). However, ectopic p27 expression induced increased c-Myc turnover even in the absence of Fbw7/Cdc4, indicating that this F-box protein is not relevant. Since Fbw7/Cdc4-mediated ubiquitylation of Myc is phospho-Thr-58-dependent [7, 8], this result is also in agreement with the ability of p27 to induce degradation of Myc-T58A (Figure 6B). The SCFSkp2 E3 ligase, which does not operate via MB1 [5, 6], also did not seem to participate in p27-induced Myc degradation (data not shown), suggesting that a novel E3 ligase is involved.

IFN-γ treatment reduces phosphorylated and total Myc at chromatin and represses Myc-driven transcription of target genes

To study if IFN-γ affected the status of Myc at target promoters, quantitative chromatin immunoprecipitations (Q-ChIP) was performed using phospho-Myc and pan-Myc Ab and primers for the cyclin D2 promoter [24]. The presence of total and in particular phosphorylated Myc decreased substantially at the promoter in response to IFN-γ (Figure 7A, upper panel). Acetylation of histone H4, a well-established function associated with Myc, decreased after treatment (Figure 7A, lower panel). The same Abs gave very low signals from the non-E-box region of the Fas ligand gene promoter used as a reference gene, and preimmune serum gave only background signals.

To examine the effect of IFN-γ on Myc-driven transcription, a minimal tk promoter driven by four E-boxes in front of a luciferase reporter, m4mintk-Luc, was transfected into U-937-myc6 cells. IFN-γ and IFN-γ+TPA treatment strongly repressed the reporter activity (Figure 7B, upper panel). Similar results were obtained with an α-prothymosin Myc target promoter/reporter construct, containing two Myc-binding E-boxes in the first intron (Figure 7B, lower panel). Constructs lacking (mintk-Luc and a-proTDE) or with mutated E-boxes were insensitive to IFN-γ treatment.
We next measured the mRNA expression of three endogenous Myc target genes, hTERT and ODC, which are activated by Myc, and p21Cip1, which is repressed by Myc, using RT-qPCR. IFN-γ+TPA treatment substantially decreased the expression of hTERT and ODC and strongly induced the expression of p21 (Figure 7C). These results are consistent with the ChIP results and suggest that IFN-γ represses Myc-regulated transcription by reducing Myc association with chromatin.

Discussion

The function and stability of the oncoprotein Myc is controlled by posttranslational modifications. Here we uncovered a new mode of regulation involving phosphorylation by cyclin E/Cdk2 during the cell cycle, in addition to the previously described Erk kinase that phosphorylates Ser-62 in response to serum, Ras and stress signaling [13, 25]. We further demonstrate signal-mediated regulation of this interaction by the cyclin-dependent kinase inhibitor p27.

What is the role of cyclin E/Cdk2-mediated phosphorylation of Ser-62? Since the Cdk2/Cdk1-inhibitor roscovitine accelerates Myc turnover (Figure 4), one role of Ser-62 phosphorylation could be to stabilize Myc, thereby increasing its time of association with chromatin. The phosphate group at Ser-62 could for instance inhibit binding of an as yet unidentified E3 ligase to Myc, either directly or by recruiting a third protein that prevents this binding. The regulation of Myc stability via Ser-62 phosphorylation is, however, complex, since it “primes” Thr 58 phosphorylation and thereby promotes turnover via the GSK3/Fbw7/Cdc4-pathway (Figure 3A). In agreement with this, the relative effect of roscovitine treatment on Myc turnover is more striking for v-Myc, which carries a Thr 58 mutation.

The identification of cyclin E/Cdk2 as Ser-62 kinase was based on sensitivity to small molecule kinase inhibitors and to p27 in vivo, on in vitro kinase assays, and on the correlation of Ser-62 phosphorylation and Cdk2 activity during the cell cycle. We did observe some reduction in Ser-62 phosphorylation upon inhibition of Mek1, an upstream activator of Erk, which is compatible with Erk being a Ser-62 kinase [13], although this reduction was not as efficient as after inhibition of Cdk2/Cdk1. This, however, could be related to cell type or culture conditions. Recently, Cdk1 was suggested to phosphorylate Ser-54 of N-Myc, the equivalent amino acid of Ser-62 in c-Myc, during mitosis in neural precursor cells undergoing differentiation [26]. Even though more selective inhibitors of Cdk1 did not have as strong effects as roscovitine in our system, it is possible that Cdk1 plays a role in phosphorylating c-Myc during mitosis. The fraction of mitotic cells would have been rather low under our experimental
conditions and thus possible CDK1-specific effects might have remained undetected.

An important finding presented here is that growth inhibitory signaling by IFN-γ can interfere with Myc function, an activity that seems to be linked to inhibition of Ser-62 phosphorylation (Figures 3 and 5). This correlates with inhibition of Cdk2 activity and IFN-γ-induced expression of p27 (Figure 5). Further, ectopic expression of p27 inhibited Ser-62 phosphorylation (Figure 6), suggesting that IFN-γ can impinge on the Myc/cyclin E/Cdk2 cooperativity by inducing the expression of p27. This coincides with reduced Myc phosphorylation, loss of Myc, and reduced histone acetylation at the promoter (Figure 7). In addition, IFN-γ repressed Myc-regulated transcription from target promoters in an E-box-dependent manner in agreement with the chromatin binding data (Figure 7).

Our results further demonstrate that IFN-γ stimulates ubiquitylation and degradation of Myc in a Thr-58-independent but Ser-62-dependent manner (Figure 1, 3 and 5), and that p27 is both required and sufficient for this process (Figure 6). Ser-62/cyclin E/Cdk2 therefore seems to regulate at least two distinct pathways that control Myc ubiquitylation and degradation, one involving Thr-58/GSK3/Fbw7/Cdc4, the other a signal-mediated pathway engaging p27 that is independent of both Fbw7/Cdc4 and Skp2 and implicates an as yet unidentified E3 Ub ligase. The identification of this E3 ligase will be an important task for the future.

It is unclear whether the loss of Myc from chromatin in response to IFN-γ treatment is due to turnover at target promoters, as has been proposed for Skp2-induced Myc turnover [6], or whether degradation occurs independent of DNA. From the fluorescence studies using Myc-GFP fusions and BiFC, there was, however, no clear evidence that Myc or Myc:Max were redistributed within the cell in response to IFN-γ treatment prior to degradation (Figure 2). In our previous studies, we concluded that IFN-g destabilized Myc:Max complexes and thereby reduced the DNA-binding capacity of Myc [18]. Of interest is a report that suggested enhanced c-Myc recruitment to the g-GCS promoter as a consequence of Erk-mediated Ser-62 phosphorylation in response to oxidative stress [25]. Reduced Ser-62 phosphorylation could therefore have the opposite effect. It is, however, not entirely clear from this work or from our previous study [18] whether the increased binding of c-Myc to DNA reflects increased Myc stability and thus higher Myc levels or whether Ser-62 phosphorylation modulates the recruitment or the affinity to DNA. This remains to be determined.

Myc is known to promote Cdk2-mediated degradation of p27 in response to proliferative signals, thereby promoting cell cycle progression. Here we demonstrate that p27 in turn is able to regulate Myc in response to anti-proliferative signals such as IFN-γ by inhibiting Cdk2. It is notable that
this negative regulation of c-Myc also applies to the stabilized Myc with mutated Thr-58, which is common in Burkitt’s lymphoma. Thus our findings establish an additional link between Myc and cyclin E/Cdk2. It is worth mentioning that the function of Mad1, a Myc antagonist, cooperates with p27 to promote granulocytic differentiation [27] and is repressed by cyclin E/Cdk2 [28], further supporting the intimate relationship of the Myc/Max/Mad network with cyclin E/Cdk2/p27 in the control of the cell cycle. While Myc and cyclin E are well established oncoproteins, a role of p27 in tumor formation has been postulated but has only become apparent recently [29]. Thus the Myc/Cyclin E/Cdk2/p27 axis may provide a target for intervention. Indeed, many cytokines or other agents that stimulate p27 expression [30] as well as specific inhibitors of Cdk2/Cdk1 [31, 32] are already in clinical use or under development, and could in the light of our new findings be of relevance for combined multi-drug therapies for patients with aggressive Myc–driven tumors.

Experimental procedures

Cell culture and transfections

Cells were cultured in RPMI-1640 (U-937, 2FTHG, Fbw7/Cdc4-/-/HCT116) or Dulbecco’s modified essential medium (DMEM) (Cos 7, HeLa and U2OS) medium supplemented with 10% fetal calf serum and antibiotics. The U937 clone myc6 expresses the OK10 v-myc gene [17]. Exponentially growing U-937 cells (10^5/mL) were treated with 1.6 x 10^-8 mol/L TPA (Sigma, St Louis, MO) and/or 100 U/mL IFN-γ (generously provided by Dr G.R. Adolf, Ernst-Boehringer Institute, Vienna, Austria). To block cells in S-phase, 10 μg/ml aphidicolin was applied for 16-24 hrs. For gene transfer, subconfluent cells were transfected using FuGENE6 (Boehringer Mannheim) or Superfect (Qiagen) according to manufacturers instructions, or were electroporated as described [18]. The following plasmids were used in transfections: CMV-Myc, CMV-MycT58A and CMV-MycT62A, pCIneo-p27, pCMV-cyclin E and pCMV-Cdk2. In luciferase assays the m4mink-Luc, mink-Luc, α-proT-Luc, α-proTAE and pEQ176 (β-gal) reporter plasmids were used.

Protein, mRNA and ChIP assays

Pulse chase, immunoprecipitation and immunoblot analyses were performed as described previously [6, 18]. The following antibodies were used in these assays: α-c-Myc (C33X), α-c-Myc (N262), α-Max (C-17X), α-9E10HRP, α-p27 (C-19), α-cyclin A (BF683X), α-cyclin E (C-19), α-Cdk2 (D-12), (all from Santa Cruz Biotechnology Inc. (SCB, Santa Cruz, CA, USA), phospho-Thr-58/Ser-62 specific Ab (Cell Signaling), phospho N-Myc (S54) Ab A300-206A, which also recognizes c-Myc phosho-Ser-62
(Figure 3B) (kindly provided by Dr. A-M. Kenney and purchased from Bethyl Laboratories Inc), α- PP2A (05-421, Upstate), α-Flag M2 (Sigma), α-Ub clone FK2 (Affiniti) and IG-C rabbit pan-Myc antiserum.

The proteasome inhibitor Z-leu-leu-leu-H aldehyde (MG115) (Peptides International) and N-acetyl-leucinyl-leucinyl-norleucinal-H (LLnL) (Sigma) or vehicle were added to the cells 2 hrs before harvest at a concentration of 50 μM. Roscovitine (Sigma), kenpaullone, PD98059, AR-A014418 and Y27632 (all from Calbiochem) were used at a concentration of 10 μM, fasculysin (Calbiochem) at 0.5 μM, wortmannin (Sigma) at 0.1 μM and SB203580 (Calbiochem) either at 10 or 50 μM. The block protein synthesis, cycloheximide (CHX) was applied at a concentration of 100 μg/ml.

