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INTERPLAY BETWEEN THE MYC ONCOPROTEIN, CYCLIN-DEPENDENT KINASES AND E3 UBIQUITIN LIGASES

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ABSTRACT

Mammalian cells grow, divide, and die in a precise and orderly fashion. For cells to grow and maintain their integrity they need to communicate at both intracellular and extracellular level. This communicative circuit is maintained by means of many different factors functioning at different level to for example receive, transmit and respond to the delivered message. Among these factors are transcription factors whose function is to regulate expression of genes downstream of transmitted signals. Myc is a transcription factor that is estimated to regulate 10%-15% of the genes in the genome and that plays an essential role in various cellular processes required for cell growth, cell division and survival. Often communication between the factors involved in signaling involves addition of small molecule moieties such as phosphate, acetyl, ubiquitin or methyl groups that is exerted through the function of enzymes like kinases, histone acetyltransferases, E3 ubiquitin ligases or histone methyltransferases.

E3 ubiquitin ligases tag their substrates by mono or polyubiquitin chain molecules that will dictate the fate of the targeted protein. Ubiquitylated proteins are usually degraded by a cellular degradation machinery called the proteasome. However, depending how ubiquitin chain is formed it can serve other roles than for protein degradation, for instance in transcription.

The work presented in this thesis provides insights into underlying the role of a kinase, Cdk2 as well as two E3 ubiquitin ligases, SCF^{FBXO28} and VHL in the regulating the function of Myc transcription factor/proto-oncoprotein.

Deregulated function of Myc plays an important role in the development of many different cancer types. Despite extensive studies on the function of Myc the mechanism underlying its deregulation is still elusive. Another oncoprotein whose deregulation is involved in development of different cancers is Ras. Myc and Ras are known to cooperate in cellular transformation but the mechanism underlying their cooperation is unclear. In paper I we provided a mechanism by which these two oncogene work together in tumor development. We show that Cdk2 kinase phosphorylates Myc at Ser-62 and that this phosphorylation is important for Myc-mediated regulation of genes involved in cellular senescence. We show that Myc in this way suppresses Ras-induced senescence, which is one of the barriers for tumor development. This unique role of Cdk2 provides a potential therapeutical advantage to combat Myc and /or Ras-driven tumors.

In paper II we identify a new E3 ubiquitin ligase, SCF^{FBXO28} that was found to target Myc for ubiquitylation and to play a critical role in regulation of Myc function in tumor progression. Our data further suggest that SCF^{FBXO28} plays an important role in transmitting Cdk activity to Myc function during the cell cycle, emphasizing the Cdk-FBXO28-Myc axis as a potential molecular drug target in Myc-driven cancers.

In paper III we show that Myc interacts with the tumor suppressor protein von Hippel Lindau (VHL) which is part of an E3 ubiquitin ligase complex. VHL was found to promote ubiquitylation of Myc in a non-proteolytic fashion. We showed that VHL associates together with Myc at a subset of Myc target genes throughout the genome and activate or repress the expression of bound genes with functions in cancer development, gene expression, metabolism and other function. We also found that

VHL and Myc bind to the MYC locus and regulate the *MYC* gene expression. Our data reveal novel functions and new modes of regulation of two of the most important oncoproteins and tumor suppressor proteins in human cancer, c-Myc and VHL, respectively. We anticipate that this work will have preclinical and potential clinical implications for cancer biology and treatment.

LIST OF PUBLICATIONS

Hydbring P, Bahram F, Su Y, Tronnersjö S, Högstrand K, von der Lehr N, **Sharifi HR**, Lilischkis R, Hein N, Wu S, Vervoorts J, Henriksson M, Grandien A, Lüscher B, Larsson LG. Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation.

Cepeda D, Ng HF, **Sharifi HR**, Mahmoudi S, Cerrato VS, Fredlund E, Magnusson K, Nilsson H, Malyukova A, Rantala J, Klevebring D, Viñals F, Bhaskaran N, Zakaria SM, Rahmanto AS, Grotegut S, Nielsen ML, Szigyarto CA, Sun D, Lerner M, Navani S, Widschwendter M, Uhlén M, Jirström K, Pontén F, Wohlschlegel J, Grandér D, Spruck C, Larsson LG, Sangfelt O. CDK-mediated activation of the SCF(FBXO) (28) ubiquitin ligase promotes MYC-driven transcription and tumourigenesis and predicts poor survival in breast cancer.

Hamid Reza Sharifi[1*],Sara Fahlén[1,2*], Helén Nilsson[1]*, Alina Castell[1], Natalie von der Lehr[3], Fan Zhang[1], Ara Rofoogaran[1], Per Hydbring[1], David Lindgren[4], Mikael Huss[5], Lorenz Poellinger[3], Olle Sangfelt[3] and Lars-Gunnar Larsson[1]. Regulation of c-Myc expression and Function by the von Hippel-Lindau E3 Ubiquitin Ligase/ Tumor Suppressor Protein

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LIST OF ABBREVIATIONS

Apc	Adenomatous polyposis coli
AREs	AU-rich elements
bHLH-Zip	Basic helix-loop-helix leucine zipper
CPSF	Cleavage and polyadenylation specificity factor
CstF	Cleavage stimulatory factor
CREB	cAMP response element-binding protein
CBP	CREB-binding protein
CBC	Cullin-elongin B-elongin C
CDKIs	Cyclin dependent kinase inhibitors
Cdks	Cyclin-dependent kinases
DISC	Death-inducing signaling complex
DSIF	DRB sensitivity-inducing factor
EMT	Epithelial-Mesenchymal Transition
EPO	Erythropoietin
FADD	Fas-associated death domain
GSK-3β	Glycogen synthase kinase-3β
HATs	Histon acetyltransferases
HDAC	Histon deacetylases
HECT	Homologous to the E6AP Carboxyl Terminus
HREs	Hypoxia-response elements
mTOR	Mammalian target of rapamycin
MMPs	Matrix metalloproteinases
MCM	Minichromosome maintenance
NELF	Negative elongation factor
OIS	Oncogenic induced senescence
PABP	Negative elongation factor
pTEFb	Oncogenic induced senescence
PIC	Poly (A) binding proteins
PHDs	Positive transcriptional elongation factor b
RBPs	Pre-Initiation Complex
SA-Bgal	Prolyl hydroxylase domain proteins
SAHF	RNA binding proteins
	Senescence-associated β-galactosidase

SASP	Senescence-associated heterochromatin foci
SID	Senescence-associated secretory phenotype
SPRF	Single polypeptide RING-finger
TBP	TATA-binding protein
TAFs	TBP-associated factors
TGF-B	Transforming growth factor beta
TPO	Thrombopoietin
TRADD	TNFR-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
TRRAP	Transcativation/Transformation Associated Protein
TNFR	Tumor Necrosis Factor Receptor
UPS	Ubiquitin/proteasome system
SCF	Skp2-Cullin-F-box
VHL	von Hippel-Lindau tumor suppressor gene

INTORDUCTION

Cellular signaling

In order to communicate with their surrounding environment and maintain proper tissue architecture cells must be able to receive signals from extracellular space and into the cell interior. Upon binding their cognate ligands cell surface receptors become activated and transduce received signals through the plasma membrane into the cytoplasmic intracellular environment and into the cell nucleus. These signals dictate cell fate; whether a cell should proliferate or differentiate, remain attached or migrate, survive or die etc.. Many of these signals comprise growth factors, cytokines and hormones.

Receptor tyrosine kinases (RTKs) constitute a large group of similarly structured receptors that are specialized to transmit signals delivered by growth factors (Shaw and Cantley 2006). RTKs share cytoplasmic tyrosine kinase domains and structurally highly variable ectodomains that protrude into the extracellular space to bind a wide range of extracellular ligands. Upon ligand binding the monomeric RTK dimerizes and the tyrosine kinase of each receptor monomer transphosphorylates the cytoplasmic tyrosine kinase of the other monomer. The phosphorylated cytoplasmic tails of receptor then recruit adaptors and signaling molecules that activate downstream signal transmitters such as Ras, which allow the signaling to proceed (See below) (Lemmon and Schlessinger 2010).
Tyrosine phosphorylation is primarily used by mitogenic signaling pathway. Here, in addition to some of the RTKs mediated pathways I will briefly discuss below few other signaling pathways that all together are among the most frequently deregulated pathways in human cancers.

The MAPK and PI3/Akt signaling pathway

The MAPK pathway is activated upon binding of growth factors, hormones and chemokines to their cognate RTKs leading to phosphorylation of C-terminal tyrosine residues of the receptor. Through the function of adaptor proteins such as Grb2, plasma membrane-localized and inactive GDP-bound Ras protein is converted to GTP-bound active form of Ras. Activated Ras protein then activate serine/threonine kinases of the Raf protein kinase family. Raf phosphorylates and activates MEK which in turn phosphorylates and activates the mitogen-activated protein kinase (MAPK) Erk1/2 (Extracellular signal Regulated Kinase 1/2) protein kinases. Activated Erk1/2 have both cytoplasmic and nuclear substrates. In the cytoplasm Erk1/2 activates factors involved in translation hence regulating protein synthesis. Once translocated into the nucleus, Erk1/2 can regulate transcription through phosphorylation of transcription factors such as CREB, c-Fos and c-Jun. (Downward 2003, Katz, Amit et al. 2007, Finocchiaro, Hayes et al. 2008).

Another pathway activated through the RTKs and Ras is the phosphatidyl inositol-3 kinase (PI3K) pathway. Upon activation by Ras, PI3K phosphorylates

phosphatidylinositol 4,5-biphosphate (PIP2) to PIP3, which in turn is recognized by the Akt/PKB kinase. Activated Akt signaling promotes cell growth and survival, and inhibits apoptosis by phosphorylating a number of substrates, including MDM2, Bad, caspase 9 (Cantley 2002, Engelman, Luo et al. 2006) GSK-3β (Frame and Cohen 2001) and components of the mammalian target of rapamycin (mTOR). FoxO transcription factors that negatively regulate cell cycle progression are other phosphorylation targets of PI3/Akt pathway. It has been shown that the PI3/Akt pathway by suppressing FoxO proteins promotes Myc-mediated proliferation and transformation (Bouchard, Marquardt et al. 2004).
The PI3K pathway is negatively regulated by tumor suppressor phosphatase and tensin homolog PTEN by dephosphorylating PIP3 to PIP2 (Keniry and Parsons 2008). The components of the MAPK and the PI3/Akt signaling pathways can interact and cross-talk with each other to promote the growth and survival of transformed cells.

TGF-β signaling

The cytokine TGF-B has its own unique receptor that belongs to serine/threonine kinase rather than tyrosin kinase family. Binding of TGF-B ligand to the type II receptor brings the type II and type I receptor together resulting in the phosphorylation of type I receptor, which in turn phosphorylates the cytosolic Smad 2 or Smad 3 proteins. Either of these phospho-Smads then associates with Smad 4 forming a heterodimeric Smad complex that migrates to the nucleus and functions as a transcription factor. Some of the most important targets of TGF-B signaling are cell cycle regulatory proteins such as Myc, $p15^{INK4B}$ and $p21^{Cip1}$ CDKIs (cyclin dependent kinase inhibitors). The Smad complex together with transcription factor Miz1 induce transcription of $p15^{INK4B}$ and $p21^{Cip1}$ (Seoane, Pouponnot et al. 2001, Wu, Cetinkaya et al. 2003). Smad 3 forms a complex with E2F4 or E2F5 and the pocket protein p107 to repress expression of the Myc gene. While TFG-B signaling plays a major growth inhibitory signal in normal cells and early stage development of many cancer types, it enables cancer cells to become anchorage independent, invasive and to promote angiogenesis (for review (Massague 2012).

Wnt/β−catenin signaling

Receptors of the Wnt proteins are members of the Frizzled family of transmembrane proteins that upon binding and activation by Wnt proteins transmit a signal to the phosphoprotein Dishevelled that is located in the cytoplasm and act to suppress the activity of glycogen synthase kinase-3β (GSK-3β). GSK-3β phosphorylates several key substrate proteins such as β-catenin, Cyclin D, Myc and Cyclin E, which are thereby targeted for ubiquitin/proteasome-mediated degradation (He, Sparks et al. 1998, Tetsu and McCormick 1999).In the absence of Wnt signaling β-catenin will form a multiprotein complex including adenomatous polyposis coli (Apc), Axin and GSK-3β resulting in phosphorylation and subsequent destruction of β-catenin. Hence Wnt signaling results in accumulation of β-catenin and its translocation to the nucleus where it associates with the T-cell specific transcription factor/lymphoid enhancer-binding factor (Tcf/Lef) transcription factors to drive the expression of genes including those

involved in cell growth and proliferation such as cyclin D1 and Myc (for review see (Gordon and Nusse 2006)).

Jak/STAT pathway

The receptors of the Jak/STAT pathway are stimulated by many cytokines including IFN-γ, IL-6, erythropoietin (EPO) and thrombopoietin (TPO). The Jak/STAT signaling pathway is activated through the function of the Janus kinas (Jak) class of the tyrosine kinases and the signal transducers and activators of transcription (STATs).

Following ligand binding the Jak enzyme, which is associated with each receptor, phosphorylates tyrosines on the cytoplasmic tail of the partner receptor similar to transphosphorylation seen in the RTKs. The resulting phosphotyrosine tail will then recruit STAT proteins followed by phosphorylation, homodimerization and nuclear translocation of the STATs where they function as transcription factors. The STATs activate target genes that are important for cell proliferation and survival such as the Myc, cyclin D and Bcl-X_L genes (Bromberg 2001). However, it has been shown that some STAT family member negatively regulate the cell cycle by inducing CDK inhibitor $p21^{cip1}$ (Vainchenker and Constantinescu 2013).