Chromatin immunoprecipitations were performed as detailed [6]. Briefly, cells were crosslinked with 1% formaldehyde on ice for 6 minutes. Nuclear chromatin was sonicated on ice to fragments from 0.3 kb to 0.5 kb. Nuclear chromatin equivalent to 2.5x10^7 cells was immunoprecipitated with 2μg antibody. The following antibodies were used in CHIP: α-pan-Myc (IG-C), α-Ac-H4 (606-866)(Upstate), α-Phospho-Thr58/Ser62-Myc (#9401S)(Cell Signaling), and pre-immune serum (IG-0). The primer sequences for the cyclin D2 promoter (E-box binding site region) and the Fas ligand gene, (non-E-box MEF2 binding site region) used in Quantitative real-time PCR are provided upon request.

Expression and purification of human cyclin A or E/Cdk2 complexes from insect cells and in vitro kinase assays were carried out as described [33]. For PP2A phosphatase assays, 400 μg of cell lysate was IP-ed with α-PP2A antibody as described above, after which a non-radioactive, malachite green-based phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY) based on phosphate release was used according to manufacturer’s instructions.

RT-qPCR analysis of mRNA expression of was performed as described [6]. The primer sequences are provided upon request.

Fluorescence microscopy

2F1G1 cells were cotransfected with constructs containing Myc-GFP together with CFP. BiFC was performed essentially as described [6] using constructs containing c-Myc fused to the C-terminal fragment of YFP (MycYC), Max fused to the N-terminal fragment of YFP (MaxYN) or Ub fused to the N-terminal fragment of YFP (YNUb) (kindly provided by T. Kerppola, Ann Arbor, Michigan). Fluorescence emissions were observed in living cells using an inverted microscope (Zeiss Axiovert 200M, Goettingen, Germany) together with software from Improvision (OpenLab 4.0.1), and black and white images were captured with a Hamamatsu ORCA-ER,
digital camera, where non-treated cells were used as reference for exposure settings. CFP, GFP and YFP fluorescence was measured by excitation at 436, 470 and 513 nm, respectively, and emission at 470, 505 and 535 nm, respectively. Quantification of fluorescence intensities was performed via software from Improvision (Volocity 3.0), on images captured and processed as described above.

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References


Figure Legends

Figure 1. IFN-γ treatment increases the rate of Myc degradation and ubiquitylation

(A) IFN-γ reverses the v-Myc block of monocytic differentiation. Schematic picture describing the U-937 differentiation model system.


(C-D) Combined IFN-γ and TPA treatment (24 hrs) increases degradation of v- and c-Myc in U937-myc6 cells and of c-Myc in c-myc-amplified Colo320 cells (D, right panel). v-Myc contains a point mutation at Thr-58, resulting in its stabilization. 35S-Met pulse-chase was followed by IP using a pan-Myc antiserum and SDS-PAGE. (D) Quantitation of c-Myc turnover by phosphor imager. Note the logarithmic scale of the Y-axis.

(E) IFN-γ-induced c-Myc degradation is JAK/STAT1 dependent. Parental 2fTGH fibrosarcoma cells and U3A STAT-deficient and U4A JAK1-deficient sublines were treated with IFN-γ for 24 hrs, pulse chased and analyzed as above.

(F) IFN-γ increases c-Myc ubiquitylation. 2fTGH cells were treated as indicated for 24 hours and cell lysates were IP-ed with Myc Ab followed by WB using Ub Ab. Note that endogenous proteins are analyzed and none of the components was ectopically expressed.

(G) IFN-γ-induced Myc degradation is proteasome-dependent. Pulse chase analysis was performed in U-937-myc6 cells treated with IFN-γ+TPA for 24 hours in the presence or absence of the proteasome inhibitor LLnL as indicated.

Figure 2. Visualization of c-Myc turnover and ubiquitylation in living cells.

(A-C) IFN-γ treatment increases c-Myc and c-Myc/Max degradation in living 2fTGH cells. The micrographs show a time lapse for the indicated time points after IFN-γ treatment of 2fTGH cells expressing a c-Myc-eGFP fusion protein together with an eCFP construct (A), and a BIFC experiment (B) with c-Myc-YC and Max-YN. Quantification is shown in C with mean values and standard deviations of 50 cells analyzed.

(D and E) IFN-γ treatment increases c-Myc ubiquitylation in living 2fTGH cells as determined by BiFC (c-Myc-YC and YN-Ub). The micrographs show 2fTGH cells pretreated with the proteasome inhibitor MG115 followed by IFN-γ treatment for 4 hours or left untreated. Quantification is shown in E with mean values and standard deviations of 10 cells analyzed.
Figure 3. IFN-γ decreases Myc Ser-62 phosphorylation and induces Myc degradation in a Ser-62 dependent manner

(A) Schematic picture showing the function of the phosphorylation sites in MB1 and their regulation. (1) Phosphorylation of Ser-62 by a priming kinase enables (2) GSK3 to phosphorylate Thr-58 (3) that is recognized by SCFFbw7/Cdc4 leading to ubiquitylation and degradation.

(B) Specificities of the phospho-Ser62 and phospho-T58/S62 Myc Abs. Cos-7 cells were transfected with FLAG-tagged wt c-Myc, T58A, S62A and T58/S62A Myc mutants as indicated. The c-Myc proteins were IP-ed with FLAG Ab, followed by WB analysis using pan-Myc Ab (upper panel), phospho-T58/S62-Myc (middle panel) or phospho-S62-Myc Ab (lower panel).

(C) Immunoblot analysis of v-Myc and c-Myc expression in v-Myc-expressing U-937-myc6 and parental U-937-GTB cells using a mini-gel system.

(D) IFN-γ decreases Myc-Ser-62 phosphorylation. v-Myc expressing U-937-myc6 cells were treated as indicated for 24 hours and for the last two hrs with the proteasome inhibitor MG115. Cell extracts were IP-ed with pan-Myc Ab followed by WB analysis using phospho-T58/S62 (upper panel) or pan-Myc Ab (lower panel).

(E) IFN-γ induces Myc degradation in a Ser-62 dependent manner. 2fTGH cells were transfected with FLAG-tagged T58A or S62A c-Myc mutants, treated with or without IFN-γ for 24 hr, and CHX added for the indicated times. c-Myc was IP-ed with Flag Ab followed by WB analysis using pan-Myc Ab. Quantification was performed using a CCD camera.

Figure 4. Cdk2 is a major c-Myc Ser-62 kinase regulating c-Myc turnover.

(A) Effect of kinase inhibitors on the phosphorylation of c-Myc Ser-62. U2OS cells expressing FLAG-tagged c-Myc-T58A were treated with the indicated inhibitors (4 hrs) and MG115 (2 hrs). c-Myc proteins were IP-ed with FLAG Ab, followed by WB analysis using phospho-T58/S62-Myc Ab (upper panel). WB analysis of total cell lysates using FLAG Ab (lower panel). The following kinase inhibitors were used (targets within brackets): Roscovitine (Cdk2 and Cdk1), kenpaullone (Cdk1 more efficiently than Cdk2), fasculysin (Cdk4 more efficiently than Cdk1 and Cdk2), PD98059 (Mek1), SB203580 (p38 MAPK and less efficiently Jnk), AR-A014418 (GSK3), wortmannin (PI3K) and Y27632 (ROCK).

(B) Cdk2 phosphorylates Myc at Ser-62 in vitro. Purified wt and mutant Myc GST-fusion proteins were phosphorylated using recombinant cyclin E or A/Cdk2 in presence of ³²P-γ-ATP. Histone H1 was used as a control for Cdk2 activity.

(C) Roscovitine inhibits phosphorylation of c-Myc during S phase. U-937 cells were arrested in S-phase by treatment with aphidicolin for 24 hrs. Roscovitine or vehicle was added during the last two hours of aphidicolin
treatment. Myc phosphorylation was measured using P-S62 (upper panel) and P-T58/S62 (middle panel) Ab and compared with total c-Myc using pan-Myc Ab (lower panel).

(D) Roscovitine induces v- and c-Myc degradation. U937-myc6 cells were treated with roscovitine for 2 hrs prior to CHX chase. Myc was IP-ed with pan-Myc Ab followed by WB analyses with pan Myc Ab. Quantitation was performed as in Figure 3E. Note the logarithmic scale of the Y-axis.

Figure 5. IFN-γ-induced p27<sup>kip1</sup> expression correlates with Cdk2 inactivation, reduced Myc phosphorylation and increased Myc turnover

(A) IFN-γ inhibits cyclin E/Cdk2. Lysates from U-937-myc6 cells stimulated as indicated were IP-ed with cyclin E Ab and assayed in vitro for histone H1 phosphorylation. Rosc, roscovitine.

(B) Kinetics of p27 expression following stimulation with IFN-γ+TPA. p27 expression was determined by immunoblot analysis.

(C) Kinetics of reduced Myc phosphorylation. The assay was performed as in Figure 3D using indicated cell extracts.

(D) Kinetics of induced Myc turnover. A CHX chase experiment with quantitation was performed as in Figure 3E using cells treated with IFN-γ+TPA for 0, 4 or 8 hrs as indicated.

Figure 6. p27 promotes reduced phosphorylation and Ser-62 dependent degradation of Myc and is required for IFN-γ-induced Myc turnover

(A) p27 reduces phosphorylation of Myc Ser-62. Flag-tagged wt c-Myc or Myc-T58A were cotransfected with p27 into HeLa cells. c-Myc was IP-ed with Flag Ab followed by WB analysis using phospho-Myc (upper panel) or pan-Myc Ab (lower panel). A T58A/S62A double mutant was used as a negative control.

(B) p27 reduces c-Myc levels in a Ser-62 dependent manner. Flag-tagged wt c-Myc and indicated mutants were cotransfected with p27 into HeLa cells. The total c-Myc levels were determined as described in (A).

(C) IFN-γ-induced degradation of c-Myc is p27-dependent. wt and p27<sup>-</sup> MEF cells were stimulated with murine IFN-γ for 24 hrs and the steady state level of c-Myc was determined as above.

(D) Fbw7/Cdc4 is not required for p27-induced turnover. p27 or a control vector were transfected into HCT116 Fbw7/Cdc4<sup>-</sup> cells after which Myc turnover was determined by CHX chase as described in Figure 3.
Figure 7. IFN-γ treatment reduces phosphorylated and total Myc at chromatin and represses Myc-driven transcription of target genes.

(A) IFN-γ regulates association of phosphorylated and total Myc as well as acetylated histone H4 with chromatin. U-937-myc6 cells treated with IFN-γ+TPA for 6 hrs were analyzed by ChIP using the pan-Myc and phospho-Myc Ab (upper panel), acetylated histone H4 Ab (lower panel) and primers specific for an E-box-containing region of the cyclin D2 promoter. Preimmune serum (pre-Im) was used as negative control. The non-E-box region of the Fas ligand gene was used as a reference gene.

(B) IFN-γ and IFN-γ+TPA repress Myc-driven transcription of target promoter/reporter gene constructs. m4mintk-Luc (containing four E-boxes in front of a minimal tk promoter) and mintk-Luc (lacking the E-boxes) (upper panel) and α-prothymosin containing two Myc-responsive E-boxes in the 1st intron (lower panel) were electroporated into U-937-myc6 cells, and the day after divided into four aliquots and treated as indicted. The next day cells were harvested and analyzed for luciferase activity.

(C) mRNA expression of the indicated Myc target genes were analyzed by Q-RT-PCR in U-937-myc6 cells +/-24 hrs of IFN-γ+TPA treatment.
Supplemental Information

Supplementary Figure 1. Analysis of Myc turnover.
(A) IFN-g+TPA treatment increases c-Myc degradation in Colo-320 colon carcinoma cells. Gel corresponding the experiment described in Figure 1D, right panel.
(B) IFN-g-induced c-Myc degradation is JAK/STAT1 dependent. Gel corresponding the experiment described in Figure 1E.
(C) IFN-g induces c-Myc degradation in a Thr-58-independent manner. Gel corresponding the experiment described in Figure 1F.
(D) Roscovitine induces v- and c-Myc degradation. Gel corresponding the experiment described in Figure 3D.
(E) Kinetics of IFN-g-induced Myc turnover. Gel corresponding the experiment described in Figure 4E.
(F) Fbw7/Cdc4 is not required for p27-induced turnover. Gel corresponding the experiment described in Figure 5D.

Supplementary Figure 2. Kinetic analysis of p21Cip1 expression, cycle distribution and PP2A activity after IFN-g+TPA treatment of U-937-myc6 cells.
(A) IFN-g+TPA, but not IFN-g alone, upregulates p21Cip expression. 35S-Met-labeled cell lysates from cells treated as indicated were IP-ed with p21Cip1 Ab followed by SDS-PAGE and phosphor imager analysis.
(B) Kinetics of IFN-g + TPA-induced G1 cell cycle arrest. The cell cycle distribution was determined by FACS analysis. Data are presented as percentage of cells in the G1/G0, S and G2/M phases of the cell cycle.
(C) IFN-g does not affect PP2A activity. The cells were treated with TPA+IFN-g for indicated times. Phosphatase activity in cell extracts measured as phosphate release was assayed as described in Materials and Methods.
Figure 1 Bahram et al.

Figure 2 Bahram et al.
Phosphorylations in Myc box 1

1. GSK3
2. "Priming" kinase
3. Fbw7 (Ubiquitylation and degradation)

A

Phospho-antibody specificities

B

c-Myc

Ab

pan-Myc

phospho-T58/S62

phospho-S62

C

U-937-myc6

U-937-GTB

c-Myc

v-Myc

pan-Myc western (minigel)

D

U-937-myc6

Contr

TPA

IFN-\gamma

+TPA

P-S58/62-Myc

Total Myc

+ MG115

E

Turnover of c-Myc phospho-mutants

% remaining protein

0 30 60 120 min

T58A Control

T58A IFN-\gamma

S62A Control

S62A IFN-\gamma

Figure 3 Bahram et al.
Figure 4 Bahram et al.
Figure 5 Bahram et al.
Figure 6 Bahram et al.
Figure 7 Bahram et al
Supplemental Figure 2 Bahram et al
Specific requirements for activation of late G1/S phase genes by c-Myc - potential coactivating roles of cyclin E/Cdk2 and Skp2

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Running title: Roles of cyclin E/Cdk2, p27Kip1, and Skp2 for cell cycle-regulated transcription by c-Myc

Abstract
The c-Myc transcription factor/oncoprotein regulates a large number of genes involved in cell growth and proliferation, apoptosis, metabolism, differentiation and many other processes in the cell. c-Myc expression is turned off in quiescent cells but reactivated in response to mitogenic signals and plays an important role in the activation of cells from G0 to G1 and in the progression of the cell cycle from G1 into S phase. We and others have shown previously that the E3 ubiquitin ligase Skp2 interacts with and ubiquitylates c-Myc at the G1/S border, stimulates c-Myc-induced S-phase entry and is required for c-Myc-driven transcription of certain genes. We have further shown recently that the cell cycle regulator cyclin E/Cdk2 phosphorylates c-Myc at Ser-62 during the G1/S transition. This phosphorylation is negatively regulated by the Cdk inhibitor p27Kip1 in response to interferon (IFN)-γ signaling. Using P439-6 lymphoma cells with Tet-regulatable Myc, we confirm that induction of c-Myc in quiescent cells results in its rapid association with target promoters and activation of genes like ODC. In contrast, we show here that S-phase-specific direct Myc targets, such as RFC4, were not induced until late G1 despite early c-Myc binding to its promoter. Interestingly, we demonstrate that Skp2, which is
induced in mid G1, binds to the promoter of the late G1 RFC4 but not to the early ODC promoter, correlating kinetically RFC4 gene activation. We also show that cyclin E/Cdk2 binds to c-Myc target gene promoters and enhances c-Myc-driven transcription. IFN-γ treatment leads to binding of p27Kip1 to c-Myc target promoters, decreased association of total Myc, phosphorylated Myc and cyclin E/Cdk2 as well as strong repression of transcription. These findings suggest that regulation of S-phase genes by c-Myc seems to involve an intricate interplay between the cell cycle regulators and potential cofactors p27Kip1, Skp2 and cyclin E/Cdk2.

Introduction

The c-myc proto-oncogene encodes a short-lived transcription factor that plays an important role in cell proliferation, apoptosis and cancer development (for review see Oster et al., 2002). Two regions are required for transcriptional regulation and oncogenic transformation by c-Myc, the N-terminal transactivation domain (NTAD) and the C-terminal basic/helix-loop-helix/leucine zipper (bHLHZip). The bHLHZip domain mediates dimerization with the cofactor Max, which is required for the binding of c-Myc to E-box elements at target gene promoters and therefore for activation of transcription. The highly conserved region Myc-box 2 (MB2) within the NTAD is required for interaction with a number of proteins, including the coactivator TRRAP, which is a component of histone acetyltransferase (HAT) complexes that participates in the regulation of chromatin structure. c-Myc can also repress transcription by interacting with the initiator-binding zinc finger protein Miz-1. A growing number of target genes regulated by c-Myc have been identified, and recent estimations suggest that these cover 10-15 % of the genome, including genes involved in the cell cycle, cell growth, apoptosis, metabolism, differentiation and immortalization (Oster et al., 2002).

c-Myc is degraded via the ubiquitin/proteasome pathway (for review see Amati 2004). Ubiquitylation of c-Myc involves recognition by at least three E3 ubiquitin ligases, SCFSkp2, SCFFbw7/hCdc4 and HectH9 (Adhikary et al., 2005, Kim et al.2003, von der Lehr, 2003, Welcker et al., 2004). HectH9 conjugates K63-linked ubiquitin chains on the C-terminal domain of c-Myc, which does not lead to proteolysis. SCFFbw7/hCdc4 interacts with phosphorylated residues in MB1 at the N-terminal domain of c-Myc, leading to ubiquitylation and degradation of c-Myc. SCFSkp2 interacts with c-Myc through MB2 and the C-terminus. In contrast to SCFFbw7/hCdc4, SCFSkp2 does not only ubiquitylate and degrade c-Myc but also enhances c-Myc induced transcription. Skp2 is in fact recruited by c-Myc to target gene promoters, correlating with ubiquitylation of chromatin-associated proteins (that could be c-Myc and/or other proteins) (von der Lehr et al., 2003). The proteasome is also found associated with promoters in a c-Myc-dependent manner. The 19S regulatory subunit and possibly the whole proteasome seem to play a positive role in transcription since siRNA-
mediated knockdown of the proteasomal subunit Sug1, like knockdown of c-Myc or Skp2, reduces c-Myc-driven transcription (von der Lehr et al., 2003a, 2003b, for review see Jin and Harper, Lipford and Deshaies, 2003). Skp2 is a cell cycle regulated protein that interacts with c-Myc from late G1 (von der Lehr et al., 2003, Fuji et al., 2006). Skp2 was originally identified as a cyclin/Cdk-interacting protein, and plays a role in activating cyclin E/Cdk2, which is the cyclin/Cdk complex that normally triggers the G1/S transition. The substrates of cyclin E/Cdk2 include the retinoblastoma protein Rb and the cyclin-dependent kinase inhibitor p27Kip1. The Rb family proteins play a crucial role in the cell cycle by controlling the expression of E2F-dependent genes that are necessary for S phase entry. p27Kip1 is a tumor suppressor that controls G1/S transition by inhibiting cyclin E/Cdk2 activity. Phosphorylation on Thr187 of p27Kip1 by cyclin E/Cdk2 leads to its degradation mediated by SCFSkp2 (Kossatz et al., 2006). Skp2, p27Kip1, cyclin E/Cdk2 and Rb therefore constitute an autoinduction loop: cyclin E/Cdk2-induced phosphorylation of p27Kip1 triggers its degradation, which leads to further activation of cyclin E/Cdk2, resulting in further Rb phosphorylation, release of E2F, and further E2F-dependent cyclin E and Skp2 expression. (Yung et al., 2007, Ungermannova et al., 2005, for review see Assion and Yung, 2008). It has been shown that the different functions of c-Myc during cell cycle progression are entangled in a variety of ways with the cyclin E/Cdk2 kinase complex. First, c-Myc binds to E-boxes in the promoters of cyclin D1, cyclin D2, and CDK4 (Bouchard et al., 2001, Fernandez et al., 2003, Hermeking et al., 2000, Menssen and Hermeking, 2002). The cyclin D/Cdk4 complex functions to activate the cyclin E/Cdk2 complex by sequestering p27Kip1. Second, c-Myc induces the phosphatase Cdc25, which dephosphorylates and activates Cdk2 (Galaktionov et al., 1996). Third, c-Myc directly represses transcription of Cdk inhibitor genes such as p15Ink4b, p21Cip1, and p27Kip1 (Claassen and Hann, 1999, Gartel et al., Yang et al., 2001).

It is known that c-Myc induces transcription of both genes early in G1 and genes that are first expressed in late G1/S phase. However, it is at present unclear whether the late genes are indirect targets of c-Myc, and the mechanisms behind these differential inductions therefore remain to be investigated. We know that Skp2 is expressed at late G1 phase and enhances c-Myc induced transcription (von der Lehr et al, 2003). This might indicate that Skp2 is a transcriptional cofactor for a subpopulation of c-Myc target genes of relevance for the G1/S transition of the cell cycle. Cyclin E/Cdk2 has been well known to play a crucial role in G1/S transition and has recently been shown to be a kinase for c-Myc (Bahram et al., paper II). In this study, we are addressing the possibility of interplay between c-Myc, Skp2, p27Kip1 and cyclin E/Cdk2 in the regulation of c-Myc-induced transcription during G1/S cell cycle transition.
Results

Induction of c-Myc target genes at early and late G1 phase

In order to study c-Myc-induced transcription during the cell cycle, the P493-6 human lymphoid B cell line carrying a conditional tetracycline (Tc)-regulatable c-myc construct was used. Proliferation of these cells is strictly dependent on the expression of c-myc. In presence of Tc, c-myc is turned off and the cells go into quiescence. When Tc is removed, c-myc is activated within a few hrs, after which the cells will enter the cell cycle synchronously (Schuhmacher et al., 1999).