Transcription factors and Transcription

Genetic information in the form of DNA molecules reside within the tightly packed and highly organized structure of chromosomes in the nuclei of cells. For the enormously large size of the eukaryotic genome to fit into the small volume of the nucleus, the strand of DNA is wrapped around an octamer of core histone H2A, H2B, H3 and H4 proteins to form nucleosome, the basic unit of chromatin, which in turn is organized into higher order of chromatin structure . The densely packed nucleosomes must, however, be accessible for proteins involved in transcription, replication and DNA repair (Woodcock and Ghosh 2010).

The information encoded in the DNA molecules is first transcribed into RNA molecules and the protein-encoding mRNAs are then translated into functional proteins. Transcription is achieved by the elaborate function of three different types of RNA polymerase enzymes in eukaryotes, namely RNA polymerase I-III (RNA pol I-III). This process through which expression of genes occur is in many cases the end point of signal transduction pathways.

Each RNA polymerase is responsible for synthesizing a specific class of cellular RNA. RNA pol I transcribes the ribosomal RNA (rRNA) subunits 28S, 18S and 5.8S rRNAs. RNA pol II transcribes all protein-coding RNAs and most non-coding RNAs including microRNAs (miRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). RNA pol III transcribes the 5S rRNA and tRNA. Amongst these RNAs, only products of RNA pol II are translated into proteins.

The transcription cycle by RNA polymerase includes three coordinated multistep processes; transcription initiation, elongation and termination. Here, I will briefly discuss these steps focusing mostly on the more extensively studied RNA pol II transcriptional regulation.

All three RNA polymerases exhibit overall structural similarity with about half of their subunits being identical and the rest being homologous. The active center and core enzymes subunits are similar among all three RNA polymerases and it is the pronounced structural differences among the cofactor-interacting subunits that account for gene class specific function (Cramer, Armache et al. 2008).
The C-terminal domain (CTD) of the large subunit of RNA pol II, Rpb1, play an essential role in transcription and contains a unique 52 heptapeptide tandem repeat of the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 which is absent from RNA pol I and III (Dahmus 1994, Suh, Hazelbaker et al. 2013). The phosphorylation status of Rpb1 CTD tail regulates recruitment of RNA Pol II-interacting factors that promote mRNA capping, splicing, 3' untranslated region (UTR) end processing and chromatin modification (Buratowski 2009).

Two general types of specific proteins/ transcription factors are required for the initiation of the RNA pol II-mediated transcription; the general transcription factors (GTFs) including TFIIB, TFIID, TFIIA, TFIIE, TFIIF and TFIIH which together with RNA pol II form the pre-initiation complex (PIC) and constitute the minimal set of proteins necessary for directing the polymerase to the transcription start site (TSS). Secondly, DNA binding transcription factors that bind to specific promoter or enhancer DNA sequences in target genes and activate or repress the expression of individual genes in response to intra-, or extra-cellular signals.
The promoters of many genes transcribed by RNA pol II contain a TA-rich DNA sequence called TATA box, which is recognized by TFIID through its subunits TATA-binding protein (TBP) and TBP-associated factors (TAFs). In addition to TATA box, a second important sequence element called initiator (Inr) sequence spans the transcription start site (Moqtaderi, Bai et al. 1996, Kornberg 2005, Malik, Baek et al. 2005).
Other crucial sequences are enhancer elements that reside at proximal or distal chromosomal location from the core promoter. Upon binding of an activator, enhancers promote the recruitment of GTFs and RNA pol II to the target gene promoters (Thomas and Chiang 2006). The Mediator, is a multiprotein complex that an essential function in transcription initiation by connecting activators bound to promoters or enhancers with the PIC at the promoter (Kornberg 2007).
Despite RNA pol II's capability of unwinding/rewinding DNA and synthesizing RNA it requires participation of the GTFs for promoter recognition and transcription initiation. Two models exist to describe how the transcribing complex is formed. Both models explain how the duplex DNA is melted, bent and inserted in the Pol II active core center enabling the initiation of transcription. In one model the PIC is first formed in the absence of DNA interaction with subsequent loading of the full complex onto the promoter of the target gene. In contrast to this model, the stepwise assembly model explains the sequential recruitment of the PIC to the promoter. The three steps of transcriptions are discussed below describing the stepwise assembly model of PIC formation.

Transcription initiation

Transcription starts with the binding of an activator to the enhancer DNA elements, which are then brought together with the PIC via the mediator complex (Shandilya and Roberts 2012).

Upon activation and binding of TFIID and its subunits TBP and TAFs, TBP bends the TATA-sequence containing promoter DNA and facilitates the ordered assembly of other GTFs. The recruitment of TBP can be positively or negatively be regulated by activators or repressors. Next TFIIA stabilizes the TBP/DNA binding at the promoter. TFIIB then recognizes the sequences flanking TATA box and form a stable ternary complex with TBP-bound DNA. Subsequently, TFIIF recruit the RNA pol II to the promoter and further stabilizes the PIC formation. At the same time, histone modifying enzymes such as histon acetyltransferases (HATs), histone methyltransferases (HMTs) as well as ATP-driven nucleosome remodeling enzymes are recruited and favor an open chromatin structure for transcription (Shandilya and Roberts 2012).

Further, TFIIE enters the complex and recruit TFIIH and its subunits including an ATPase/helicase subunit, which introduces negative superhelical tension that together with thermal unwinding produces a transient transcription bubble. TFIIF then captures the nontemplate strand facilitating the single stand DNA bending and entering the pol II active center. After the initiation steps and transcribing few nucleotides, transcription machinery pauses, an important mechanism of regulating transcription seen in higher eukaryotes also known as promoter-proximal pausing (Nechaev and Adelman 2011). The RNA pol II pause is mediated by negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF). Another subunit of TFHII contains Cdk7 that phosphorylates Ser5 residue of the CTD of Rpb1 large subunit and is thought to contribute to promoter clearance and transition from transcription initiation to elongation (Hengartner, Myer et al. 1998, Conaway, Florens et al. 2005, Espinosa 2010).

Transcription elongation

As mentioned above NELF and elongation factor DSIF are involved transition of the inititating/pausing transcription machinery into the elongating phase of the transcription.

During elongation the positive transcriptional elongation factor b (P-TEFb) complex, which consist of Cdk9 and cyclin T, phosphorylates NELF and DSIF as well as Ser2 of the CTD tail, leading to dissociation of NELF and DSIF from RNA pol II. Further, TFIIH-mediated CTD phosphorylation at Ser5 contributes to recruitment of capping enzyme to the 5’ end of nascent mRNA, which also triggers RNA pol II escape from the promoter to the gene body.

Following RNA pol II promoter clearance and switching to elongating mode, a part of the PIC remains associated at the promoter to form the reinitiation complex for the next transcription cycle. While RNA pol II moves along the genes towards the 3’ region the level of phosphor-Ser5 of CTD tail decreases with the concomitant increase in the level of phosphor-Ser2 by Cdk9 (Buratowski 2003). Phospho-Ser2 promotes recruitment of the mRNA splicing complex, the 3’ processing and termination factors (Ahn, Kim et al. 2004, Meinhart and Cramer 2004).

Transcription Termination

Transcription termination is the least understood step of transcription cycles and still very elusive. At the end of most of the protein coding genes elongating RNA pol II reaches the highly conserved sequence 5'-AATAAA-3' (5'-AAUAAA-3' in the transcribed mRNA molecule), also known as the poly (A) signal that is followed by a G/U rich sequences. When RNA pol II transcribes the poly (A) sequences its elongating rate is reduced followed by downstream pausing of transcription and endoribonucleolytic cleavage of the mRNA transcript (Nag, Narsinh et al. 2007, Kazerouninia, Ngo et al. 2010). Subsequently, the mRNA transcript is polyadenylated, which is the process of addition of multiple adenosine monophosphates that builds up the poly (A) tail to the 3' end of the mRNA transcript by a specific polymerase (Kuehner, Pearson et al. 2011). Several protein complexes facilitate this process including the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulatory factor (CstF). The phospho-Ser2 CTD tail of elongating RNA pol II act as a scaffold for the recruitment of CPSF and CstF (Ahn, Kim et al. 2004). When the poly (A) sequences are transcribed, the CPSF complex interacts with them on the mRNA transcript inducing RNA pol II pause (Glover-Cutter, Kim et al. 2008). Further, CstF binds to the downstream GU-rich sequences followed by sequestering of CPSF after which Pol II is released and subsequent CPSF-mediated cleavage of mRNA transcript ocuurs. Next the upstream cleavage product is polyadenylated by polyadenylate polymerase (PAP) whereas the downstream cleavage product is degraded (for review see (Kuehner, Pearson et al. 2011, Shandilya and Roberts 2012).

The transcriptional machinery including some of the RNA pol II subunits can also influence posttranscriptional fate of the nascent mRNA, such as cytoplasmic localization, translation initiation efficiency and decay (Dahan and Choder 2013).

After poly (A) addition to the 3' end, deadenylases trim the 3' end to the proper length after which it is exported to the cytoplasm. Generally, changes in poly (A) tail length occur throughout the mRNA lifetime and play essential role in its stability. AU-rich elements (AREs) within 3'UTR of mRNA are regulatory sequences that are recognized by RNA binding proteins (RBPs) such as HuRs, a ubiquitously expressed member of the Hu family of RBPs (Brennan and Steitz 2001). All mammalian HuR proteins, positively regulate the stability and translation of their target transcript (Fan and Steitz 1998).

Also miRNAs can bind to 3'UTR region of transcript and control their translation and stability (Garneau, Wilusz et al. 2007).

Further, poly (A) binding proteins (PABP) also interact with the 5'- cap-bound translation initiation factors eIF-4E and eIF-4G and enhance translation initiation.

Cell cycle

In order to proliferate, cells must duplicate and segregate their genome and organelles to produce daughter cells in a precisely and timely regulated process called the cell division cycle, or the cell cycle. The eukaryotic cell cycle consists of four discrete phases. The two major cell cycle phases are synthesis (S) phase, where the cell DNA content duplicates, and mitosis (M) where cell divides. The two less dramatic but

equally important gap phases G1 and G2 separate the S and M phases. The G1, S and G2 phases make up the period of the cell cycle called interphase. Different organisms have different cell cycle durations. Even within an organism the duration of the cell cycle can vary depending on the cell type. The cell cycle of human somatic cells take approximately 24 hours. The interphase typically takes between 18-22 hours. M phase is the shortest phase and last only 2-3 hors. The G1 phase consist the most variable and often the longest period of the cell cycle.
During late G1, the cell passes through a critical checkpoint (restriction point), which monitors whether environmental conditions are favorable for replication. If not and in the absence of growth factors, the cell may enter a resting state known as G0 where it stays until the proper mitogenic signaling stimulate the cell cycle entry again.
During the G2 phase another important checkpoint scrutinize the duplicated DNA to ensure that it is properly replicated and structurally intact to proceed to M phase. Another critical checkpoint takes place during mid-mitosis to determine whether the chromosomes have properly attached to the mitotic spindle (Nurse 2011).

The discovery of two classes of proteins, cyclins and their binding partner cyclin-dependent kinases (Cdks), was a big step forward for our understanding of the cell cycle in eukaryotic cells (for review see (Murray 2004)). The level of the cell cycle regulatory cyclin proteins, oscillate dramatically during the cell cycle (Evans, Rosenthal et al. 1983). Cdks are serine/threonine kinases the activity of which require appropriate cyclin binding (Morgan 1997). Upon cyclin binding, a significant conformational change on the kinase catalytic site occurs and subsequent phosphorylation by the Cdk activating kinase (CAK) complex results in Cdk activation. CAK complex includes Cdk7 and cyclin H, which also play a regulatory function on RNA polymerase II transcriptional activity (see "Transcription factor and Transcription" section).
The activity of cyclin/Cdk complexes can be inhibited in different ways. For instance, during the S phase and DNA replication, the Cdk activity can be inhibited by addition of an inhibitory phosphorylation mediated by Wee1 kinase (Heald, McLoughlin et al. 1993). However, this inhibitory phosphorylation can be reversed by the function of phosphatase Cdc25 (Perry and Kornbluth 2007).
Among the Cdks and cyclin proteins that have been discovered only a certain subset of cyclin/Cdk complexes are directly involved in driving the cell cycle. These include Cdk2, Cdk4 and Cdk6 and the mitotic Cdk1 that form combinatorial complexes with ten cyclins belonging to four different classes A-, B-, D, and E-type cyclins.
The activities and functions of cyclin/Cdk complexes are inhibited by two families of Cdk inhibitors. The inhibitors of Cdk4 (INK4) family including $p16^{INK4a}$, $p15^{INK4b,}$ $p18^{INK4c}$ and $p19^{INK4d}$ specifically bind to Cdk4 and Cdk6 and inhibit binding of D-type cyclins. Secondly, the Cip/Kip family of inhibitors including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which target Cdk1 and Cdk2 and inhibit E-, A- and B-type cyclins (Toyoshima and Hunter 1994, Aprelikova, Xiong et al. 1995).
Different Cyclin/Cdk complexes regulate different phases of the cell cycle. During much of the G1 phase, Cdk4 and Cdk6 associate with the D-type of cyclins including cyclin D1, D2 and D3. Near the restriction point in late G1, the E-type cyclins cyclin

E1 and E2 associate with Cdk2 to phosphorylate substrates required for S phase entry. As cells enter into S phase, the A-type cyclins including cyclin A1 and A2 replace E-type as the partners of Cdk2, enabling S phase to progress. Later in S phase, the A-type cyclins switch partners and associate with Cdk1(Pagano, Pepperkok et al. 1992). As the cell move into G2 phase the B-type cyclins, cyclin B1 and B2, replace the A-type and associate with Cdk1 triggering prophase, metaphase, anaphase and telophase, the constituent steps of the complex program of mitosis. A third B-type cyclin, cyclin B3 has also been shown to interact with Cdk2 (Gallant and Nigg 1994).
The ubiquitin/proteasome system play very crucial role in degrading and controlling the level of cyclins and other cell cycle regulatory proteins (See Ubiquitin/proteasome system" section).