We first investigated the expression of Skp2 and cyclins during cell cycle progression in the P493-6 cells after c-Myc activation from quiescent cells. Both protein and mRNA levels of c-Myc were strongly induced within 4 hrs after Tc removal (Fig. 1, 2). Expression of cyclin A increased after 20 hrs (Fig. 1), indicating the progression of cells into S phase at this time point, in agreement with previous reports (Pajici et al., 2000). Skp2 mRNA and protein expression started to increase around 12 hrs and kept increasing until 32 hrs post Tc removal (Fig.1, 2), consistent with previous reports that Skp2 expression starts in mid/late G1 and reaches its maximum during the S and G2 phases of the cell cycle (for review see Nakayama and Nakayama, 2005).

We next addressed the question whether there are direct c-Myc target genes that are specifically activated late in G1 phase or whether all c-Myc targets are induced shortly after c-Myc induction. Schuhmacher et al. reported that the replication factors RFC4, MCM4, and the E2F dimerization partner DP-1 were induced late after c-Myc activation in P493-6 cells (Schuhmacher et al., 2001), making these genes our candidates, although it was not clear if these were direct c-Myc targets. Q-PCR was therefore performed to measure RFC4 mRNA expression levels compared to the previously well characterized direct c-Myc target genes ornithine decarboxylase (ODC) and cyclin D2 (Fig.2). As shown in Figure 2, ODC mRNA levels increased at the latest 6 hrs after Tc removal, i.e. immediately after c-Myc is activated in early G1. Interestingly, the expression of RFC4 did not start to increase until around 12 hrs after c-Myc-induction, and was maximal at 18 hrs and thereafter, which is in agreement with Schuhmacher’s observations. Thus the induction of RFC4 followed the expression pattern of Skp2. cyclin D2 mRNA levels exhibited a biphasic expression pattern during G1 (data not shown), suggesting that the regulation of this gene is quite complex.
Skp2 is found at the promoter of the late G1 c-Myc target gene RFC4 but not at the early gene ODC

The relatively late induction of Skp2 expression in combination with our previous finding that Skp2 acts as a cofactor for c-Myc-induced transcription at the cyclin D2 promoter (von der Lehr et al., 2003a) prompted us to raise the question whether Skp2 could play a role in the induction of late c-Myc target genes as opposed to early genes. To answer this question, CHIP experiments were performed in P493-6 cells. As shown in Figure 3, c-Myc bound strongly to the E-box-containing promoter regions of both ODC and RFC4 already at 6 hrs after Tc removal, although the expression of RFC4 was not activated until several hrs later. In contrast, ODC was activated with the same kinetics as the c-Myc binding to its promoter. Interestingly, Skp2 exclusively bound to the RFC4 promoter and not to the ODC promoter. Skp2 binding to the promoter of RFC4 occurred only at the later time points, thus correlating with Skp2 expression. These results are compatible with a role of Skp2 as cofactor for c-Myc induced activation of late G1 genes such as RFC4.

Cyclin E/Cdk2 binds to c-Myc target gene promoters and acts as a cofactor that enhances c-Myc-driven transcription

The cyclin E/Cdk2 complex plays a key role in the promotion of cell cycle progression into S-phase that is intertwined with c-Myc and Skp2 (Bahram et al, Paper II, Assion and Yung, 2008). Cyclin E/Cdk2-mediated phosphorylation of c-Myc occurs in late G1 (Bahram et al., paper II), we therefore wanted to investigate a possible direct involvement of cyclin E/Cdk2 in c-Myc-induced transcription of late G1 genes. We first examined whether the interaction between c-Myc and cyclin E could be detected by communoprecipitation (coIP) in U-937 cells, the system where we conducted most of our previous phosphorylation studies (Bahram et al, Paper II). U-937 cells are human histiocytic lymphoma cells, that can be induced to differentiate from monoblasts to monocytes/macrophages with substances such as the phorbol ester TPA and interferon (IFN)-γ. As shown in Figure 4A, low but measurable amount of c-Myc specifically coIP-ed with cyclin E. A substantial amount of total c-Myc coIP-ed with Max as expected. This indicates a relatively weak or more transient interaction between c-Myc/cyclin E than between c-Myc/Max. It is conceivable that cyclin E/Cdk2 “touches” c-Myc during the process of Ser-62 phosphorylation (Bahram et al., paper II).

To investigate whether c-Myc and cyclin E/Cdk2 also interact on chromatin, we performed CHIP experiments in U-937 cells using antibodies against cyclin E or Cdk2 respectively. c-Myc was detected on the cyclin D2 promoter (Bouchard et al., 2001) (Fig.4b). Importantly, specific signals were also detected using two different antibodies each for cyclin E and Cdk2, while the two negative control antibodies gave only background
signals. This suggests that cyclin E and Cdk2 indeed associate specifically with E-box regions in the cyclin D2 promoter. To investigate whether c-Myc associates directly with these factors at the cyclin D2 promoter, re-ChIP experiments were performed, where protein/DNA complexes IP-ed with anti-c-Myc antibody were released and reprecipitated with anti-Cdk2 or pre-immune serum. Fig 4c demonstrates that Cdk2 antibody produced signals above background, suggesting that c-Myc indeed forms complexes with Cdk2 at the cyclin D2 promoter. Unfortunately, CHIP signals for cyclin E/Cdk2 were too weak to be picked up in P493-6 cells.

These results indicate that cyclin E/Cdk2 may act as a transcriptional cofactor for c-Myc at certain promoters. To measure the effect of cyclin E/Cdk2 overexpression on c-Myc-induced transcription, we cotransfected a c-Myc-driven promoter/luciferase reporter (m4mintk-Luc) together with c-Myc and/or cyclin E/Cdk2 into U2OS cells. Cotransfection with cyclin E/Cdk2 substantially enhanced c-Myc-driven transcription (Fig.4d), thus supporting the hypothesis that cyclin E/Cdk2 could be a cofactor for c-Myc-driven transcription.

IFN-γ treatment decreases c-Myc and cyclin E binding and increases p27Kip1 binding at c-Myc target gene promoters

We have reported previously that the combination of IFN-γ and TPA treatment overrides the transforming activity of c-Myc and induces differentiation and G1 arrest in v-Myc-transformed U-937 cells (Bahram et al., 1999). Recently, we showed that IFN-γ+TPA treatment induces the expression of the Cdk-inhibitor p27Kip1, which inhibits cyclin E/Cdk2-mediated phosphorylation of c-Myc at Ser-62, including chromatin-associated c-Myc (Bahram et al., Paper II). Our results show that p27Kip1 interacts with c-Myc in U937 cells from communoprecipitation experiments (Fig. 4a), and CHIP experiments also demonstrate that p27Kip1 indeed associates specifically with the E-box region of the cyclin D2 promoter (Fig.4b). The interaction between c-Myc and p27Kip1 is also visualized in HL-60 cells treated with IFN-γ, IFN-γ+TPA and to a lesser extent in TPA-treated cells, but was hardly detectable in untreated cells, in agreement with the expression pattern of p27Kip1 (Fig.5a). We therefore next investigated whether IFN-γ+TPA treatment of these cells affects the association of c-Myc and cyclin E with the early and late G1 c-Myc target genes, ODC and RFC4, respectively. Fig. 5b shows that not only c-Myc but also cyclin E associates with the promoters of both genes in non-synchronized cells, whereas preimmune serum gave only background signals. After IFN-γ+TPA treatment, the presence of c-Myc at the promoters of both genes decreased. Interestingly, also the cyclin E binding to these promoters decreased. The same antibodies gave very low signals from the non-E-box region of the Fas ligand gene promoter used as a reference gene (Fig. 5b, lower panel). Correspondingly, IFN-γ+TPA treatment resulted in the reduction of the mRNA levels of both ODC and
RFC4 (Fig 5c). Consistent with this, the association of p27Kip1 with the cyclin D2 promoter also increases upon IFN-γ+TPA treatment (Fig. 5d). Interestingly, re–CHIP using antibodies against c-Myc and p27Kip1 demonstrates that p27Kip1 antibodies produce signals above background, suggesting that c-Myc indeed forms complexes with p27Kip1 at the cyclin D2 promoter (Fig. 5e).

Together with the results above, these data suggest that binding of cyclin E/Cdk2 to c-Myc target gene promoters during cell cycle progression enhances c-Myc-induced transcription, and that p27Kip1 inhibits cyclinE/Cdk2 kinase activity at the promoter, thereby repressing transcription.

Discussion

c-Myc-induced transcription, like the activity of other DNA-binding activator proteins, requires cofactor activity. For example, TRAAP is known to be necessary for transactivation of some c-Myc target genes, such as hTERT. Other c-Myc target genes, such as CAD, are transcribed independently of TRAAP. This phenomenon suggests the possibility that c-Myc may induce transcription of different genes by cooperating with different coactivators. Former work performed in our group has demonstrated that Skp2 interacts with c-Myc in the nucleus, associates with c-Myc target gene promoters in a c-Myc-dependent manner, and is required for c-Myc-induced transcription and S phase entry (von der Lehr et al., 2003). Here we show that Skp2 is recruited specifically to the promoter of the c-Myc target gene RFC4 during late G1 and S phase of the cell cycle, but does not associate with the promoter of the immediate early c-Myc target gene ODC, not even late after c-Myc induction when this gene is still expressed and Skp2 expression is also high. Other cofactors, such as TRRAP, may be involved in ODC transcription. Taken together, we suggest that c-Myc binds to its target gene promoters soon after its activation, and that some target genes such as ODC are transcribed immediately. Other genes, like RFC4, are kept silent until a certain cofactor, in this case Skp2, is recruited at late G1 phase.

What could be the role of Skp2 in c-Myc induced transcription of late G1 genes, such as RFC4? To answer these questions, more experiments need to be performed. For instance, siRNA could be used to investigate the effects of knockdown of Skp2 on the transcription of RFC4. It should also be investigated whether Skp2 binds to promoters in complex with c-Myc. However, at this point we can show that Skp2, in a still unclear way, is correlated to c-Myc-induced transcription during G1/S transition.

The cyclinE/Cdk2 complex is another important player during G1/S transition. Here we show that cyclin E interacts with c-Myc and enhances c-Myc-induced transcription. Importantly, cyclin E/Cdk2 interacts with
chromatin-bound c-Myc, thereby possibly participating in the regulation of
c-Myc target genes. We recently showed that c-Myc is phosphorylated at
Ser 62 by cyclin E/Cdk2 during late G1 and S-phase, and that cyclin
E/Cdk2-mediated phosphorylation of Ser62 is likely to occur at c-Myc
 target gene promoters (Bahram et al., Paper II). In addition, we have
demonstrated that the growth inhibitory cytokine IFN-γ represses c-Myc-
regulated transcription from target promoters in an E-box dependent
manner (Bahram et al., Paper II), in agreement with our present finding that
both c-Myc and cyclin E binding to c-Myc target gene promoters decrease
whereas p27Kip1 binding increases upon IFN-γ treatment.

Put together, these results imply several possibilities of how Skp2 and
cyclin E/Cdk2 could be involved in c-Myc driven transcription during
G1/S transition. One possibility is that Skp2 functions as a transcriptional
cofactor of c-Myc; i.e. that c-Myc binds to target gene promoters but
cannot induce transcription until Skp2 is recruited to the promoter.
Recently, Skp2 has been found to antagonize the binding of p53 to p300,
thus suppressing p300-mediated acetylation of p53 and the transactivation
ability of p53 (Kitagawa et al., 2008). This brings up a potential pathway in
which Skp2 may function in a similar way to remove the repression on c-
Myc, thereby enhancing c-Myc induced transcription. We also show that
p27Kip1, which is known as a substrate of Skp2-mediated degradation,
binds to c-Myc target gene promoters in complex with c-Myc. One
interesting hypothesis is that Skp2 ubiquitylates and degrades p27Kip1 at the
promoter, thereby releasing its inhibition of cyclin E/Cdk2, which in turn
would assist the coactivating function of cyclin E/Cdk2 on c-Myc induced
transcription. This, however, remains to be investigated. The mechanism by
which cyclin E/Cdk2 carries out a coactivator function for c-Myc is also
unclear. One possibility is that the c-Myc transactivation function is
enhanced through Ser62-dependent phosphorylation. Cyclin E/Cdk2-
mediated phosphorylation of Ser62 stabilizes c-Myc, thereby increasing its
time of association with chromatin (Bahram et al., Paper II). Cyclin E/Cdk2
is also able to phosphorylate other components in the Myc transcriptional
complex such as the histone acetyl transferase cofactor CBP (Ait-Si-Ali et
al., 1998, Vervoorts et al., 2003). Finally the kinase itself may serve as an
adaptor for recruitment of other factors. It is therefore conceivable that
cyclin E/Cdk2 assists c-Myc in the regulation of a subpopulation of genes of
relevance for G1/S cell cycle transition. In conclusion, further investigations
are required to fully understand the possible role Skp2 and cyclin E/Cdk2
in the complex mechanisms by which the activation of different subsets of
c-Myc target genes are regulated.
Methods and materials

Cell culture

Cells were cultured in RPMI-1640 (U-937-myc6, p493-6) or DMEM (HL-60, U2OS) medium supplemented with 10% fetal calf serum and antibiotics. To synchronize the P493-6 cells, exponentially growing cells were treated with 0.1mg/ml tetracycline in RPMI-1640 supplemented with 0.25% serum for 72hr. Thereafter, in order to activate c-Myc, cells were washed with cold PBS and re-cultured in RPMI-1640 medium supplemented with 10% serum without tetracycline and harvested at the indicated time points. Exponentially growing U-937 cells were treated with 1x10^{-7} mol/L TPA (Sigma, St Louis, MO) and 100 U/mL IFN-γ for 6 hrs.

Transfections and reporter activity analysis

Subconfluent U2OS cells were transfected using FuGENE6 (Boehringer Mannheim). The plasmids used are CMV-Myc, pCMV-cyclin E and pCMV-CDk2. The m4mintk-Luc reporter plasmids were used in luciferase.

Protein and ChIP assays

Immune precipitations and immunoblot analyses were performed as described previously (Bachrati et al., 1999; von der Lehr et al., 2003). The following Abs were used: α-c-Myc (C33X), α-c-Myc (N262), α-Max (C-17X), α-cyclin E (C-19), and α-cyclin A (BF683X), (all from Santa Cruz Biotechnology Inc. (SCB, Santa Cruz, CA, USA), and pre-immune serum (IG-0).

Chromatin immunoprecipitations were performed as described (von der Lehr et al., 2003). Briefly, cells were crosslinked with 1% formaldehyde on ice for 6 minutes. Nuclear chromatin was sonicated on ice to fragments of 0.3 kb to 0.5 kb. Nuclear chromatin equivalent to 2.5x10^7 cells was immunoprecipitated with 2μg antibody and analyzed by PCR. In the re-ChIP assay, 50μl release buffer (1%SDS, 10mM DTT, TE pH 7.5) was added to the beads after the first immunoprecipitation step, and incubated 30 min at 37 °C. The supernatant was then used for immunoprecipitation with a second antibody as in the ChIP assay.

The following antibodies were used in ChIP: α-pan-Myc (IG-C), α-Skp2 p45 (H-435X), α-cyclin E (HE-111X), α-cyclin E (M-20), α-CDK2 (M-2), α-CDK2 (H-298), α-P27 (C-19), α-P27 (F-8), α-Gal4(DBD)(sc-577) (all from Santa Cruz) and pre-immune serum (IG-0). The primer sequences for the cyclin D2, ODC, and RFC4 promoters (E-box binding site region) and the Fas ligand gene (non-E-box MEF2 binding site region) used in Quantitative real-time PCR are provided upon request.
RT-qPCR assay

Total RNA was extracted from P493-6 or U-937-myc6 cells using QIAshredder spin columns and RNeasy mini kit (Qiagen Sciences). Samples were DNase-treated before cDNA synthesis was performed with oligo(dT) and RevertAid M-MuLV Reverse transcriptase from Fermentas. cDNA was used as template in quantitative PCR reactions with DyNAmo HS SYBR Green qPCR kit from Finnzymes. The comparative Ct method was used for relative quantification of expression levels. The expression levels of the genes of interest were normalized to the expression level of β-actin. The primer sequences are provided upon request.

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References


Figure Legends

**Figure 1.** Expression of c-Myc, Skp2, and cyclin A protein levels after c-Myc activation in P493-6 cells.

Cells were harvested at different time points after Tc removal and analyzed by western blot using specific antibodies as indicated.

**Figure 2.** Kinetics of c-Myc-induced expression of ODC and RFC4 target genes.

Q-PCR analysis showing relative mRNA levels of c-myc, skp2 and the c-Myc target genes ODC and RFC4 was performed in P493-6 cells harvested at indicated time points after Tc removal.

**Figure 3.** c-Myc and Skp2 occupancy at RFC4 and ODC c-Myc target promoters.

ChIP experiments were performed with chromatin prepared from P493-6 cells harvested at indicated time points after removal of Tc using indicated antibodies and primers. pre-Im, preimmune serum. The non-E-box region of the Fas ligand gene was used as a reference gene.

**Figure 4.** CylinE/Cdk2 interacts with c-Myc on the cyclin D2 promoter and enhances c-Myc-driven transcription.

A) Cyclin E interacts with c-Myc. c-Myc was coIP-ed from extracts from U-937-myc6 cells treated with IFN-γ for 24 hrs using indicated antibodies, after which Western blot analysis was performed using pan-Myc antibodies.
B) Cyclin E, Cdk2 and p27 associate with an E-box-containing region of the cyclin D2 promoter. Exponentially growing U-937-myc6 cells were used for ChIP using the indicated cyclin E, Cdk2, p27Kip1 and c-Myc antibodies. Preimmune serum (PI), GFP and Gal4 antibodies were used as negative controls.
C) c-Myc interacts with Cdk2 at the cyclin D2 promoter. Re-ChIP analysis using the indicated antibodies was performed as described in Materials and Methods.
D) CylinE/Cdk2 enhances c-Myc-driven transcription. The reporter construct m4mintk-Luc, containing four E-boxes in front of a minimal tk promoter, was cotransfected with c-Myc and/or cyclin E/Cdk2 expression vectors into U2OS cells. Luciferase activity was measured 48 hrs later.
Figure 5. IFN-γ regulates the association of c-Myc, cyclin E and p27Kip1 with target promoters.

A) The interaction between c-Myc and p27Kip1 increases after stimulation with IFN-γ and/or TPA. CoIP of c-Myc and p27Kip1 was performed using extracts from HL-60 cells treated with IFN-γ for 24 hrs.

B) IFN-γ + TPA treatment reduces association of c-Myc and cyclin E with RFC4 and ODC promoters. U937-myc6 cells were treated with IFN-γ (100 U/ml) and TPA (10^{-7} M) for 6 hrs. ChIP experiments were performed with indicated antibodies and pre-immune serum.

C) IFN-γ+TPA represses the expression of ODC and RFC4. RT-qPCR was performed using mRNA extracted from U973-myc6 cells treated with IFN-γ+TPA for 24 hrs.

D) IFN-γ regulates association of p27Kip1 with chromatin. U-937-myc6 cells treated with IFN-γ+TPA for 6 hrs were analyzed by ChIP using antibodies against p27Kip1 and preimmune serum (pre-Im). The non-E-box region was used as a reference gene as in Fig 3.

E) c-Myc interacts with p27Kip1 at the cyclin D2 promoter. Re-ChIP analysis using the indicated antibodies was performed as described in Materials and Methods.
Figure 3

Figure 4
Figure 5
Characterization of cell cycle regulated bHLH genes in Arabidopsis

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Running title: Cell cycle-regulated bHLH genes in Arabidopsis

Abstract

Transcription factors (TF) of the basic region helix-loop helix (bHLH) family constitute the second largest TF family in the plant Arabidopsis thaliana (At) and play role in many developmental processes and in signal transduction, for instance in response to light and stress. Little is, however, known about the transcriptional regulation of cell division in plants. In animals, bHLHZip genes of the MYC family play important roles in cell proliferation by regulating, for instance, cell cycle genes such as cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. The MYC family genes are conserved throughout animal evolution and are regulated in a cell cycle-dependent manner. We asked whether there exist MYC-like genes in plants with similar functions as in animals. Using the very conserved bHLHZip domains of animal Myc-family proteins, we searched the Arabidopsis genome and found 84 bHLH genes belonging to the same ancient B subgroup of eukaryotic bHLH genes as MYC. These could be further subdivided into 15 subfamilies that were evolutionary separated from the animal bHLH genes. Examination of a Myc-family “fingerprint” of 21 conserved amino acids in the bHLH domain revealed that 18-20 of these amino acids were conserved at the identical positions in most of the 15 At bHLH subfamilies, suggesting that they are “Myc-like” to some extent. To gain insight into the potential functions of the At bHLH genes, we
examined the expression profile of these genes in proliferative tissues of Arabidopsis. Seven different cell division systems were studied, including shoot apex, flowers, leaves, siliques/seeds, root as well as auxin-induced early lateral root initiation and sucrose-induced cell cycle progression in suspension cultures. We found that one gene in particular, At1g26260, was auxin-induced and regulated in a cell cycle-dependent manner in six out of the seven systems. Four other genes, At1g10120, SPATULA, At3g25710 and At3g1950 were cell cycle regulated in five systems, and a total of 15 At bHLH genes scored positive in at least two of the systems. This suggests that some of these genes may potentially have a role in cell proliferation in general, while others may have more cell type-specific proliferative functions. Interestingly most of these genes clustered in 3 subgroups of At genes. Homozygous disruption by T-DNA insertion did, however, not result in phenotypic aberrations in six of these genes, this possibly due to redundancies. In conclusion, we have identified a number of “Myc-like” bHLH genes that are regulated in a cell cycle-dependent manner and that may potentially play a role in cell division in plants.