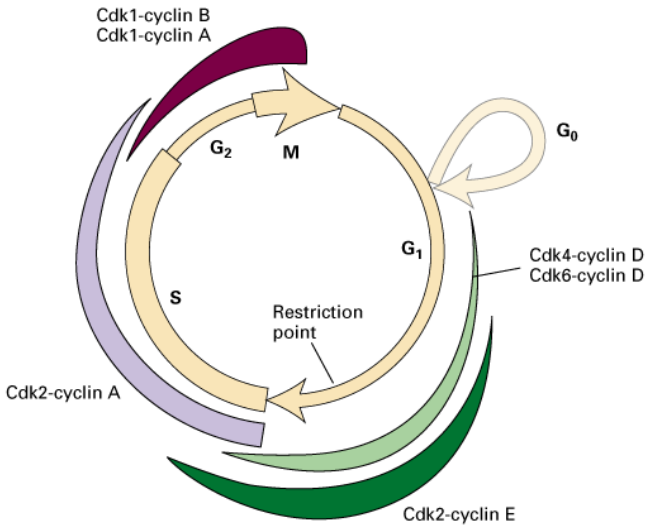

Figure 1. Schematic presentation of human cyclins and their levels through the cell cycle. Associated Cdks with correspondent type of cyclins for each phase of the cell cycle are shown.

The G1/S phase transition

CyclinD-Cdk4/6 and cyclin E-Cdk2 are two major G1 phase cyclin/Cdk complexes that regulate the events during G1 phase involved in G1/S phase transition. In early G1 phase and upon mitogenic signals, upregulated cyclin D-types and their associated Cdk4/6 initiate phosphorylation of "pocket proteins" and tumor suppressor RB family protein including pRB, p107 and p130 (Hatakeyama and Weinberg 1995, Garriga, Limon et al. 1998, Ho and Dowdy 2002). RB proteins are in a complex with and suppress the E2F family transcription factors that regulate genes of importance for cell cycle progression and S phase entry (Weinberg 1995). E2F family members include transcriptional activators E2F1, E2F2, and E2F3 as well as the transcriptional repressors E2F4, E2F5 and E2F6, although recent studies show that they can switch function in different contexts (Chong, Tsai et al. 2009)). Phosphorylation of RB

proteins enhances dissociation from E2F, derepressing their transcriptional activity. Among early E2F-regulated genes are the genes encoding E-, and A-type cyclins, Cdk1/2 as well as minichromosome maintenance (MCM) family of protein involved in DNA replication initiation. Initial phosphorylation of RB leads to dissociation of histone deacetylases (HDAC) from hypophosphorylated RB and derepression of target genes such as cyclin E (Harbour, Luo et al. 1999). The cyclin E/Cdk2 complex is also repressed by p27 ^{Kip1} that also interact with cyclin D-Cdk4/6 usually without repressing it (Hwang and Clurman 2005, Welcker and Clurman 2005). As the level of cyclin D increases over the cell cycle, more p27 ^{Kip1} is sequestered to the complex liberating cyclin E/Cdk2 from its suppression. This creates a positive feedback loop leading to the formation of even more active cyclin E/Cdk2 complexes that will further phosphorylate RB on additional sites. Hyperphosphorylated RB will then liberate the E2F transcription factor leading to further transcription of the genes needed for S phase entry.
p27 ^{Kip1} itself is a phosphorylation target of cyclin E/Cdk2 complex leading to ubiquitylation by the SCF E3 ligase S-phase kinase-associated protein 2 (Skp2) (Carrano, Eytan et al. 1999) (for SCF Skp2 see "Ubiquitin/proteasome system" section). The SCF regulatory subunit Skp2, is another E2F-regulated gene.

The S/G2 and M phase

After entry to S phase A-type cyclins play the central role and support DNA replication and lead the cell cycle through the G2 and into the M phase. During late G1 and early S phase, cyclin E/Cdk2 together with the assembly factor CDC6 support the recruitment of the MCM proteins to the pre-replication complex and sequentially cyclin A/Cdk2 phosphorylate and inactivate CDC6, terminates pre-replication complex assembly formation and activates DNA replication (Su and O'Farrell 1998, Furstenthal, Kaiser et al. 2001). In early S phase, cyclin E is phosphorylated by Cdk2 and subsequently recognized by SCF^{Fbw7} E3 ligase for ubiquitylation and proteasomal degradation (Welcker, Singer et al. 2003). Thereafter cyclin A form a complex with Cdk2, which phosphorylates additional substrates regulating DNA replication. Later in S phase, cyclin A switches from Cdk2 to form complex with Cdk1. Cyclin A/Cdk1 regulates the events needed for initiation of prophase during G2-M phase transition. Thereafter and just after nuclear envelope breakdown cyclin A is degraded, an step seemingly necessary for cell to enter anaphase (den Elzen and Pines 2001).
While cyclin A1 knock out mice are viable and develop normally, cyclin A2 deletion results in early embryonic lethality indicating the vital role of cyclin A2 during early embryogenesis. Cyclin A2 has been reported to be required for S phase (Girard, Strausfeld et al. 1991) as well as the G2/M transition and to co-localize with sites of DNA replication in mouse cell lines (Cardoso, Leonhardt et al. 1993).
CDC25 phosphatase removes the inhibitory phosphorylation from both Cdk2 and Cdk1, hence promoting the G1/S and G2/M entry respectively.

B-type cyclins play the major role during the M phase in complex with Cdk1. Among B-type cyclins, cyclin B1 plays the more essential role in mitosis. Both cyclin B1 and B2 associate with Cdk1 but it is the cyclin B1/Cdk1 complex that play important role in

nuclear envelope break down, chromosome condensation and mitotic spindle assembly (Ookata, Hisanaga et al. 1993).
Studies on Cdk knock out mice indicate that Cdk1 is the only essential Cdk to drive the cell cycle, whereas Cdk2, Cdk4 and Cdk6 knockout mice are viable (Berthet, Aleem et al. 2003, Malumbres and Barbacid 2009). Single elimination of mitotic Cdk1 arrests the cell cycle and prevents mouse embryonic development. Cdk1 can also bind to all types of cyclins and mouse embryonic fibroblasts (MEFs) derived from mouse lacking other interphase cyclins, can proliferate in the presence of Cdk1 alone (Santamaria, Barriere et al. 2007).

The anaphase-promoting complex/cyclosome (APC/C) complex regulates degradation of both B-type and A-type cyclins by the help of the substrate recognition subunit cell division cycle 20 (CDC20), which also triggers chromosome segregation (for review see (Murray 2004)). Cdk1 phosphorylates and activates CDC20 subunit of APC/C, hence activating APC/C proteolytic function. APC/C also degrades securin, the anaphase inhibitor, leading to segregation of chromosomes. Inactivation of CDC20 is therefore crucial during the spindle assembly checkpoint until all chromosomes are attached to both spindle poles (Hagting, Den Elzen et al. 2002)

Another important kinase family that is expressed and active during the late G2 and M phase is the Aurora kinase family (Aurora A, B and C in mammals) that are essential for proper execution of mitotic events and maintaining genomic integrity. Aurora kinases have been shown to regulate mitotic events such as centrosome maturation/separation, mitotic entry, bipolar spindle assembly and cyctokinesis (for review see (Marumoto, Zhang et al. 2005).

Ubiquitin/proteasome system

After discovery of lysosome it was suggested that cellular protein turn over occurs in this organelle. However, a series of experiments strongly indicated the existence of a non-lysosomal machinery that specifically regulate the process of protein turn over together with the identification of the ubiquitin protein (Goldstein, Scheid et al. 1975) led to the discovery of the ubiquitin/proteasome system (UPS) (for review see (Ciechanover, Heller et al. 1980, Ciechanover 2005). Ciechanover and Hershko found that the small 76 amino acid ubiquitin protein attached to certain proteins before degradation. Today we know that ubiquitin is transferred to the target substrate in a sequential process of enzymatic reactions called ubiquitylation. This process engages the function of three main enzymes; E1 ubiquitin activating enzyme, a ubiquitin conjugating enzyme E2 and a substrate-specific ubiquitin-protein ligase E3 (Hershko and Ciechanover 1998). An E4 ubiquitin chain assembly enzyme has also been suggested for efficient processive ubiquitylation.

There are only two known mammalian E1 enzymes. E1 enzyme activates the ubiquitin molecule in an ATP-dependent process. The activated ubiquitin is then transferred to

one of about forty E2 conjugating enzymes. Further, E3 facilitates the transfer and conjugation of ubiquitin from the E2-ubiquitin complex to the substrate protein (Haas, Warms et al. 1982, Ardley and Robinson 2005). The role of the E3 enzymes is critical in this process in that they specifically and selectively bind to the substrate. About 1000 different E3 enzymes in mammalian cells comprising multisubunit families provide a high degree of substrate specificity (Reinstein and Ciechanover 2006). Based on structural and biochemical features, E3 enzymes are divided into subclasses that will be described in more detail below.

When the E2 enzyme has transferred the first ubiquitin to the lysine residue on the target protein, further ubiquitin can be attached through any of the seven lysine residues on the surface of the ubiquitin (K6, K11, K27, K29, K33, K48 and K63) forming a polyubiquitin chain (Kim, Kim et al. 2007). However, recent studies have challenged this model by evidence indicating the existence of already formed polyubiquitin chain on E2 and E3 enzymes before substrate conjugation (Ben-Saadon, Zaaroor et al. 2006, Li, Tu et al. 2007). The substrate target can also be monoubiquitylated.

Polyubiquitin chains can be formed in different orders. The chain can be homotypic using the same lysine residue for conjugating the ubiquitin molecule or several distinct lysines can be used to form a mixed-linkage polyubiquitin chain. Other ubiquitin-like molecules such as SUMO can also be conjugated forming a heterologous polyubiquitn chain (for review see (Ikeda and Dikic 2008)).
Depending on the specific lysine residue and the length of the ubiquitin chain the substrate fate and function varies a lot. Substrates labeled with the K48-linked polyubiquitin chains are generally degraded through the proteasome (Hershko and Ciechanover 1998). While K63-linked polyubiquitin chain has been implicated in the regulation of different cellular processes such as transcriptional activation (Adhikary, Marinoni et al. 2005) DNA repair (Spence, Sadis et al. 1995) ribosome modification (Spence, Gali et al. 2000) it has also been shown to mediate proteasomal degradation but less efficiently (Kim, Kim et al. 2007). In addition, heterologous polyubiquitin chain consisting K63, K11 and K48 have been reported to promote proteasomal degradation even in the absence of K48 (Kirkpatrick, Hathaway et al. 2006).
Individual E3 ligases can promote formation of different polyubiquitin chains on different substrates, leading to different biological outcomes. An interesting example is HectH9 E3 ligase that promotes the synthesis of K63-linked polyubiquitin chain on c-Myc leading to transcriptional activation (Adhikary, Marinoni et al. 2005) while it was also reported to assemble K48-linked polyubiquitin chain on N-Myc promoting proteasomal degradation (Zhao, Heng et al. 2008). Certain polyubiquitin chains maybe favored in different context depending on the presence of particular cofactors. K63 and K11-linked polyubiquitin chains were predominantly formed rather than K48-linked polyubiquitin chain on the presence of Auora A kinase during mitosis (Otto, Horn et al. 2009). The variety of E2 and E3 enzymes and their combination with different cofactors in different contexts potentially provide enormous combination by which the synthesis of diverse type of ubiquitin chain can be directed.

Mono-ubiquitylation also play role in different cellular processes including DNA repair and receptor transport (for review see (Hicke 2001)).

Based on their structural motifs E3 ubiquitin ligases are categorized into four major groups. The largest class is the group of Really Interesting New Gene (RING) finger, which will be discussed more in detail below for review see (Deshaies and Joazeiro 2009).
The homologous to the E6AP Carboxyl Terminus (HECT), which includes the first identified E3 ligase that mediated ubiquitylation of p53. Through C-terminal domain HECT accepts the ubiquitin from bound E2 conjugating enzyme and the amino-terminal part of the HECT acts as substrate recognition region and regulate subcellular localization (for review see (Rotin and Kumar 2009, Scheffner and Kumar 2013)). The U-box E3 ligases and the PHD (Plant Homeo-Domain)-finger E3 ligases that are less studied consist the other two classes of the E3 ligases (for review see (Hatakeyama, Yada et al. 2001)). Here I will describe briefly the RING class of the E3 ligases of relevant for this thesis.

The role of RING finger domain-containing proteins in ubiquitylation became apparent after identification of a small RING finger protein, Rbx1/Roc1and its essential role for SCF E3 ligase activity (see below) (Kamura, Koepp et al. 1999, Ohta, Michel et al. 1999, Skowyra, Koepp et al. 1999). The RING finger E3 ligases comprise among other subgroups Cullin-RING the largest subclass, APC/C and single polypeptide RING-finger (SPRF). The two more extensively studied subclass of the RING-finger E3 ligase family are the SCF (Skp1-Cullin1-F-box protein) and CBC (Cullin 2/5-elongin B-elongin C) E3 ligases. Both SCF and CBC E3 ligases contain Rbx1 as the RING finger subunit. There are seven Cullin-based E3 ligases depending on the Cullin type in the E3 complex structure.
The SCF E3 ligase substrate specificity is conferred through the function of their variable component, the F-box protein. Approximately seventy F-box proteins have been identified in human genome that can be divided into three categories based on their interaction domain; The F-box proteins containing interaction domain such as WD40 repeats (FBXW), those with leucin-rich repeats (FBXL) or other domain (FBXO). As a component of SCF E3 ligase, the F-box protein interact with Skp1 adaptor protein through its F-box domain whereas the substrate recognition and interaction is conferred through one of its interaction domain type.
Three important SCF E3 ligases have been shown to play important role during the cell cycle; the S-phase associated kinase associated protein 2 (Skp2/FBXL1), FBW7 (FBXW7) and β-transduction repeat-containing protein (β-TRCP/FBXW1/11) (See "Myc post-translational modifications and degradation" section).