Introduction
Transcription factors of the bHLH-family play important regulatory roles during neurogenesis, myogenesis, hematopoiesis, cell proliferation, differentiation, cell lineage determination, sex determination and other important processes in animals. The common bHLH domain of these proteins is around 60 amino acids long and consists of a basic region (b), which binds specifically to E-box DNA recognition sequences in regulatory regions of target genes, and the HLH domain, which mediates protein-protein interactions, leading to the formation of homo- or heterodimers. The HLH domain forms two amphipathic a-helices separated by a small loop, where helix I forms a continuous a-helix with the basic region (for review see (Lüscher and Larsson, 1999).

The bHLH-family can be subdivided into six different groups, A-F, based on their sequence similarity, DNA-binding specificity and the presence or absence of additional domains (Atchley and Fitch, 1997; Ledent et al., 2002; Ledent and Vervoort, 2001). The A-group proteins, which are only found in animals, typically bind E-boxes with the sequence CAGCTG or CACCTG and include factors involved in animal neurogenesis, myogenesis, hematopoiesis and other developmental processes. The prototype of this subgroup is MyoD, a factor involved in muscle differentiation. bHLH B-group have been found in animals, fungi and plants, and is thought to be a evolutionary more ancient group (Atchley and Fitch, 1997). The c-Myc oncoprotein is the proto-type of this group, which further includes the animal SREBP, USF, TFE, and fungal Esc, Cbf and Ino and a very large group of plant genes (see below). These proteins bind E-boxes (called G-boxes in plants) with the sequences identical or similar to CACGTG. The B-group can be subdivided into members containing or
lacking a leucine zipper (Zip) motif, which is involved in homo- and heterodimerization and is characterized by leucines or other hydrophobic residues at every 7th position. Group C contains PAS domain proteins, such as hypoxia inducible factor (HIF) and the Drosophila SIM protein, that bind ACGTG or GCGTG DNA sequences, while group D contains HLH proteins lacking the DNA-binding region, including the Id proteins that negatively regulates many bHLH proteins by forming unproductive heterodimers unable to bind DNA. Recently two new subgroups have been found, E, including the Drosophila Hairy and Enhancer of split genes, which contains an orange domain and a WRPW motif and preferentially bind to so called N-box sequences (CACGCG or CACGAG), and F, which contains a COE-domain involved in dimerization and DNA binding (Ledent et al., 2002; Ledent and Vervoort, 2001).

The plant *Arabidopsis thaliana* contains up to 160 bHLH genes that can be further divided into a number of subgroups (Bailey et al., 2003; Buck and Atchley, 2003; Heim et al., 2003; Riechmann et al., 2000; Toledo-Ortiz et al., 2003). This constitutes the second largest transcription factor family in *Arabidopsis* after the MYB superfamily of around 190 members. Most of the plant bHLH genes belong to the B group and are involved in a number of different processes. Among these are the R subfamily gene *TT8*, which regulates the expression of genes required for flavonoid/anthocyanin biosynthesis (Nesi et al., 2000), *GL3* and *EGL3*, regulating trichome and root hair initiation (Bernhardt et al., 2003; Payne et al., 2000; Zhang et al., 2003), *SPATULA*, involved in carpel development (Heisler et al., 2001), *ALCATRAZ* and *INDEHISCENT*, regulating fruit opening (Liljegren et al., 2004; Rajani and Sundaresan, 2001), *SPEECHLESS*, *MUTE* and *FAIMA*, involved in stomata development (MacAlister et al., 2007; Pillitteri et al., 2007). A number of bHLH proteins take part in positive or negative regulation of light signaling, either by direct interaction with phytochrome A and/or B photoreceptors, including *PIF3* (Ni et al., 1998), *HFR1* (Fairchild et al., 2000), *PIF4* (Huq and Quail, 2002), *PIF1/PIL5* (Huq et al., 2004; Oh et al., 2004; Yamashino et al., 2003), *PIF5/PIL6*, PIF6/PIL2 (Khanna et al., 2004; Yamashino et al., 2003), and PIL7 (Leivar et al., 2008), or as downstream transmitters of light signals such as PIL1 (Khanna et al., 2006; Yamashino et al., 2003), for reviews see (Duek and Fankhauser, 2005; Monte et al., 2007). bHLH proteins also play a role in other types of signaling, for example responses to drought stress and the plant hormone abscisic acid (ABA) (AtMyc2, AtMyc3 and AtAIG1) (Abe et al., 2003; Kim and Kim, 2006; Smolen et al., 2002), in jasmonate signaling (AtMyc2) (Boter et al., 2004), cold stress (ICE) (Chinnusamy et al., 2003) and to brassinosteroids (BEE genes) (Friedrichsen et al., 2002). Most of these bHLH proteins cooperate with other transcription factors, in particular members of the Myb-family that play important roles in a variety of processes.
The knowledge about transcription factors regulating the cell cycle in plants is relatively limited. The basic mechanisms of cell cycle regulation in plants are similar to those in animals (for review see (De Veylder et al., 2007)). There are two major cyclin-dependent kinases, CDKA and CDKB, which regulate the transition from the G1 to S and G2 to M phases of the cell cycle, respectively. CDKA associate with G1 cyclins of the cyclin D group (CYCD) and later in S phase with cyclin A (CYCA), whereas CDKB associates with G2/M cyclins CYCA and cyclin B (CYCB). There are several CDK inhibitors (CKIs) related to the animal p27Kip1 family, called Kip-related proteins, KRP (or ICK). The animal Rb/E2F pathway is also conserved in plants. The E2F transcription factor that controls expression of S-phase specific genes associates with the retinoblastoma protein-related corepressor RBR in G1 to keep these genes silenced. In late G1, CYCD/CDKA phosphorylates RBR resulting in release of E2F and activation of S-phase genes (for review see (De Veylder et al., 2007)). The AP2-related factor AINTEGUMENTA (ANT), the KNOTTED-like HOMEOBOX (KNOX) proteins and the WOX homebox protein STIP are among transcription factors that have been implicated in coordinating cell division and cell growth in meristematic tissues and to directly or indirectly regulate expression of D-type cyclins (for reviews see (Gegas and Doonan, 2006; Traas and Bohn-Courseau, 2005)). Further, TCP and MYB-type transcription factors have been reported to regulate B-type cyclins.

In animals, the B-group bHLHZip proteins of the MYC-family (including c-, N- and L-Myc among others), play an important role in regulating the cell cycle, cell growth and metabolism and are thought to be key coordinators of these processes (Adhikary and Eilers, 2005; Oster et al., 2002). The Myc-family proteins cannot homodimerize but needs the obligatory bHLHZip protein Max to form DNA-binding heterodimers. Max also forms heterodimers with the members of the Mad/Mnt-family, which all are transcriptional repressors that compete with and antagonize Myc. The MYC genes are conserved throughout animal evolution and some similar proteins have also found in fungi (Atchley and Fernandes, 2005). It controls 10-15 % of all genes in mammalian cells, including D- and B-type cyclins, G1 CKDs, several CKIs, E2F, Cdc25 and E3 ubiquitin ligases involved in cell cycle control. Since many regulators of the cell cycle are fairly conserved between animals and plants, we were interested to see if Myc-related bHLH proteins could be found in plants and if they would carry out similar functions. The bHLH domain of MYC contains a number of highly conserved residues that can be used as a “fingerprint” for Myc-like genes in distantly related species (Atchley and Fernandes, 2005; Lüscher and Larsson, 1999), and we therefore used this domain for screening the genome of Arabidopsis. Further, since the expression of the animal Myc genes are cell cycle regulated, we investigated the expression of Arabidopsis bHLH genes in several cell cycle and cell growth systems. Our results demonstrate that 84 B-group bHLH genes exist in Arabidopsis that evolutionary separated from...
animal bHLH genes but are still “Myc-like” to a certain extent. 15 of these
genes were expressed in a cell cycle-dependent manner in one or more
proliferative tissues.

Results

Classification of Arabidopsis B-group bHLH genes.

In an attempt to identify MYC-like B-group genes in Arabidopsis thaliana
(At), the bHLHZip domains of a number of animal Myc, Max and Mad
proteins from different species (Supplemental Fig. 1S) were used to screen
At databases utilizing the BLAST Psi- and Phi-BLAST programs. We
identified 84 B-group At bHLH sequences out of the total number of
approximately 160 bHLH genes. This group is characterized by a
characteristic H-E-R “fingerprint”, namely H at position five, E at position
nine and R at position thirteen out of the 13 amino acids in the basic region
(Ref). These amino acids are all found on same side of the alpha helix of the
basic region and are involved in recognition of the CACGTG E-box
sequence. A neighbor-joining phylogenetic tree was constructed with
amino acid sequences of the bHLHZip domains of these At genes using
MEGA 4.0. As shown in Fig. 1, the tree divides the At B-Type bHLH
genes into 15 phylogenetic subfamilies (which we named from 1-15) with
high predictive values (bootstrap support of 50 or greater) and 8 orphan
genes that could not be grouped into any of these subfamilies. These
subfamilies are overlapping but not entirely identical to some the 15, 12 and
21 subfamilies of At bHLH genes proposed by Buck et al., Heim et al. and
Toledo-Ortiz et al., respectively (Buck et al., 2003, Heim et al., 2003,
Toledo-Ortiz et al., 2003). The reason for this could be that we used also
the Zip regions in our search and may have used slightly different criteria
for the selection of the genes. In agreement with these reports, we find that
the 15 plant B-group bHLH subfamilies are all separated evolutionary from
the animal MYC/MAX/MAD family genes. Also after inspecting the amino
acid “fingerprints” typical of the Myc/Max/Mad family proteins as a whole
or of the individual subfamilies of Myc, Max and Mad proteins,
respectively (Supplemental Fig. 1S), it was not possible to identify any
particular At B-group bHLH subfamily that was much more MYC, MAX or
MAD-like than the other, although all of them had many features in
common (data not shown). Among the 21 amino acids that are conserved
in the basic region, helix one, loop and helix two during animal evolution
of MYC/MAX/MAD family genes (Supplemental Fig. 1S), most of the At
subfamilies have 18–20 of these amino acids conserved in the same positions.
Many of these genes also seems to contain a leucine zipper (Zip), or at least
parts thereof, continuous with the bHLH domain, another hallmark of the
animal Myc/Max/Mad family proteins. From these points of view one
could argue that the At B-group bHLH family genes are all more or less
“Myc-like”. However, these similarities do not go beyond the bHLHZip
domain, and we have for instance not found any sequences in the plant genes resembling the conserved Myc-boxes in the transactivation domains of the animal genes.