The second class of the RING-finger E3 ligase is the CBC E3 ligase. One of the well studied CBC E3 ligases is the CBC VHL (von Hippel-Lindau) tumor suppressor/E3 ligase. The SCF and CBC VHL are structurally related families of E3 ligases. Like F-box proteins for SCF E3 ligase, VHL function as the substrate recognition subunit of the CBC VHL E3 ligase complex. In contrast to SCF E3 ligases that many of which

function transiently during the cell cycle or in response to certain signals, CBC VHL E3 ligase continuously regulates the level of hypoxia-inducible transcription factors (HIFs) in normoxic cells (Maxwell, Wiesener et al. 1999). (See "The Von Hippel-Lindau (VHL) protein/E3 ligase" section).

Beside the SCF and CBC E3 ligases, APC/C is the other multisubunit RING-finger E3 ligase complex. Like SCF E3 ligases, APC/C E3 ligase function to regulate the cell cycle. APC/C complex consist of the invariable core components APC1 and APC2 and number of other components for which the function is not fully understood. The APC/C substrate specificity is conferred by the cell division cycle 20 (CDC20) variable activator subunit similar to F-box proteins in the SCF complex (Castro, Arlot-Bonnemains et al. 2002).

The process of polyubiquitylation of proteins is best known for degradation of targeted protein into smaller peptides through the 26S proteasome. The 26S proteasome is a large chambered multicatalytic protease consisting of two subcomplexes. The 20S core particle which is a barrel-shaped structure containing the catalytic sites with different proteolytic activities and the 19S regulatory particle. The 20S complex can be capped at one or both sides by the 19S regulatory particle. The 19S particle is composed of two multisubunit compartments called base (also known as APIS) containing six ATPases and the lid that includes deubiquitylating (DUBs) enzymes. The 19S particle recognizes polyubiquitylated proteins, unfold them and open a gap in the opening of the 20S particle allowing the unfolded substrate to enter the proteolytic chamber. The DUBs mediate the removal and processing of ubiquitin from the substrate that will be degraded by the proteasome (for review see (Ciechanover 2005). The 19S particle may have additional functions and have been detected on promoters in yeast cells, where it has been suggested to play a role in activating transcription (Ferdous, Gonzalez et al. 2001, Gonzalez, Delahodde et al. 2002).

As mentioned before many of the cell cycle regulatory proteins are degraded by the UPS system. Generally G1 phase cyclins, cyclin D and E-types are ubiquitylated by SCF E3 ligase complex whereas M phase cyclins, cyclin B-types are ubiquitylated by the APC/C (Yamamoto, Iwabuchi et al. 2005). Skp2 E3 ligase has been shown to target many of the cell cycle regulatory factors including $p21^{cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ and E2F1 (Yu, Gervais et al. 1998, Tsvetkov, Yeh et al. 1999, Nakayama, Nagahama et al. 2000, Kamura, Hara et al. 2003).

The Von Hippel-Lindau (VHL) protein/E3 ligase

The von Hippel-Lindau tumor suppressor gene (VHL) encodes a multifunctional protein that contains 213 amino acid residues. Germ line mutations in VHL that occurs at a frequency of 1 in 36000 in the general population predispose the patients to several highly vascularized benign and malignant tumors, including clear cell renal cell carcinoma (ccRCC), hemangioblastoma of the central nervous system (CNS) and retina, and pheochromocytoma of adrenal glands. Somatic mutation of VHL is associated to the development of sporadic ccRCC (for review see (Maher 2011)).

Beside the full length VHL a second isoform also exist as a result of internal translational initiation at amino acid 54. The two VHL isoforms are called VHL 30 and VHL 19 after their apparent molecular weigh and behave similarly in the biochemical and functional studies. Both isoforms appear to retain tumor suppressor activity. The VHL shuttles between the nucleus and this shuttling is important for its tumor suppressor function. The VHL 30 isoform predominantly reside in the cytoplasm while the VHL 19 isoform reside primarily in the nucleus (Iliopoulos, Ohh et al. 1998, Kaelin and Maher 1998).
The VHL protein structure consists of two important regions; a β-domain, which spans amino acid residues 64–154 and the c-terminal α-domain that lies between amino acid 155-193. After its identification in 1993, the VHL gene was later mapped to chromosome 3 (Latif, Tory et al. 1993) and in a series of subsequent studies VHL was found to function as the substrate binding subunit of a CBC E3 ubiquitin ligase. Through its α-domain VHL was found to interact with cullin 2, elongin B, elongin C, Rbx1 and to promote proteasomal degradation of hypoxia-inducible factor (HIF) transcription factors, to which it binds via the β-domain (Lonergan, Iliopoulos et al. 1998, Maxwell, Wiesener et al. 1999).

Regulation of HIF in normal physiological conditions is the most known function of VHL E3 ligase. HIFs belong to the PAS (Per-ARNT-Sim) family of heterodimeric basic helix–loop–helix transcription factors and regulate gene expression by binding to hypoxia-response elements (HREs), specific DNA recognition sequences located in hypoxia enhancer-containing regulatory regions. The HIF heterodimer is composed of a stable β subunit (also known as ARNT) and either of three oxygen-regulated HIFα subunits, HIF1α, HIF2α or HIF3α. At normal oxygen level, HIFs are hydroxylated at two proline residues, Pro-402 and Pro-564, within an oxygen-dependent degradation domain (ODD) (for review see (Kaelin and Ratcliffe 2008)). The prolyl-hydroxylated HIFs are recognized by VHL E3 ligase, leading to polyubiquitylation and proteasomal degradation. The hydroxylation reaction is mediated by the prolyl hydroxylase domain proteins (PHDs) (Berra, Benizri et al. 2003). Another oxygen-dependent hydroxylation at a asparagine residue at the carboxy-terminal transactivation domain of HIFα subunits by factor inhibiting HIF (FIH) prevents CBP/p300 recruitment, thereby repressing HIFs transcriptional activity. Other residues within the N-TAD domain of HIF1α , such as Tyr-564 and Ilo-565 reported to be involved in HIF protein stability and transcriptional activity were also identified (Pereira, Zheng et al. 2003).
Under hypoxic conditions (typically below 3%–5% O_2), the prolyl hydroxylases are inactive and HIFs become stabilized and translocated to the nucleus where they activate transcription of target genes involved in adaptation to hypoxia, such as genes stimulating angiogenesis (VEGF), production of red blood cells (EPO), glucose transporters (Glut1) and glycolytic enzymes (LDHA). The HIF1α, HIF2α and HIF3α subunits are all targeted by VHL for proteasomal degradation. The role of HIF1α, HIF2α heterodimers as transcriptional activators has been well established, whereas the role of HIF3α in transcriptional activation is less clear (for review see (Kaelin 2007, Zhang, Gao et al. 2007)).

Regulation of HIFs is found to be disabled by mutations in the VHL β-domain or α-domain, disrupting either the interaction with HIFs or the elongin C complex, respectively, or both. The effect of mutations within each domain has been reported to be residue- and substitution-specific. For example it has been shown that within the β-domain the N78S mutant abrogated HIF binding but retained elongin C interaction, whereas S65W mutant disrupted both proteins interaction, most likely because of major structural effects on VHL. Similarly, within the α-domain, R167Q greatly reduced elongin C binding but retained HIF binding in some assays, whereas L158P abrogated both interactions. Studies show that it is difficult to predict the functional effects of point mutations from site alone, and that direct testing in functional assays will be necessary to understand the disease. VHL disease displays complex genotype–phenotype correlations. The disease phenotype has been grouped clinically into two subtypes depending on the presence or absence of pheochromocytoma. Whether a VHL patient develop RCC, hemangioblastomas, pheochromocytomas or a certain combination of tumors, depends on where in the VHL protein a mutation has occurred and if it is a point mutation or truncation. Mutations in type one disease are more commonly deletions or truncating mutations are associated with hemangioblastomas and RCC but not with pheochromocytomas. Mutations in type two disease are more common missense mutations predisposing to pheochromocytomas. Type two mutations are further subdivided to type 2A, 2B and 2C. Type 2A mutations, such as Y98H, more commonly predispose to hemangioblastomas and pheochromocytomas and rarely RCC,. Type 2B mutations, for example the R167Q, is associated with hemangioblastomas, RCC and pheochromocytomas. Type 2C mutation, such as L188V predispose only to pheochromocytomas (for review see (Kaelin 2007)).

Some VHL mutants are still able to bind and ubiquitylate hydroxylated HIFs indicating that HIF-independent biological functions may contribute to the pathogenesis of VHL disease. Indeed many HIF-independent activities of VHL have been reported. The role of VHL in microtubule dynamics, primary cilium maintenance, cell proliferation, neuronal apoptosis, extracellular matrix deposition and responses to DNA damage has been shown. Based on results from different studies it has been suggested that tumor initiation requires not only VHL mutation but also the alteration of additional cooperating cancer pathways (for review see (Frew and Krek 2007)). This mutation-dependency of tumor phenotype suggests multiple and/or tissue-specific functions for VHL, and the existence of specific mutations in VHL disease which are associated with differential tumor risks provides tools to dissect the relationships between VHL functions and tumor susceptibility.

Apoptosis

Apoptosis as one form of programmed cell death is highly conserved throughout evolution and generally characterized by distinct morphological characteristics and energy-dependent cascade of molecular events that allow the cells to commit suicide cell death, (Hotchkiss, Strasser et al. 2009). The distinct morphological changes that was first described by (Kerr, Wyllie et al. 1972) comprise cell shrinkage, smaller cell

size, membrane blebbing, dense cytoplasm with tightly packed organelles and so called pyknosis or condensation of chromatin are the most characteristic feature of apoptosis (Kerr, Wyllie et al. 1972). The biochemical changes associated with apoptosis are largely explained by the activation of caspases, a family of cysteine proteases that are wildly expressed in an inactive proenzyme form in most cells. The caspases form a protease cascade activated by stimuli either at the cell membrane upon cytokine-receptor binding, or within a cell and generated by micro-environment of a particular organelle.

Two main apoptotic pathways have been extensively studied and described; the extrinsic pathway mediated by a sub-group of tumor necrosis factor Receptor (TNFR) superfamily including TNF, Fas and TNF-related apoptosis inducing ligand (TRAIL) receptors or so called death receptors. And, the intrinsic or cell autonomous pathway which is largely centered around mitochondria.
As mentioned above a cascade of caspases act as common effector molecules in both intrinsic and extrinsic apoptosis pathways. Among them caspases 8, 10 stimulated by extracellular ligands and caspase 9 activated at the mitochondrial membrane by intracellular signals are the initiator caspases. Subsequently, the initiator caspases by cleavage at their specific target site activate a set of so called executioner caspases, notably caspases 3, 6 and 7. The executioner caspases will synchronously cleave proteins in many cell compartments.

Upon stimulation of death receptors the extrinsic pathway of apoptosis is induced by the formation of a death-inducing signaling complex (DISC). In this complex, Fas-associated death domain (FADD) adaptor protein is recruited upon Fas/FasR binding. TNF/TNFR binding results in sequential formation of two complexes, complex I and II. Complex II among other proteins include TNFR-associated death domain (TRADD) as well as FADD adapter proteins. FADD and/or TRADD complexes then associate and activate procaspse 8 and/or 10 leading to activation of the downstream executioner caspases3, 6 and 7. Activated executioner caspases are then responsible for the cleavage of a number of so called death substrates that lead to the characteristic hallmarks of apoptosis.

The intrinsic pathway is activated by various stimuli, such as DNA damage, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals, and acts through the mitochondria, which are controlled by the Bcl-2 family of proteins. The Bcl-2 family members possess a homologous domain responsible for protein-protein interaction. Bcl-2 itself and Bcl-xl, the two closely related members possess four such domains namely BH 1-4. While these members of the family support cell survival they interact with the other two pro-apoptotic member of the family namely Bak and Bax through the BH domains. In addition to interacting with Bcl-2 and Bcl-xl proteins and antagonizing their anti-apoptotic function, Bak and Bax can also form homo-oligomers. In this configuration Bak and Bax can create a wide diameter pore through the mitochondrial membrane through which cytochrome- c and ATP are released into the cytosol. Cytochrome c then binds and and activates Apaf-1 and together with ATP

form a platform for recruitment and activation of procaspase 9, known as apoptosome . The apoptosom activates the initiator caspase 9, which then leads to the activation of the executioner caspase 3. This leads to the same type of apoptotic response also observed in the extrinsic pathway.

In homeostatic conditions, the anti-apoptotic Bcl-2 members maintain mitochondrial membrane integrity by preventing the pro-apoptotic Bak and Bax. However, during cellular stress beside Bak and Bax, other members of the Bcl-2 family that possess only BH3 domain are activated. Among BH3-only proteins are Bim, Bid, Bad, PUMA and Noxa. During stress insult a rise in BH3-only protein concentration and their interaction with anti-apoptotic Bcl-2 members provides conditions that favour Bak/Bax oligomer formation and mitochondrial permeability.

There are other pro-apoptotic proteins released from the mitochondria that contribute to apoptosis. For example, Smac/Diablo antagonize the action of inhibitor of apoptosis proteins (IAPs) and relieves its inhibitory interaction with caspase 9, 3 and 7.
Activation of the intrinsic pathway can occur following the activation of the extrinsic pathway. Hence, the mitochondrial permeabilization in the Fas pathway of apoptosis is mediated by the caspase 8 cleavage of the pro-apoptotic Bid to its activated form. This relatively simple example of cross-talk between the intrinsic/extrinsic pathways further serves to illustrate the complex interrelationship between the two pathways.
The tumor suppressor protein p53 induces transcriptional activation of pro-apoptotic factors such as PUMA, and BAX. Noxa is also a mediator of *p53*-induced apoptosis. It has been shown that Noxa can localize to the mitochondria and interact with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9. Induction of Puma and Noxa by *p53* suggest that these factors might mediate the apoptosis that is elicited by geno-toxic damage or oncogene activation.
Myc has also been reported to potentiate apoptosis through both *p53*-dependent and -independent mechanisms.
The apoptosis mechanism results in the controlled breakdown of the cell into apoptotic bodies which are subsequently recognized and engulfed by surrounding cells and phagocytes. As apoptotic cells do not release their cellular constituents into the surrounding tissues and are quickly phagocytosed there is essentially no inflammatory reactions associated with the process of apoptosis.

Cellular senescence

The observation by Hayflick that primary human fibroblast cells in culture do not proliferate indefinitely but instead gradually come to a complete halt was the first evidence of so called cellular senescence (Hayflick and Moorhead 1961). Today we know that telomeres, the repetitive DNA sequences that protect the end of linear chromosomes are subject to shortening by each cell cycle. Due to the biochemistry of DNA replication, DNA polymerase is unable to completely replicate the full telomeric DNA end (Olovnikov 1971, Watson 1972). Hence, telomere erosion occurs progressively by each cell cycle until telomeres reach a critical minimal length or the so called “Hayflick limit” (Counter, Avilion et al. 1992) Consequently, DNA damage

response (DDR) signaling cascade including DDR-associated factors ATM/ATR and CHK1/CHK2 kinases is triggered. Several cell cycle regulatory proteins and mediators of growth arrest such as RB, $p16^{INK4a}$, p53 and $p21^{cip1}$ are subsequently induced through which a stable cell cycle arrest or senescence phenotype is stabilized (for review see (Rodier and Campisi 2011)). By definition, cellular senescence is irreversible cell cycle arrest, which is associated with certain changes in morphology and gene expression. Senescent cells are usually flattened out in shape and despite being arrested in the cell cycle they are metabolically active and express a complex secretome known as senescence-associated secretory phenotype (SASP) (Kuilman, Michaloglou et al. 2010). An increased senescence-associated β-galactosidase (SA-Bgal) activity due to increased number of lysosomes is very often seen in senescent cells.Senescence cells also undergo changes in chromatin structure giving rise to so called senescence-associated heterochromatin foci (SAHF) enriched in histone H3 trimethylated at lysine 9 (H3K9me3), which have been linked to of silencing pro-proliferative genes such as E2F family target genes (Narita, Nunez et al. 2003)).
The abovementioned senescence phenotype associated with telomere erosion, referred to as replicative senescence, can be bypassed by ectopic expression of the catalytic subunit of the telomerase holoenzyme hTERT which elongates eroded telomeres. However, senescence can be induced prematurely in the absence of any telomere loss by a variety of conditions such as oncogenic stress, tumor suppressor loss, DNA damage, oxidative stress and therapeutic drugs. Here I will focus on oncogenic induced senescence (OIS) and tumor suppressor loss-induced senescence of relevance for this thesis.

Several oncogenes such as Ras, Raf, Akt and STAT5 have been reported to induce cellular senescence. Introducing mutant oncogenic Ras into primary fibroblasts caused phenotypic changes, including proliferative arrest, resembling replicative senescence as first reported by Serrano et al (Serrano, Lin et al. 1997). Two main tumor suppressive mechanisms, the $p16^{INK4a}$/RB and p53/$p21^{Cip1}$ pathways, were shown to be responsible for the observed cell cycle arrest. As indicated above, theses pathways are also responsible for the senescence phenotype seen in cells undergoing replicative senescence. In murine cells, however, p53 seems to play a more prominent role than $p16^{INK4a}$ for induction of senescence compared with human cells, where loss of p16INK4a is commonly linked to escape/bypass of senescence. Inactivation of p53 was reported to be sufficient to bypass OIS induced by the mutant activated Ras^{V12} in murine cells (Kamijo, Zindy et al. 1997, Serrano, Lin et al. 1997), whereas human mammary epithelial cells and keratinocyte cells were shown to be solely dependent on $p16^{INK4a}$. However, this is not a universal rule as various studies suggest that the combination of both pathways is needed to induce and maintain the senescence phenotype in certain cell types, and the relative importance of each individual pathway seems to be context dependent.
OIS is now a known to be not only an in vitro phenomenon but has been demonstrated also in vivo. Among the first reported examples of OIS in vivo utilized a mouse model with conditional expression of K- Ras^{V12} that upon induction, causes development of multiple premalignant lung adenomas as well as some malignant lung

adenocarcinomas. By using senescence markers including upregulation of $p16^{INK4a}$, $p15^{INK4B}$, SA-Bgal activity and formation of SAHFs the authors demonstrated the existence of senescent cells in premalignant tumors but not in the malignant ones (Collado, Gil et al. 2005).
The oncogenic $B\text{-}Raf^{V600E}$ mutation is a frequent in both human benign naevi and melanoma. $B\text{-}Raf^{V600E}$-transduced human melanocytes as well as a panel of specimens of human naevi were shown to express elevated level of $p16^{INK4a}$ and displayed increased SA-Bgal activity associated with cell cycle arrest (Michaloglou, Vredeveld et al. 2005). Further, mice that were induced to express conditional $B\text{-}Raf^{V600E}$ developed benign lung adenoma followed by growth arrest associated with a senescence-like phenotype. Genetic inactivation of the INK4A locus, containing p16 and $p19^{Arf}$, or p53 in these mice led to tumor development (Dankort, Filenova et al. 2007).
In another study mice with conditional melanocyte-specific expression of $B\text{-}Raf^{V600E}$ developed benign melanocyte that fail to progress to melanoma. Upon depletion of PTEN these mice developed metastatic melanoma (Dankort, Curley et al. 2009)

The histon methyltransferase Suv39h1 is involved in trimethylation of H3K9, which is another marker of senescence as mentioned above. In both Eμ-NRAS and Eμ-myc transgenic mouse models of lymphomagenesis Suv39h1 was shown to have tumor suppressive role associated with maintaining the senescence barrier against tumor development (Braig, Lee et al. 2005).

In addition to OIS, loss of tumor suppressor loss-mediated senescence has also been shown in both mouse and human cells. Conditional complete inactivation of tumor suppressor PTEN obtained by using $PTEN^{loxP/loxP}$ and Cre-recombinase technique in transgenic mice was associated with high-grade prostatic intraepithelial neoplasia displaying characteristics of senescence accompanied by induction of p53. Upon concomitant inactivation of p53 these lesions progress to malignancy indicating cooperative tumor suppression where p53 acts as a failsafe protein in PTEN deficient tumors (Chen, Trotman et al. 2005). Interestingly the oncogenic condition of PTEN/p53 deficiency can be reversed by depletion of SCF^{Skp2} E3 ligase leading to tumor regression (Lin, Chen et al.).
Inactivation of VHL tumor suppressor was also shown to induce senescence in an Rb- and p400-dependent manner that was associated with decreased mRNA level of Skp2 and increased level of $p27^{Kip1}$ (Young and Kaelin 2008).

As mentioned senescent cells are characterized by secretion of a complex mixture of secreted molecules (SASP) that can both act as pro-oncogenic or tumor suppressive factors in an autocrine or paracrine fashion (for review see (Rodier and Campisi 2011). Among these factors are many cytokines such as interleukins IL-6, IL-8 and TGF-β as well as VEGF and matrix metalloproteinases (MMPs).
For example, the cytokine growth-related oncogen (GRO) level was elevated in prostate fibroblast senescent cells, and direct co-culture as well as conditioned medium from these cells stimulated proliferation of premalignant epithelial cell (Bavik, Coleman et al. 2006).

Similarly, elevated level of IL-6 and IL-8 that are secreted by senescent cells were associated with induction of epithelial-mesenchymal transition (EMT) and invasiveness in cultured premalignant epithelial cells (Coppe, Patil et al. 2008). On the other hand, upregulation of IL-8 receptor was shown to be essential for reinforcing growth arrest in Ras-induced and replicative senescent cells (Acosta, O'Loghlen et al. 2008). An IL-6 mediated inflammatory response was also shown to be required for senescence entry and maintenance in an autocrine fashion (Kuilman, Michaloglou et al. 2008). Further, Myc inactivation in a model of in T-cell lymphoma triggered TGF-β signaling that was shown to be essential to induce senescence, also here by an autocrine mechanism (van Riggelen, Muller et al. 2010).

Further, secretion of cytokines by senescent tumor cells has been shown to stimulate and attract the innate immune system leading to clearance of senescent cells (Xue, Zender et al. 2007). Altogether the SASP mediated cytostatic or pro-mitogenic functions seems to be context dependent, and more studies are required to carefully examine the consequences of SASP in different tumor models

In a recent study and using the Eμ-*myc* mouse lymphoma model the authors demonstrated that chemotherapy-induced senescent cells both increased glucose utilization and ATP production. They further showed that strong SASP-mediated proteotoxic stress required high energy production for increased ubiquitylation and adaptaiton to toxic conditions, involving autophagy-dependent destruction of toxic proteins. Upon blocking glucose utilization and autophagy an apoptosis response was induced accompanied with tumor regression in vivo (Dorr, Yu et al. 2013).

Myc/Max/Mad network

The *Myc* proto-oncogene encodes a transcription factor that plays a central role in the genesis of many different human cancers. *Myc* belongs to the family of genes that also includes *MYCL* and *MYCN*. The proteins encoded by the MYC genes are members of a large group of proteins referred to as the Myc/Max/Mad network that function as transcriptional factors controlling the expression of a large number of different genes. The set of distinct target genes modulated by this network play key role in different aspect of cellular behavior including regulation of cell cycle, cell growth, proliferation, differentiation, apoptosis and metabolism (for review see (Prendergast 1999, Rottmann and Luscher 2006).

Members of the Myc/Max/Mad network share a related basic helix-loop-helix leucine zipper (bHLH-Zip) domain in their structure. The bHLH-Zip domain-containing proteins need to homo or heterodimerize for DNA binding. This domain is the common structural element of the network and is responsible for specificity and stability of dimer formation (Murre, McCaw et al. 1989). The bHLH-Zip domain contains two α-helices, helix H1 and H2 that are separated by a loop. A basic DNA binding region preceding the first α-helix H1 specifies the interaction with DNA major groove. The helix-loop-helix together with the leucine zipper region at the carboxy terminal to the second α-helix H2, facilitate the dimerization (for review see (Luscher and Larsson 1999)). The basic DNA binding region, specifically binds to the 5'-CACA/GTG-3' DNA sequences which is a subset of the general E-box sequence CANNTG bound by all bHLH-Zip proteins (Blackwell, Kretzner et al. 1990). The Myc proteins in the

network comprise a transcriptional activation domain (TAD) in their N-terminal 150 amino acids through which recruit different cofactors and function as a transcriptional regulators (Henriksson and Luscher 1996).
The search to find the dimerization partners led to the break-through identification of a closely related bHLH-Zip protein Max (Blackwood and Eisenman 1991, Prendergast, Lawe et al. 1991)).
Max as Myc proteins obligatory heterodimerization partner is essential for various biological activities, including transformation, apoptosis and transcriptional activation (Amati, Littlewood et al. 1993, Luscher 2001). Max is evolutionary the most conserved component of the network with particularly high conservation in the bHLH-Zip domain. Members of the Myc family heterodimerize with Max in order to bind to DNA (Blackwood and Eisenman 1991). Initial studies show that the Myc/Max heterodimer can also bind non-canonical sequences that are variants of the canonical E-boxes (Blackwell, Huang et al. 1993). Although Myc/Max heterodimerization is required for Myc-mediated transcription in mammals, a Max-independent role of Myc in transcription has been reported in Drosophila (Steiger, Furrer et al. 2008).

Shortly after the discovery of Max, a second class of bHLH-Zip proteins called Mad/Mnt proteins were identified. The members of this family of proteins include four different Mad proteins, Mad1, Mxi1, Mad3, Mad4 and the two rather diverged proteins Mnt and Mga (Ayer, Kretzner et al. 1993, Zervos, Gyuris et al. 1993, Hurlin, Ayer et al. 1994). The four Mad proteins are highly homologous proteins with functional similarities and specifically interact with Max but not with each other nor with Myc proteins.
Mad/Mnt proteins except for Mga contain an N-terminal region that mediates interaction with the transcriptional corepressor mSin3 known as mSin3-interacting domain or SID (Schreiber-Agus, Chin et al. 1995, Ayer, Laherty et al. 1996, Eilers, Billin et al. 1999). mSin3 is a component of a repressor multiprotein histone deacetylase complex HDAC1/2 (Schreiber-Agus and DePinho 1998, Knoepfler and Eisenman 1999).
The E-box sequences can also be bound by heterodimers of Max and Mad proteins resulting in repression of corresponding genes. Thus Max plays a central role of a transcriptional activator-repressor network. While the expression level of Max appear to be fairly constant, Myc expression is very low in quiescent G0 cells, increasing in response to mitogenic signals and continually expressing in cycling cells (Oster, Ho et al. 2002). In contrast, Mad expression increases during differentiation (Ayer, Kretzner et al. 1993, Amati and Land 1994, Larsson, Pettersson et al. 1994). Hence the relative levels of Myc and Mad proteins determine the transcriptional status of target genes. Myc/Max gene occupancy and associated coactivators in growing cells switches to Max/Mad associated with co-repressors in differentiated cell (Bouchard, Dittrich et al. 2001). Little is known about Mad deregulation in tumors. When overexpressed, Mad proteins strongly repress cell proliferation and antagonize transformation. Knock out of the individual Mad genes in mice is not associated with any strong phenotype suggesting that the Mad protein members may compensate for each other (Luscher 2012).

The other member of the Myc/Max/Mad network Mnt, is a larger protein relative to the rest of the network which is ubiquitously expressed. Beside the bHLH-Zip and SID domains Mnt share no extensive homologies to other network members (Hurlin, Queva et al. 1997). Knockdown of Mnt is associated with accelerated proliferation and apoptosis as well as tumorigenesis and together with Ras is sufficient to transform mouse embryonic fibroblast (MEFs), similar to over expression of Myc (Hurlin, Zhou et al. 2003, Nilsson, Maclean et al. 2004). In addition, Mnt is regulated during the cell cycle (Popov, Wahlstrom et al. 2005) altogether suggesting an important tumor suppressive role of Mnt for cellular proliferation.