Identification of *Arabidopsis* bHLH genes expressed in a cell cycle-regulated manner in shoot apex, flowers, leaves and/or siliques/seeds

We have thus identified 84 B-group *At* bHLH genes that to some extent exhibit similarities with the animal MYC/MAX/MAD family genes. As described above, the MYC- and MAD-family gene products are involved in regulation of cell cycle genes such as cyclins and CKIs and are themselves also usually differentially regulated during the cell cycle. MYC and MAD family expression in animal cells often correlates with G1 cyclin and CKI expression, respectively, whereas the common heterodimeric partner MAX is ubiquitously expressed. To investigate whether *At* bHLH genes might show functional similarities with the animal MYC/MAX/MAD family genes, we investigated the expression patterns of the 84 *At* genes with respect to proliferative tissues of the plant by searching the AtGenExpress microarray database (for address see Supplemental Table 1S). Supplemental Table 1S shows the retrieved data for 15 *At* bHLH genes that showed expression profiles correlating with proliferative status in shoot apex, flowers, leaves and/or siliques/seeds (Jacqmard et al., 2003; Mordhorst et al., 1998; Zhang et al., 2005). Among these were four genes described previously, *SPATULA* (*SPT*), *ALCATRAZ* (*ALC*), *EGL3* AND *PIF3* (see above). The details of developmental stages etc of the tissues studied are found in Supplemental Table 2. As reference genes, four genes encoding cycle regulators genes were used; the G1 cyclins *CYCD3;3* and *CYCD3;1*, the transcription factor *E2Fa*, and the Cdk inhibitor *KRP1*. The expression of these reference genes showed very good correction with proliferative status of the tissues, *CYCD3;3*, *CYCD3;1* and *E2Fa* being highly expressed in proliferative tissues and vice versa for *KRP1*.

As shown in Table 1, three of these genes, such as *At1g10120*, *At1g68810* and *At3g25710*, were coregulated with *CYCD3;3*, *CYCD3;1* and *E2Fa* in all four tissues. Others were coregulated with these cell cycle genes in three out of four tissues, while others had similar expression patterns as the cyclins in some tissues, but resembled more the expression of *KRP1* in other tissues. Some of the genes were differentially expressed between tissues or during the development of a certain tissue, but in a way that seemed unrelated to the cell cycle. This suggests that some of these genes may have roles in cell proliferation in general, while others may have more cell type specific proliferative functions, others may have more complex roles and finally, some genes are not likely to play a direct role in cell division.
Expression of At bHLH genes during the proliferative stage of root development

To investigate whether any of these bHLH genes are coregulated with cell cycle genes in other tissues as well, we utilized published microarray expression profiling data from *Arabidopsis* root (Beemster et al., 2005; Birnbaum et al., 2003). Root developmental can be divided into three stages that roughly correspond to the distance from the apical meristem. The first stage of active cell proliferation and differentiation originating from the apical meristem occurs in the dividing zone in the root tip, the second stage is when longitudinal expansion begins in the elongating zone, and at stage 3, cells and root hairs are fully elongated and found in the mature region. As expected, highest expression of CYCD3;1 and CYCD3;2 and lowest expression of KRP1 and KRP2 was found in stage 1 in the dividing zone and vice versa in the stage 3 in the mature region (Fig. 2 and Supplemental Table 3S). Nine of the bHLH genes showed expression patterns that were similar to the cyclins, including At3g25710 that were amongst the genes that were coregulated with cyclins in all of the previous tissues, and At1g26260, *SPT*, *EGL3* and At3g61950 that were coregulated in three out of four of the previous tissues. Two of the genes showed an expression pattern that was similar to *KRP 1* and 2 (Fig. 2 and Supplemental Table 3S, summarized in Table 1).

Expression of At bHLH genes during auxin-induced early lateral root initiation and cell cycle progression

We next looked at the expression of the bHLH genes in an auxin-induced cell division system, early lateral root initiation (Himanen et al., 2004). In this system pericycle cells are blocked in G1 by germination in the presence of 1-N-naphthylphthalamic acid (NPA), resulting in roots lacking lateral root initiation sites. By transfer to media containing auxin, lateral root initiation and cell cycle progression is induced synchronously. Here we utilized published microarray expression data from Vanneste et al. (Vanneste et al., 2005), where both wt *Arabidopsis* plants and the dominant auxin signaling mutant solitary root (slr1) were analyzed using this system. The mutant plants are defective in auxin-induced lateral root initiation, which is linked to defective induction of key cell cycle components, including CYCD3;1 (Vanneste et al., 2005). Interestingly, the bHLH gene At1g26260, which was coregulated with CYCD3 in four out of the five tissues studied above (Table 1), was strongly induced after 6 hrs of auxin treatment in wt plants but not in the slr1 mutant, suggesting that it plays a role in auxin-signaling and further implicates it in cell cycle regulation (Fig. 3 and Supplemental Table 3S, summarized in Table 1). The expression of two bHLH genes, At3g25710 and PIF3, was repressed by auxin signaling in wt but not in mutant plants.
Expression of At bHLH genes during sucrose-induced cell cycle progression in suspension cultures

To further investigate the expression of the selected At bHLH genes during the cell cycle, we utilized an Arabidopsis suspension cell culture system in which the cells are synchronized in G0 by sucrose starvation. After re-addition of sucrose to the medium the cells enter and traverse the cell cycle synchronously (Menges and Murray, 2002). Cultures were harvested at different times after sucrose addition, after which total RNA was prepared. The mRNA expression of the selected At bHLH genes was analyzed by Q-RT-PCR. Fig. 4a shows that the expression of CYCD3;3 was induced and reached a peak already within two hours, while the induction of CYCD3;1 was slower and reached its maximum level 6 hrs after sucrose treatment in agreement with previous reports (Menges et al., 2005; Menges et al., 2003). The expression of KRP2 slightly increased transiently at 2 hrs and then declined as reported (Menges et al., 2005). Analysis of the expression of the At bHLH genes showed that they fell into three different categories (Fig. 4). Four genes, At3g07340, At1g10120, At5g50915 and ALC, exhibited an expression pattern that were similar to the CYCD3 genes, i.e. they were induced with different kinetics, reached their peaks at different time points and gradually declined back to basal level (Fig. 4b). With the exception of At3g07340, these genes are all coregulated with CYCD3 genes in 4-5 different systems (Table 1). At3g07340 has in three systems shown similar expression patterns as CYCD3 genes and in siliques/seeds. Another group of three genes, At1g26260, SPT and At3g61950 were all induced very rapidly and reached high peak levels already at 2 hrs post treatment, after which they rapidly fell back to basal levels (Fig. 4c), and we refer to these as immediate early transiently expressed genes. They are all genes that are coregulated with the cyclins in 5-6 other systems (Table 1). Finally, we have a category of three genes, PIF3, EGL3 and At1g68810, that shows an expression pattern similar to KRP1/2 (Fig. 4d). They all drop to levels lower than the basal level after sucrose induction, PIF3 after an initial slight and transient increase that also resembles KRP2. PIF3 has shown KRP-like expression patterns in two other systems, whereas EGL3 and At1g68810 have rather been expressed in a cyclin-like pattern in most other systems (Table 1).

After microarray analysis of gene expression in a similar sucrose starvation/re-addition suspension culture system in Arabidopsis, Menges et al (Menges et al., 2003) obtained similar results as we did for At3g07340 and At5g50915, but had opposite results for EGL3 and At1g10120. The reason for this discrepancy is unclear at present, but we have used different methods for measuring mRNA expression, utilized different cell suspension systems and included sorbitol in the medium to avoid osmotic stress.
T-DNA insertion mutants of cell cycle regulated At bHLH genes do not display gross phenotypic aberrations

To approach the question of the biological function of the above studied bHLH genes, we identified and obtained *Arabidopsis* lines with T-DNA insertions in six of the bHLH genes from the SALK Institute. These were verified by PCR analysis and predicted to knock out the functions of At1g26260, At1g10120, At3g61950, EGL3, ALCATRAZ, and SPATULA. We obtained homozygous mutants that were seeded out for phenotypic examination. Under normal culture conditions, no gross phenotypic aberrations were observed. We also obtained spt and alc homozygous mutants, which exhibited the expected gynoecia phenotypes. When crossing these homozygous mutants with each to obtain double mutants in all combinations, our preliminary data suggests that these double mutants do not display phenotypic aberrations under these culture conditions (data not shown). Exceptions were as expected any mutants containing spt and alc, which, however, did not show any further phenotypic alternations other than those of the single mutants (data not shown).

Discussion

The overall aim of the present study was to try to explore whether MYC-like bHLH genes could be found in the plant *Arabidopsis thaliana* and if so, whether such genes might carry out similar functions as in animals, i.e. control genes involved in cell proliferation. We identified 84 B-type bHLH genes, likely to encode proteins recognizing the Myc binding site CACGTG. The genes could be subclassified into 15 subgroups, but these were all separated evolutionary from animal bHLH genes and none of these were more similar to animal MYC/MAX/MAD-family genes than the other. On the other hand, when comparing 21 conserved amino acid residues in the bHLH domain that are conserved during animal evolution of the Myc/Max/Mad-family, 18-20 were conserved at identical positions in most of the 15 At subfamilies, suggesting that they are “Myc-like” to a certain extent. Many of these subgroups also seem to contain a functional Zip-domain, which is always found in Myc/Max/Mad proteins, in particular in group 1, where most of our cell cycle regulated genes were found. These observations suggest that the function to bind CACGTG E (G)-box sequence elements by forming hetero- or homodimers is conserved. However, no similarities were found to other regions of Myc, including the conserved Myc boxes in the transactivation domain. This was on the other hand not so expected, since transactivation domains are much less conserved in evolution.

The question remains if any of these At bHLH genes could be involved in control of cell proliferation similar to the MYC-network genes? Investigation of the expression patterns of the genes in proliferative tissues of the plant could give some indications of their functions. Indeed, most
Myc/Max/Mad-family genes, with the exception of Max, are cell cycle regulated. Expression profiling in shoot apex, flowers, leaves and siliques/seeds indicated that 15 of the 84 bHLH genes seemed to be differentially regulated in proliferative tissues in at least some of these systems. Further analysis of root development, auxin-induced early lateral root initiation and sucrose-induced cell cycle progression in suspension cultures narrowed down the selection to around 10 genes of interest. Summary of the analysis in the seven different cell proliferation systems revealed that one gene, At1g26260, is auxin-induced and coregulated with the cyclins in 6 systems, four genes, At1g10120, SPT, At3g25710 and At3g61950, are coregulated with cyclins in five systems, two genes, At5g50915 and ALC, in 4 systems, two other genes, At1g68810 and EGL3 also coregulated with cyclins in four systems but sometimes with KRP1/2 in other systems (Table 1). Other genes are more often coregulated with KRP1/2, such as At3g07340 and PIF3, and the remaining only show cell cycle regulation in a few tissues. We find it likely that at least some of the former genes play a role in cell proliferation in general or at least in certain tissues and/or in response to certain signals. Interestingly most of these are clustered in 3 subgroups of genes.

To clarify the precise function of these candidate genes it will be necessary to perform knockout and/or knockdown the activity of these genes. Analysis of homozygous T-DNA insertion mutants of four of the previously non-characterized genes that would predict to render the genes inactive did not show any phenotypic aberrations under normal growth conditions. One plausible explanation for this is redundancy, i.e. that other genes in the large bHLH family can take over the function of the non-functional gene. Our preliminary results from studies of double mutants have not resolved this problem. It will therefore be necessary to generate triple or higher order mutants or to knockdown the expression of many family genes simultaneously to clarify the function of these genes. Identification of dimerization partners by biochemical means or via interaction screens may be an alternative route, since many bHLH proteins are strictly dependent on heterodimerization for function.