Myc transcription factor

The Myc family proto-oncoproteins comprising c-Myc, N-Myc and L-Myc are nuclear proteins that are required for normal cell proliferation and growth (Oster, Ho et al. 2002, Gallant 2006). Deregulation of Myc family proteins has been linked to a wide variety of human and other animal cancers (Oster, Ho et al. 2002). Myc was first discovered as the cellular homolog of the avian myelocytomatosis viral oncogene (v-myc) (Sheiness and Bishop 1979, Vennstrom, Sheiness et al. 1982). Genomewide binding studies indicate that over 10%-15% of genomic loci are bound by Myc (Dang, O'Donnell et al. 2006).

As transcription factor Myc can both activate and repress transcription (for reviews see (Adhikary and Eilers 2005, Cole and Nikiforov 2006, Herkert and Eilers 2010). During embryonic development stem cells keep high level of Myc which supports proliferation and antagonize differentiation. Myc and N-Myc knockout mice are embryonically lethal whereas L-Myc knockout mice do not exhibit any phenotypic abnormalities (for review see (Pirity, Blanck et al. 2006)). Myc is induced or downregulated under control of different signaling pathways such as Wnt, Notch, NF_KB and TGF-β exerted through the function of transcription factors such as Tcf, E2Fs, Smads, Sp1 and many other family of transcription factors (for review see (Liu and Levens 2006)).

Myc and cofactors (transcriptional activation/repression)

Both NTAD and C-terminal domain of Myc are required for Myc to exert its transcriptional activity. Recent studies show that Myc regulates promoters that pre-exist in an active or poised chromatin state further modifying them. Doing so Myc function in transcription has been suggested to function as a global amplifier of transcription (Rahl, Lin et al. 2010, Lin, Loven et al. 2012, Nie, Hu et al. 2012). Myc interacts with a number of cofactors such as histone acetyl transferases (HATs), ATP-dependent, chromatin remodeling complexes, E3 ubiquitin ligases, mediator subunits and DNA methyl transferases (Adhikary, Marinoni et al. 2005).

At the N-terminal transactivation domain (NTAD) Myc contains two highly conserved regions called Myc homology box I and II (MBI and MBII) (Amati, Dalton et al. 1992). Two additional MBIII and MBIV have also been defined (Herbst, Hemann et al.

2005). The carboxy terminal bHLH-Zip domain facilitates DNA binding and heterodimerization with Max. The basic motif in this domain is required for DNA binding and the dimerization with Max is mediated through the HLHZ motif (Murre, McCaw et al. 1989), (Blackwell, Kretzner et al. 1990), (Prendergast, Lawe et al. 1991), (Blackwood and Eisenman 1991).

Through MBII Myc interacts with Transcativation/Transformation Associated Protein (TRRAP). TRRAP is a large multiprotein complex that recruits HATs such as GCN5, TIP60, as well as ATPase/helicase complexes TIP48 and TIP49 to target gene promoters. Other HATs such as CREB-binding protein (CBP) and p300 have been shown to interact with and become recruited by Myc to target gene promoters. Myc and TRRAP interaction probably also has functions other than recruitment of HATs. For instance, the p400 histone exchanger factor that assembles with TRRAP also associates with Myc (Martinato F, Cesaroni, M, 2008). Martinato et al also showed that recruitment of HATs to target gene promoters by Myc promotes hyper-acetylation of multiple lysines on histones H3 and H4 locally.

The proteasome subunit 19S has been identified at promoter of transcriptionally active genes indicating a non-proteolytic role of UPS components in RNA pol II transcription (Gonzalez, Delahodde et al. 2002). Myc was shown to recruit Sug1, a component of the 19S particle of the UPS to cyclin D2 gene promoter resulting in transcriptional activation (von der Lehr, Johansson et al. 2003). In addition, through MBII Myc interacts with the Skp2 E3 ubiquitin ligase and recruits it to target gene promoter (Kim, Herbst et al. 2003, von der Lehr, Johansson et al. 2003) suggesting that ubiquitylation of Myc may result in recruitment of proteasomal subunits with a role in transcriptional activation. Myc also interact with the HectH9 E3 ligase and upon ubiquitylation recruit coactivator p300 that is required for transactivation and induction of cell proliferation (Adhikary, Marinoni et al. 2005)

Myc plays an important role during the transition from the initiation to the elongation phase of transcription by recruiting the kinase complexes pTEF-B (cyclin T1/cdk9) as well as TFIIH/cdk7 to target gene promoters. In addition to these factors, Myc also interacts with the mediator subunit TRAP220, altogether leading to promoter polymerase clearance and transcriptional elongation at the RNA pol II-preloaded gene promoters. These interesting initial findings were confirmed by a genome wide analysis of Myc function in embryonic stem cells (Bouchard, Marquardt et al. 2004) (Rahl, Lin et al. 2010).

Beside interacting with components of the RNA pol II transcription machinery through which Myc regulates most of its target gene expression, Myc additionally regulates transcription mediated by RNA pol I (Arabi, Wu et al. 2005, Grandori, Gomez-Roman et al. 2005) and RNA pol III (Gomez-Roman, Grandori et al. 2003)through recruitment of cofactors such as TRRAP. Upon TRRAP interaction, Myc-mediated RNA pol III transcription is associated with histone 3 acetylation and subsequent recruitment of TFIIIB (Gomez-Roman, Grandori et al. 2003).

Recruitment of TFIIH by Myc was shown to promote 5’ mRNA cap methylation on a subset of genes and that correlated with an increase in RNA pol II CTD tail phosphor-

Ser5. Only expression of N-termial part of Myc was reported to be sufficient to promote 5' mRNA cap methylation and protein abundance suggesting that Myc also has transcription-independent functions. Another transcription independent function of Myc was shown where Myc was localized at early replication origin in complex with MCM pre-replicative proteins promoting DNA replication. Full length of Myc protein was required to exert this function. Moreover, Myc plays an important role in regulation of the cellular response to hypoxia through its functional interactions with the hypoxia-inducible factors HIF1α and HIF1α and HIF2α (See below) (for review, see (Gordan, Lal et al. 2008)).

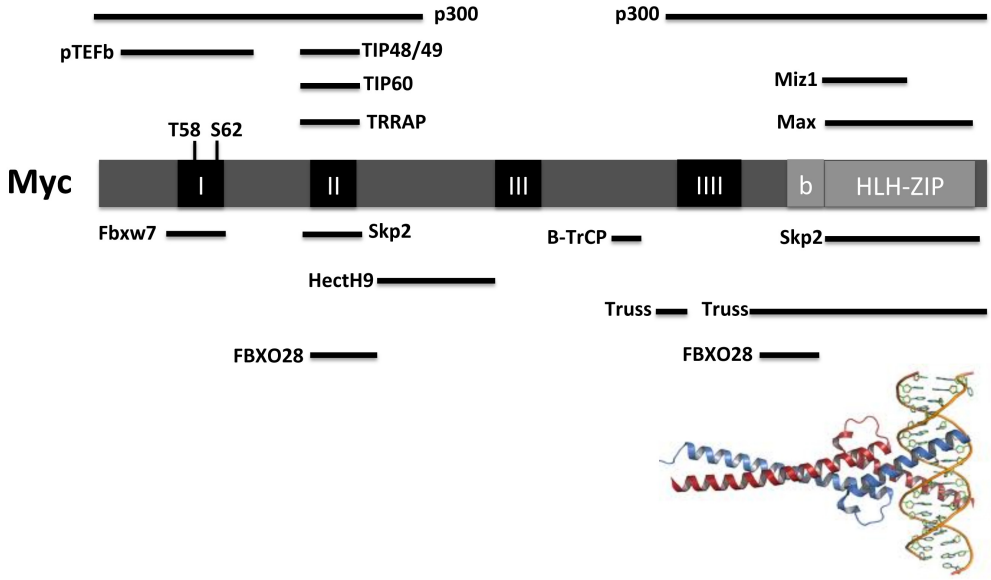

Figure 2. Schmatic representation of Myc protein structure, its functional domains and regions through which Myc interacts with some of its cofactors (shown on top of Myc structure) and E3 ubiquitin ligases (shown below).

Myc and Max heterodimerization and DNA binding is shown in the bottom.

Distinct functions have been identified for Myc homology boxes MB I- MB IV. The role of MBI has been implicated in Myc protein stability and turn over (Bahram, von der Lehr et al. 2000).

MBII plays an essential role in biological function of Myc as it is required for activation or repression of most of Myc target genes. In fact, Myc requires MBII for all its known biological functions such as apoptotic and oncogenic activities as well as for blocking differentiation. MBIII also regulates protein stability and is necessary for Myc-induced transformation but also negatively regulates Myc-induced apoptosis. It is

also required for full transactivation and transrepression of a subset of Myc target genes (Herbst, Hemann et al. 2005). MBIV plays role in Myc-induced transformation and apoptosis .

A large amount of data suggests that Myc can both activate and repress transcription indicating that Myc-mediated repression also plays an important role in Myc biological and pathological functions. The mechanism through which Myc represses transcription is far less understood. The first indications of Myc-mediated transcriptional repression was that Myc repressed itself arguing the existence of a negative feedback loop (Zindy, Eischen et al. 1998). Unlike Myc-mediated transcription activation where Myc can activate transcription also from distant sites, Myc-mediated repression seems mostly to be directed through core promoters. Two transcription factors Miz1 and Sp1 that Myc interact with are present at the core promoter of many Myc repressed genes. The best generally accepted mechanism of Myc repression is that Myc interacts with Miz1 leading to disruption of Miz1 and its transcriptional coactivator p300 and nucleophosmin (NPM), thereby repressing Miz1-activated target genes such as $p15^{INK4B}$ and $p21^{Cip1}$ (for review see (Herkert and Eilers 2010)) (Staller, Peukert et al. 2001) (Seoane, Pouponnot et al. 2001) (Herold, Wanzel et al. 2002) (Herold, Wanzel et al. 2002). The Myc/Miz1 complex also recruits the DNA methyl transferase Dnmt3a to the promoter of $p21^{Cip1}$ gene to repress transcription. Myc/Miz1 together with ARF tumor suppressor form a complex inducing repressive H3K9-trimethylated heterochromatin. The Myc/Miz1/ARF complex was associated with repression of genes involved in cell adhesion and induced apoptosis suggesting a mechanism of elimination of cells with oncogenic mutation (Herkert and Eilers 2010).

Myc post-translational modifications and degradation

The Myc protein is very unstable with a short half-life of 20-30 minutes. The mechanism underlying Myc protein stability and its prolonged half-life in certain cancers like Burkitt's lymphoma has been an interesting subject of study in Myc field.

Among many Myc phosphorylation sites T58 and S62 in MBI region of Myc have been the focus of most studies on Myc stability and activity (Bahram, von der Lehr et al. 2000); (Henriksson, Bakardjiev et al. 1993); (Pulverer, Fisher et al. 1994) ;. The phosphorylation status of these sites has been linked to Fbxw7-mediated degradation of Myc. The phosphorylation of S62 is mediated by number of candidate kinases like ERK, JNK and Cdk1 upon variety of stimuli such as mitogenic signals, cytokines, DNA damage and UV (for review see (Hann 2006)). The phosphorylation of S62 primes phosphorylation of T58 by GSK3β kinase (Lutterbach & Hann 1994 ; Sears, 2000). Fbxw7 then recognizes T58-phosphorylated Myc and mediates its ubiquitylation/proteasomal degradation (Yada, Hatakeyama et al. 2004). This was shown to be mediated by Pin1 recognition of phosphorylated T58-S62 with subsequent dephosphorylation of S62 by phosphatase PP2A followed by Fbxw7 recruitment and Myc degradation (Yeh, Cunningham et al. 2004). During G2 phase of the cell cycle Aurora A interacts with Fbxw7 and N-Myc and counteracts Fbxw7-mediated degradation of N-Myc leading to N-Myc stability (Otto, Horn et al. 2009).

At least four other E3 ligase have been identified that ubiquitylate and regulate the turn over of Myc. The first identified E3 ligase Skp2 binds MBII and bHLHZ domains of Myc and ubiquitylate Myc. Skp2 together with several subunits of proteasome were recruited to MYC target gene promoters. The Skp2-mediated ubiquitylation of Myc was suggested to provide a "licensing" mechanism that both stimulates transcriptional activity but at the same time targets Myc for proteasomal degradation (von der Lehr, Johansson et al. 2003) (Kim, Herbst et al. 2003).

As mentioned before HectH9 E3 ligase mediates K63-linked ubiquitylation associated with transcriptional activation of c-Myc (Adhikary, Marinoni et al. 2005) whereas it was also reported to assemble K48-linked ubiquitylation of N-Myc leading to increased N-Myc degradation (Zhao, Heng et al. 2008).

Another E3 ligase that was recently shown to target Myc for ubiquitylation/proteasomal degradation is tumor necrosis factor receptor-associated ubiquitous scaffolding and signaling protein/ damage-specific DNA-binding protein 1/Cullin4 (Truss/Ddb1/Cul4). Truss/Ddb1/Cul4 binds to C-terminal domain of Myc and its ubiquitylation/proteasomal-mediated destruction of Myc is associated with repression of Myc-mediated transactivation and transformation.

In addition to Skp2 and HectH9 E3, at least two other E3 ligases have been identified that promote Myc transcriptional activity. The E3 ligase β-TrCP binds N-terminal domain of Myc and mediates heterotypic polyubiquitin chain leading to stabilization of Myc. This function of β-TrCP was dependent on the role of E2 conjugating enzyme UbcH5. β-TrCP mediated ubiquitylation of Myc antagonized Fbxw7-mediated degradation that occurs during G1 phase of the cell cycle suggesting a mechanism of stabilizing Myc after G1 phase (Popov, Schulein et al. 2010).

We also recently showed that the SCF^{FBXO28} E3 ligase ubiquitylates Myc in a cell cycle and phosphorylation-dependent manner leading to recruitment of HAT p300 enhancing Myc-mediated transcription, proliferation and tumorigenesis (paper II). This will be discussed more in detail in results and discussion section.

The Myc promoter and auto-repression

As mentioned earlier, c-Myc represses its own promoter through a negative feedback loop. This represents a global homeostatic control mechanism and is needed for normal cell growth control. From work in Burkitt's lymphoma it is known that the translocated myc allele is expressed while the non-translocated allele is transcriptionaly silent. The c-myc auto-repression is operative in primary and some established cell lines and is lost in many transformed or tumor-derived cell lines.
Later studies showed that c-Myc auto-regulation is concentration dependent.
The Myc gene comprises three exons, exon1, 2 and 3 and four promoters P0, P1, P2 and P3. Exon 1 is non-coding and Myc protein is encoded from exon 2 and 3. Most of the transcription within the MYC locus initiates form promoter 1 and 2, accounting for 25% and 75% of Myc mRNA, respectively. The P2 promoter contains a TATA-box

sequence and two initiator elements and the P1 promoter includes less optimal TATA-box sequence while P0 and P3 promoters lack TATA-boxes. At the end of exon 3 there are two polyadenylation signal sequences, poly A1 and poly A2.

The mechanism of Myc auto-repression is still elusive. A mechanism of Myc autorepression was reported where binding of VHL to Myc gene promoter region through interacting with Myc was associated with recruitment of HDAC1/2 that led to repression of Myc gene (Hwang I, Roe J, 2012). The authors did not provide the mechanism behind this but suggested that might be part of the well-known Myc auto-repression. Tissue specific enhancer elements were identified in the 5' upstream region of the Myc gene. This suggest that beside upstream oncogenes and signaling molecules, Myc itself may regulate its expression through promoter/enhancer elements and that may require tissue specific cooperating factors. This could also explain organ-specific growth control and tumor-type specificity of oncogenes.

A mechanism of Myc autorepression was reported where initiator sequences as well as E2F binding sites were required.

Myc was also shown to induce the expression of microRNAs miR-17 and miR-20 that target E2F1 transcription factors. As E2F1 positively regulates Myc gene expression this may provide another mechanism of Myc autorepression (O'Donnell, Wentzel et al. 2005).

It has also been reported that Myc gene expression is regulated by blockage of transcription elongation. Although the mechanism of Myc autoregulation is still unresolved different mechanisms may exist that individually and context-dependent or in orchestrate together may be applied by Myc for self-regulation.

Myc and cellular safeguard mechanisms

By stimulating aberrant proliferation and cell growth, in addition to tumor suppressors prototypic oncogenes such as Myc and Ras are also able to trigger the self-defense mechanisms. While Myc was known as a potent inducer of apoptosis its role as an important player in triggering cellular senescence is becoming more obvious.

Myc and Apoptosis

In addition to playing an important role in cellular growth and proliferation, Myc was also found as a potent inducer of apoptosis (Askew, Ashmun et al. 1991); (Evan, Wyllie et al. 1992). Myc sensitizes cells to a wide range of death stimuli including serum starvation, hypoxia and cytotoxic drugs (for review see (Meyer and Penn 2008)). Myc exerts its apoptotic function through at least two known pathways. The tumor suppressor protein p19Arf is a target of Myc activation (Zindy, Eischen et al. 1998). Upon induction of p19Arf hence suppression of MDM2 E3 ligase, the tumor suppressor p53 will establish resulting in activation of pro-apoptotic genes such as Bax and PUMA (Nilsson, Maclean et al. 2004). Loss of either *p53* or *ARF* impairs the

apoptotic function of Myc, indicating the importance of the ARF/p53 pathway in Myc induced apoptosis (Zindy, Eischen et al. 1998).
The anti-apoptotic genes Bcl-2 and Bcl-X_L are negatively regulated by Myc resulting in activation of pro-apoptotic genes such as Bax and subsequent activation of intrinsic mitochondrial driven apoptosis (See "Apoptosis" section). The important role of Bcl-2 family member of proteins in Myc-induced apoptosis has been shown in different studies. Bax deficiency in pre-B cells is associated with resistance to Myc-induced apoptosis and promotes B-cell lymphomagenesis in Eμ-*myc* transgenic mice (Eischen, Roussel et al. 2001). In fact studies on B-cells of Eμ-*myc* transgenic mice which have both high proliferative and apoptotic rate provided some of the first evidence for Myc-induced apoptosis. The high rate of apoptosis seen in Eμ-*myc* transgenic B-cells is overridden by overexpression of Bcl-2, which cooperate with Myc to block apoptosis. This cooperation reflects a Myc-induced apoptotic pathway that selectively suppresses the Bcl-X_L or Bcl-2 in B-cells. However, more than half of the Eμ-*myc* transgenic mice develop lymphoma indicating that Myc suppression of Bcl-2 family in hematopoietic cells may be disabled. The Myc/ARF/p53 and Myc/Bcl-2 apoptosis pathways are inactivated independently in lymphomagenesis as the over expression of Bcl-2 proteins do not correlate with loss of ARF or p53 function in these tumors.
In vitro and in vivo studies have demonstrated that the level and intensity of Myc expression is a determining factor for Myc induction of apoptosis. While low level of deregulated Myc drives proliferation and may be a more efficient initiator of tumorigenesis than overexpressed Myc, the apoptotic function of Myc however seems to be solely dependent on Myc over expression (Evan, Wyllie et al. 1992) (Murphy, Junttila et al. 2008). However, after Myc-induced apoptosis is disabled for example through the function of Bcl-2 family proteins, overexpressed Myc drives tumorigenesis.

Myc and senescence
As mentioned before the overexpression of Myc causes genomic instability and sensitizes cells to apoptosis. Different studies have has also shown a role for Myc in regulating cellular senescence. Myc binds the promoter region of the human Werner syndrome gene WRN which encodes a repair-associated RecQ helicase, and directly stimulates transcription of WRN gene. The overexpression of Myc in WRN syndrome fibroblasts as well as WRN depleted fibroblasts, induced telomere-independent cellular senescence associated with enhanced Myc-induced replication stress (Grandori, Wu et al. 2003).

Short-term activation of Myc in Cdk2 knockout mouse embryonic fibroblasts, induced senescence in a $p16^{INK4a}$/RB and p53 dependent manner after an initial proliferative response. These cells also displayed a profound senescence response when exposed to ambient oxygen tension suggesting that Cdk2 may sensitize the cells to oxidative stress in addition to oncogenic insult. Moreover, Cdk2 deficient Eμ-*myc* transgenic mice showed a significant delayed lymphoma onset associated with senescence induction (Campaner, Doni et al. 2010).

Myc on the other hand is also able to repress senescence. Overexpression of Myc was shown to suppress oncogenic mutant $BRAF^{V600E}$- or $NRAS^{Q61R}$-induced senescence, which was shown to be $p16^{INK4a}$ - or p53-independent. Upon Myc depletion in $BRAF^{V600E}$ transformed melanoma cells, the senescence phenotype was reestablished (Zhuang, Mannava et al. 2008). Conditional expression of Myc was demonstrated to suppress the cell-autonomous TGF-β induced senescence in T-lymphoma cells. Continuous suppression of Miz-1 through Myc interaction hence repression of cyclin dependent kinase inhibitors including $p15^{INK4B}$ and $p21^{cip1}$ was required to antagonize TGF-β induced senescence (van Riggelen and Felsher 2010). Mirroring this study and in line with the role of TGF-β in inducing senescence it was shown that upon activation of Myc in B-lymphoma cells macrophages were activated by apoptotic lymphoma cells. In turn TGF-β was secreted from macrophages resulting in non-cell-autonomous induction of senescence. This was dependent on the function of histone methyltransferase Suv39h1 upon inactivation of senescence phenotype was abrogated associated with Myc-driven tumor progression (Reimann, Lee et al. 2010).
We have shown that Myc represses Ras-induced senescence in primary rat fibroblast and this function of Myc is dependent on phosphorylation of Myc at Ser-62 by Cdk2 (Hydbring, Bahram et al. 2010), which will be further discussed under result and discussion section.

Myc and cell cycle/cell growth

As mentioned earlier Myc expression is very low in quiescent G0 cells and upon mitogenic signals increases during the G1 phase further continually expressing in cycling cells. Expression of Myc alone is sufficient for quiescent cells to re-enter and pass through the G1/S phase of the cell cycle inducing S phase DNA synthesis. Many of the genes that contribute to cell cycle progression are direct target of Myc regulation. Among these genes are cyclin D1 and D2 and their binding kinase Cdk4, as well as E2F1-3, Cdc25a (Bouchard, Thieke et al. 1999). Drosophila cyclin A and cyclin B have also been reported as direct Myc target genes. Through Miz1, Myc is also able to represses the CDK inhibitors $p15^{INK4B}$ and $p21^{Cip1}$ (for review see (Herkert and Eilers 2010)) (Seoane, Pouponnot et al. 2001) (Wu, Cetinkaya et al. 2003). Also, $p27^{Kip1}$ is repressed by Myc in two different mechanisms; firstly Myc and FoxO3a were found to interact and to bind the $p27^{Kip1}$ promoter resulting in transcriptional repression (Chandramohan, Mineva et al. 2008). Myc also induces the Cks1 component of the SCF^{Skp2} E3 ligase, which targets $p27^{Kip1}$ for proteasomal degradation hence indirectly suppressing it.

Upon DNA damage and p53 activation downstream of CHK1 and CHK2 kinases, GADD45 is activated by p53. Gadd45 inhibits Cdk1 and consequently blocks G2/M phase of the cell cycle transition. Myc binds the promoter region of GADD45a and GADD153 and suppresses the expression of these genes, thereby counteracting p53.

For a cell to grow it needs to increase in mass and size, which requires increased protein synthesis. The Akt/mTOR signaling pathway plays a central role in regulating these processes. In addition to regulating genes involved in cell cycle, Myc contributes to cell proliferation through promoting increased protein synthesis. Myc controls the rate of protein synthesis by regulating the expression of several ribosomal proteins including L23, the BN51subunit of RNA pol III, ribosomal protein, the RNA helicase MrDb and the translation initiation factor eIF4E.
The role of Myc in cell growth and cell size, which is independent of its effect on the cell cycle, has been demonstrated most extensively in Drosophila and mouse B lymphocytes. Overexpression of dMyc in Drosophila wing cells led to an increase in cellular mass without increasing the cell number. Moreover, loss of Drosophila Myc (dMyc) was associated with slow growth and reduced cell size. Further, Myc-induced growth and increased cell size independent of the cell cycle phase was shown during B lymphocyte development.
Myc regulates several steps of ribosome biogenesis as well as regulation of all three RNA pol I-III dependent transcription. Myc interaction to sequences upstream and downstream of rDNA gene clusters has been shown by using chromatin immunoprecipitation assays (ChIP assyas) and its direct regulation of RNA pol I transcription was reported in different studies (Grandori, Gomez-Roman et al. 2005); (Arabi, Wu et al. 2005). Further, Myc also regulates RNA pol III dependent transcription through interaction with TFIIIB and binding to 5S rRNA as well as tRNA genes(Gomez-Roman, Grandori et al. 2003).Taken together, Myc has an overall control of cellular protein production by coordinating mRNA translation through the transcription of factors such as eIF4E, 5' mRNA cap methylation, as mentioned above, and it's in addition to the role in RNA pol I-, II- and III-dependent transcription, as well as processing of ribosomal RNA.

Myc and metabolism

Upon growth factor stimulation many changes including reprograming of cellular metabolism occurs to support the high demand in nutrient uptake and energy metabolism to fulfill the requirements of cell growth and proliferation. The glucose molecule is broken down to pyruvate, which can be used to fuel mitochondrial oxidative phosphorylation or be converted to lactate by lactate dedydrogenase (LDH-A) through anaerobic glycolysis. Myc promotes both anaerobic glycolysis as well as mitochondrial oxidative phosphorylation and mitochondrial gene expression.
Myc stimulates glucose uptake by regulating the glucose transporter gene Glut-1. Besides, Myc has been shown to promote glutamine uptake indirectly by repressing expression of miR-23a and miR-23b both of which downregulate glutaminase, the enzyme converting glutamine to glutamate as another source of energy (Gao, Inuzuka et al. 2009).

Transported glucose is phosphorylated by hexokinase II (HKII), which is another target of Myc. Other glycolytic genes such as enolase 1 (ENO1) and phosphofructokinase

(PFKM) that are involved in the process of converting glucose to pyruvate are also regulated by Myc (Kim, Zeller et al. 2004) (Coller, Grandori et al. 2000). Myc directly stimulates transcription of LDH-A gene hence shifting metabolism towards anaerobic glycolysis in the absence of oxygen. However, in highly proliferative cells such as tumor cells, LDH-A directs the conversion of pyruvate to lactate even in the presence of oxygen, a phenomenon termed as aerobic glycolysis or the Warburg effect.

In the presence of oxygen pyruvate is further converted to acetyl-CoA through the enzymatic reaction of pyruvate dehydrogenase (PDH). acetyl-CoA then enters mitochondria to be used in TCA cycle. Myc regulates mitochondrial biogenesis and mass as well as directing the transcription of mitochondrial genes in the nucleus. Deregulated Myc however was shown to promote pyruvate dehydrogenase kinase 1 (PDK1) which phosphorylates PDH and blocks mitochondrial oxidative phosphorylation (Kim, Kim et al. 2007). Myc has been proposed to transcriptionally coordinate both mitochondrial oxidative phosphorylation and anaerobic glycolysis (Zhang, Gao et al. 2007).
Besides increasing the synthesis of mitochondrial biosynthetic metabolites, Myc promotes nucleotide and amino acid synthesis through direct transcriptional regulation. Myc upregulates transcription of Carbamoyl phosphate synthase-Asparate transcarbamylase-Dihydroorotase (CAD) and ornithine decarboxylase (ODC) which regulate nucleotide biogenesis (Bello-Fernandez, Packham et al. 1993). ODC has been shown to be required for Myc-mediated lymphomagenesis (Nilsson and Cleveland 2003).