The previously characterized bHLH genes within our group of 15 genes included SPT, ALC, EGL3 and PIF3. Their potential role in cell proliferation has not been emphasized in the literature before. SPT is known to be involved in carpel development and has been suggested to promote proliferation of certain tissues within the gynoecium (Heisler et al., 2001). ALC and IND are involved in the development of fruit dehiscence and IND has been suggested to play a role in unequal cell divisions in layer of cells in the dehiscence zone (Wu et al., 2006). EGL3 participates in trichome and root hair initiation and has been suggested to regulate the cell division rate in early stages of epidermal specification (Bernhardt et al., 2003). PIF3 is a regulator of light signaling (Duek and Fankhauser, 2005; Monte et al., 2007; Ni et al., 1998), but does not seem to be a strong
candidate for a cell cycle regulator in the systems we have looked at (Table 1). It remains to be explored whether these or any of the other bHLH genes described here play important roles in control of cell proliferation in general or in specific tissues of the plant.

Materials and Methods

Alignment, blast and phylogenetic analysis

The basic helix-loop-helix Zip domain of 14 animal myc genes, 14 animal mad genes, and 7 animal max genes were aligned. They are used as a query to screen *Arabidopsis* gene database using blastn program. Only *Arabidopsis* bHLHZip genes with histidine (H), glutamic acid (E), and arginine (R) at the 5, 9, and 13 sites, respectively, in the basic region were considered interesting. The amino acid sequence of the bHLHZip domain of bHLH H-E-R *Arabidopsis* genes were multi-aligned and then used to make NJ (neighbor joining) tree with MEGA4.

Bioinformatic studies

Published microarray data from different tissues and different cell cycle relevant assay were used to identify *Arabidopsis* genes that have similar expression pattern to G1 cyclin, in this case cyclin D3;1 and cyclin D3;3 or opposite expression pattern to KRP, in this case KRP1 or KRP2.

Suspension culture

*Arabidopsis* thaliana ecotype Landsberg erecta cell suspension culture was maintained by weekly subculturing in MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.5mg/l NAA, 0.05mg/l kinetin. Synchronization of cell culture by sucrose starvation-readdition was done essentially as described previously (Menges and Murray, 2002) but to avoid osmotic stress of cells during sucrose withdrawal the starvation media contained 1.8% sorbitol.

RT-qPCR

Total RNA was extracted from suspension culture cells using RNeasy mini kit (Qiagen Sciences). Samples were DNase-treated before cDNA synthesis was performed with oligo (dT) and RevertAid M-MuLV Reverse transcriptase from Fermentas. cDNA was used as template in quantitative PCR reactions with DyNamo HS SYBR Green qPCR kit from Finnzymes. The comparative Ct method was used for relative quantification of expression levels. The expression levels of the genes of interest were normalized to the expression level of *Arabidopsis thaliana* actin 2 (ACT2). The primer sequences are provided upon request.
T-DNA insertion line analysis and phenotype study

Seeds of SALK T-DNA insertion lines for At1g63650/EGL3, At1g10120, At1g26260, and At3g61950 obtained from the Salk Institute for Biological Studies, CA, USA were planted out in green chamber. Leaves of each line were used for DNA extraction and seeds from each line were harvested. PCR were performed by using two primer pairs, LP+LB and LP + RP. LB is a small fragment of sequence in the T-DNA, while LP and RP are the left and right flanking sequences of the insertion site, respectively. DNA extracted from wt individuals will produce a single band with size as 900bp by LP+RP primer pair, whereas DNA extracted from homozygous insertion individuals will produce a single band with size as 400~700bp by LB+LP primer pair. Heterozygous insertion individuals will produce both bands. When no homozygous individual were found, which insertion was supposed to be lethal. Seeds produced by those identified homozygous individuals of 4 At bHLH genes, At1g63650/EGL3, At1g10120, At1g26260, and At3g61950 and from SPT and ALC knockout homozygote were planted out as before. Crossings were made between every 2 of the 6 genotypes when plants were flowering. Seeds of crossed individuals were harvested afterwards and panted out again to investigate phenotypes of each pair of double gene knockout. Besides known phenotypes of SPT and ALC, no other new phenotype was observed.

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References


Legends to figures

Figure 1. Phylogenetic tree of B-group bHLH genes in *Arabidopsis thaliana*. The B-group bHLH genes have the signature “H-E-R”, referring to conserved amino acids in the basic region (see text for further explanation). The *Arabidopsis* bHLH genes were divided into 15 subgroups and 7 orphans. The bootstrap test of all subgroup clusters are above 50 while 9 of them are above 75.

Figure 2. Expression of selected bHLH genes at different developmental stages of the root. The graphs are made based on the microarray expression data at three indicated development stages in the *Arabidopsis* root (Supplementary Table 3S) (Beemster et al., 2005; Birnbaum et al., 2003). In stage 1, the root tip has reached its full diameter (division zone); stage 2, where cells begin longitudinal expansion (elongation zone); and stage 3, where root hairs were fully elongated (mature region).

Figure 3. Expression of selected bHLH genes after auxin treatment. The graphs are based on the microarray expression data in Supplementary Table 4S (Vanneste et al., 2005). *Arabidopsis* wt and slr1 mutant seedlings were germinated on medium containing 10 mM NPA and transferred 3 d after germination under continuous light to 10 mM NAA.

Figure 4. Expression of selected bHLH genes after sucrose-induced re-entry into the cell cycle in sucrose-starved *Arabidopsis* suspension cell cultures. Sucrose-induced cultures were harvested at the indicated time points and analyzed for mRNA expression by Q-RT-PCR. The 10 genes were divided into three groups according to their expression pattern: CYCD3-like, KRP2-like, and immediate early transient expression.

Supplemental Figure 1S. Multi-alignment of animal Myc/Max/Mad network proteins. Amino acid sequences from 14 MAD-family, 14 MYC-family and 7 MAX-family proteins, representing 10 different animal species from human to sea urchin were aligned. Blue color, residues conserved in all three families; yellow color, residues conserved in two out of the three families; red color, residues uniquely conserved in only one family; green color, residues of functional importance in the leucine zipper (Zip) domain. The positions of the conserved H-E-R residues in the basic region are indicated.
Figure 2.

Figure 3.
Figure 4.
<table>
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<tr>
<th>Sub-group</th>
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<th>Leaves</th>
<th>Siliques/seeds</th>
<th>Cytochrome oxidase</th>
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<td>N.C</td>
<td>1</td>
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<td>+ (I.E)</td>
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</tr>
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Table 1. Summary of expression profiles of Arabidopsis B-group bHLH genes. +, coregulated with the G1 cyclins CYCD3;1 and/or CYCD3;3; -, coregulated with KRP1 and/or KRP2; I.E, immediate early transient expression; D.E, differentially expressed, but not cell cycle regulated; N.C, no changes in expression compared with controls; N.D, not determined; low, low/not expressed; *, based on data from Menges et al (Menges et al., 2003).
<table>
<thead>
<tr>
<th>At-code</th>
<th>Shoot apex</th>
<th>Flowers</th>
<th>Leaves</th>
<th>Siliques/seeds</th>
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**Supplementary Table 1S.** Microarray expression data for selected bHLH genes in different tissues of *Arabidopsis thaliana*. Data are adapted from the AtGenExpress database [http://www.weigelworld.org/resources/microarray/AtGenExpress/]. CYCD3;1, CYCD3;3, E2Fa and KRP1 were chosen as cell cycle regulated reference genes.
<table>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td></td>
<td>7</td>
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</tr>
<tr>
<td></td>
<td>8</td>
<td>seeds, stage 10, w/o siliques; green cotyledons embryos</td>
<td>8 weeks</td>
</tr>
</tbody>
</table>

**Supplementary Table 2S.** Tissue specification of the tissue samples in Supplementary Table 1S. The description is adopted from the AtGenExpress database [http://www.weigelworld.org/resources/microarray/AtGenExpress/](http://www.weigelworld.org/resources/microarray/AtGenExpress/)
At-code | stage 1 | stage 2 | stage 3
--- | --- | --- | ---
AT1G63650 | 273,8567509 | 149,3743954 | 14,73567101
AT1G10120 | 254,9795286 | 369,2601116 | 180,2543203
AT1G26260 | 137,4204497 | 73,96756046 | 51,96729741
AT3G07340 | 98,81518102 | 316,3702894 | 278,733134
AT5G50915 | 71,53530597 | 46,65125936 | 46,6639038
AT3G25710 | 69,35495656 | 22,5059992 | 42,45998116
AT3G61950 | 19,2145778 | 16,52271164 | 8,943153806
AT4G01460 | 11,06254943 | 10,57544325 | 3,388214869
AT1G68810 | 103,1609422 | 120,9390756 | 212,541196
AT1G09530 | 96,43158196 | 21,92464367 | 10,92153835
AT4G36930 | 370,5121455 | 152,8481599 | 86,36289713
AT5G67110 | 86,73223161 | 65,10583691 | 46,04856132
AT5G46690 | 32,85604967 | 33,10830711 | 23,11660666
AT1G22490 | 68,24089683 | 54,05950425 | 25,52175543
AT5G53210 | 5,61248608 | 3,924283374 | 4,582575695
CYCD3;3 | 913,7672351 | 220,5244884 | 267,3890237
CYCD3;1 | 87,98136166 | 59,31542801 | 51,90549104
E2Fa | 174,3965309 | 106,8951823 | 64,85869256
KRP1 | 12,8066389 | 25,55895147 | 34,21110931
KRP2 | 58,41746314 | 122,3078084 | 179,6696969

**Supplementary Table 3S.** Microarray expression data for selected bHLH genes at three development stages in the *Arabidopsis* root. At stage 1, the root tip has reached its full diameter (division zone); stage 2, where cells begin longitudinal expansion (elongation zone); and stage 3, where root hairs were fully elongated (mature region). The data is adopted from (Beemster et al., 2005; Birnbaum et al., 2003).
### Supplementary Table 4S.

Microarray expression data for selected bHLH genes during auxin-induced lateral root initiation. Data adopted from (Vanneste et al., 2005), where microarray analysis was performed using *Arabidopsis* wt Col-0 lateral roots germinated on medium containing 10 mM NPA and then transferred 3 d after germination under continuous light to 10 mM NAA. The lateral rootless phenotype of the early auxin signaling mutant slr1 was used as control.

<table>
<thead>
<tr>
<th>At-code</th>
<th>Gene name</th>
<th>Arabidopsis wt Col-0</th>
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<td></td>
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<td>0h</td>
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</table>

*Arabidopsis* wt Col-0 lateral roots germinated on medium containing 10 mM NPA and then transferred 3 d after germination under continuous light to 10 mM NAA. The lateral rootless phenotype of the early auxin signaling mutant slr1 was used as control.