Myc and hypoxia

Upon hypoxia, HIFα transcription factors become stabilized, translocate to the nucleus where dimerization with the stable β-subunit ARNT results in hypoxia- regulated gene expression. While HIF1α expression during hypoxia is ubiquitous HIF2α expression is restricted to endothelial, lung, renal, and hepatic cells, although it has been observed in tumors originating from other tissues. HIF1α and HIF2α regulate overlapping set of genes such as vascular endothelial growth factor (VEGF) and adipose differentiation-related protein (ADRP). They also regulate unique gene targets; HIF-1α mainly regulating glycolytic enzymes such as Glut1 and Glut3. and HIF2α activating other set of genes, such as the stem cell factor oct4, cyclin D1 and TGFα .

Upon hypoxic condition cell proliferation comes to a halt through the induction of CDK inhibitors. HIF1α induces cell cycle arrest through an inhibition of cyclin E /CDK2 activity via increase in $p21^{cip1}$ and $p27^{Kip1}$ expression and hypophosphorylation of the pRb. As discussed before many of the cell cycle regulated genes are directly or indirectly under the control of Myc. Several studies show a complex relationship between Myc and HIF, which regulate a partially overlapping set of genes, such as LDHA and HK2.
While HIF1α seem to repress and antagonize Myc activity for instance by displacing Myc from the $p21^{cip1}$ and Sp1 promoter (Koshiji, To et al. 2005) (Zhang, Gao et al. 2007). HIF2α have been suggested to promote hypoxic cell proliferation correlating

with enhanced binding of Myc to promoter of both activated and repressed genes through enhancing Myc/Max interaction as well as recruiting it directly to Sp1 and Miz1 and stabilizing the complex. It was shown that Myc activity is elevated in renal clear cell carcinoma (RCC) expressing only HIF2α while RCC cells with intact VHL or HIF1α/HIF2α expressing RCCs enhanced Akt/mTOR and MAPK signaling pathways (Gordan, Lal et al. 2008). On the other hand, it has been reported that dysregulated Myc and HIF1α cooperate to induce key regulatory genes involved in hypoxic adaptation such as HK2, PDK1 and VEGF (Kim, Kim et al. 2007).

Another interesting aspect regarding the relationship between Myc and the HIF is the finding that the Myc antagonist Mxi1, is induced in a HIF-dependent way during hypoxia and inhibits mitochondrial biogenesis, reprograms cellular energy metabolism and protects cells from Myc-dependent apoptosis in vitro. There also seems to occur a HIF-dependent degradation of Myc via the proteasome, but the exact mechanism behind this is unknown.
Myc therefore appears to be an important regulator of the cellular response to hypoxia through its functional interactions with the hypoxia-inducible factors HIF1α and HIF2α. Since both Myc and HIF1 subunits are critical tumor maintenance factors, their downstream target gene products could potentially be targeted for therapeutic purposes.

AIMS

The overall aim of this study was to acquire increased insights into the function and regulation of the Myc oncoprotein, in particular the role of posttranslational modifications such as phosphorylation and ubiquitylation. Part of that task has been to identify and characterize new E3 ubiquitin ligases targeting Myc, and to explore the interplay between the Myc, cofactors such as cyclin-dependent kinases and such E3 ligases in the regulation of Myc function.

In particular the three specific aims of studies presented in this thesis are as follows;

1- To clarify the role of cyclin-dependent kinase 2 (CDK2) in phosphorylation of Myc and its cofactors, and the consequences of this for Myc-driven transcription and tumorigenesis.

2- To elucidate the role of the novel SCF^{FBXO28} E3 ubiquitin ligase complex in the regulation of Myc function .

3- To investigate the role of the interaction between the Myc and the von Hippel Lindau (VHL) tumor suppressor protein/E3 ubiquitin ligase for the biological functions of Myc and VHL.

RESULTS AND DISCUSSION

PAPER I.

Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation.

Given the role of proto-oncogenes such as MYC and RAS in enhancing normal cell growth as well as tumor development when deregulated, in this study we sought to investigate the mechanism underlying the long-known cooperativity between Myc and RAS in transformation. Ras has been shown to be a potent inducer of senescence while Myc is well-known trigger of apoptosis. Ras is known to suppress Myc-induced apoptosis through the PI3K/Akt pathway (Kauffmann-Zeh, Rodriguez-Viciana et al. 1997). In this study we showed that Myc repress Ras-induced senescence and that required phosphorylation of Myc at S62 by cyclin E/ Cdk2. Further we found that Cdk2 acts as a transcriptional cofactor by interacting with and phosphorylating Myc. S62 – phosphorylated Myc was found at the promoters of genes of importance for controlling senescence such as $p16^{INK4a}$, $p21^{cip}$,*CCND2, Bmi-1*, and *hTERT*, correlating with Cdk2 activity. As described above $p16^{INK4a}$ and $p21^{cip}$ are associated with activation of senescence, and *CCND2, Bmi-1*, and *hTERT* are linked to suppression of senescence. Inhibition of Cdk2 by selective pharmacological inhibitors or induced p27 upon IFN-γ treatment abolished S62 phosphorylation. This correlated with induced expression of $p16^{INK4a}$, $p21^{cip}$ and repressed expression of *CCND2, Bmi-1*, and *hTERT* with concomitant induction of senescence. In this work we uncovered a new non-redundant role of Cdk2 as a cofactor that is highlighting this kinase as a potential target for treatment of tumors driven by Myc or Ras.

Myc has also been shown to repress $BRAF^{V600E}$- or $NRAS^{Q61R}$-induced senescence in melanocytes (Zhuang, Mannava et al. 2008). Our study provide a mechanistic insights into how Myc represses senescence, where S62 phosphorylation is an important step and where cyclin E/Cdk2 plays a crucial role as a S62 kinase and transcriptional cofactor in this process. This notion is supported by the observation that Myc-mediated repression of senescence was abrogated by the selective pharmacological inhibition of Cdk2 and not of other cell cycle-regulatory Cdks or proposed S62 kinases. Importantly, a phospho-mimicking Myc-S62D mutant could still repress senescence under these conditions. As mentioned before number of kinases like ERK and Cdk1 mediate phosphorylation of S62. The role of S62 phosphorylation in priming GSK-3 driven phosphorylation of T58 and Myc-mediated apoptosis and stability was also discussed in "Myc post-translational modifications and degradation" section.

Here we showed that the anti-senescence function of Cdk2 could not be compensated by other kinases. This may raise the question how Cdk2 plays a unique role and what

the function of phospho-S62 is in this context. It is possible that Cdk2 phosphorylates additional substrates unique to Cdk2 that are associated with Myc-regulated transcription, for instance $p27^{Kip1}$, which may mediate this specific role suppression of senescence by Myc. Further studies are required to identify other substrates of Cdk2 phosphorylation that are essential in this context.
Using a T58A mutant that did not affect the senescence phenotype we showed that S62 plays a role independent of T58 in regulating senescence. In contrast, S62A mutant completely abrogated Myc ability to suppress Ras-induced senescence.

MAPK-mediated phosphorylation of S62 has been reported to increase Myc association with chromatin. In line with this we observed reduced association of Myc to promoters upon Cdk2 inhibition indicating that S62 phosphorylation stabilizes the association of Myc to promoters. Alternatively, S62 phosphorylation may direct recruitment of cofactors with a role in regulation of senescence-related Myc target genes.
Results from this work suggest that oncoproteins like Myc and Ras complement each other by repressing the two main cellular failsafe mechanisms namely senescence and apoptosis.
Myc activation in Cdk2 knockout MEFs induced senescence but not in wt MEFs suggesting that Cdk2 suppresses Myc-induced senescence. As mentioned before, the role of Cdk2 in cell cycle is redundant and compensated by other Cdks. The senescence phenotype observed in Cdk2 knockout MEFs upon Myc activation indicates that this is a unique non-redundant role of Cdk2 in regulating senescence. The results form paper I together with Campaner et al. study show that selective pharmacological inhibitors of Cdk2 but not other Cdks induce senescence in embryonic fibroblast with deregulated Myc or Myc/Ras. Hence Myc and Cdk2 may function as a senescence switch where active Cdk2 turns Myc to a repressor of senescence whereas in the absence of Cdk2 activity Myc provokes induction of senescence in a similar fashion as Ras. These findings suggest that Cdk2 inhibition could be potentially used as a new therapeutic principle for treatment of Myc/Ras- and Myc-driven tumors. However, whether the role of Cdk2 in suppression of Myc- and Ras-induced senescence is similar or distinct is not clear at the present.

PAPER II.

CDK-mediated activation of the SCF^{FBXO28} ubiquitin ligase promotes MYC-driven transcription and tumorigenesis and predicts poor survival in breast cancer

About 70 different genes encoding F-box proteins have been identified in the human genome, but only few have been characterized (Skaar and Pagano 2009). In this study we identified an uncharacterized F-box protein, FBXO28 as a cell cycle regulated protein with a critical function in tumor cell proliferation. We found that FBXO28 assembles an SCF^{FBXO28} ubiquitin ligase whose activity is cell cycle regulated by CDK1/2-mediated phosphorylation. Phosphorylation of FBXO28 at serine 344 enables the SCF^{FBXO28} ubiquitin ligase to target Myc for non-proteolytic ubiquitylation

transcription, proliferation and tumourigenesis. We further showed that upon ubiquitylation by SCF^{FBXO28} the cofactor p300 was recruited to Myc target gene promoters.
The reduced tumor cell proliferation rate upon FBXO28 knock down correlated with downregulation of genes that are typically activated by Myc. By knock down of FBXO28 or overexpression of an F-box mutant unable to support Myc ubiquitylation we further showed that Myc-driven transcription, Myc-induced transformation *in vitro* and Myc-driven tumour growth *in vivo* was attenuated. In human breast cancer we found a strong association between poor overall-, breast-cancer specific- and relapse-free-survival and high FBXO28 protein levels in two independent cohorts, and in multivariate analysis. Further, FBXO28 expression and phosphorylation were strong and independent predictors of poor survival. In conclusion, our data suggest that SCF^{FBXO28} plays an important role in transmitting CDK activity to Myc function during the cell cycle, emphasizing the CDK-FBXO28-Myc axis as a potential molecular drug target in Myc-driven cancers, including breast cancer.

Paper II provided the first clues as to the function of the F-box protein, FBXO28. As described in section "Myc post-translational modifications and degradation" several other E3 ligases have been shown to regulate Myc. It is not clear why Myc is under regulation of so many different E3 ligases, but it is reasonable to believe that a powerful oncoprotein like Myc needs to be tightly regulated and for this reason must be under control of several different E3 ligases. For instance, Myc activity and stability may have to be regulated in response to a wide variety of different stimuli and by different cellular contexts, and the different E3 ligases targeting MYC might have distinct or cooperative functions in this respect.

The observation that phosphorylation of FBXO28 on S344 is selectively mediated by cyclin A/CDK2 or cyclin B/CDK1, but not cyclin E/CDK2, is an interesting feature of SCF^{FBXO28} that links its function to activity of certain CDKs and cell cycle-dependent functions of Myc. Based on our observations we suggested a model where CDK1/2-mediated phosphorylation of FBXO28 triggers the activation of the SCF^{FBXO28} ligase and subsequent ubiquitylation of Myc at the S- and G2/M phases of the cell cycle. This promotes the recruitment of the cofactor p300 thereby stimulating transcriptional activation of a subset of Myc target genes critical for the continued progression through the cell cycle. FBXO28 and HectH9 seem to operate in a similar manner by targeting specific lysines in the C-terminal part of Myc. Although we did not study the exact nature of the ubiquitin chains conjugated to Myc by SCF^{FBXO28}, the recruitment of p300 to Myc target gene promoter upon ubiquitylation, the non-proteolytic mode of ubiquitylation as well as the promotion of Myc-driven transcription is similar to the effect of HectH9-mediated K63-linked ubiqutylation of Myc (Adhikary, Marinoni et al. 2005). This is also resembles β-TrCP mediated ubiquitylation of Myc, which antagonizes Fbw7-mediated degradation, thereby stabilizing Myc during after G1 phase of the cell cycle. As previously mentioned the SCF^{Skp2} and HectH9-mediated ubiquitylation of Myc has been reported to play a more prominent role in the G1 or the

G1/S phase transition. The cell cycle dependent regulation of Myc by different E3 ligases may to some extent explain the redundancy of E3 ligases in regulation of Myc.

Recent studies have suggested that Myc enhances global expression from E-box containing promoters that pre-exist in an active or poised chromatin state (Lin, Loven et al. 2012, Nie, Hu et al. 2012). However, the question remains whether Myc has specific effects on different genes? This could for instance be achieved through collaboration with other transcription factors or cofactors. Hence depending on the collaborating partner Myc could function to fine tune activation or repression of transcription in a gene specific manner. Here we introduce an additional cofactor FBXO28 that cooperate with Myc to regulate transcription of a subset of Myc target genes.

We showed that Cdk-mediated phosphorylation at S344 stimulated SCF^{FBXO28} activity toward Myc. This type of regulation has been reported previously where Cdk1-mediated phosphorylation regulates APC/C E3 ligase activity (See section The S/G2 and M phase). Similarly Cdk-mediated phosphorylation affects Skp2 stability, and allows its expression in mid-G1 phase by protecting it from degradation. Here we found that phosphorylation of FBXO28 at S344 reduced its turn over rate and enhanced the protein stability. Therefore Cdks not only control SCF^{FBXO28} activity, but also impact on FBXO28 protein levels and stability presumably through a mechanism involving auto-ubiquitylation and self-destruction of FBXO28 at low S-phase CDK activity. Exactly how phosphorylation at S344 affects SCF activity towards MYC remains to be determined, but S344 phosphorylation does not appear to be essential for the interaction between FBXO28 and MYC.

As we and others have shown that Cdk1 and Cdk2 also target Myc for phosphorylation and thereby regulate its transcriptional activity (paper I ; (Sjostrom, Finn et al. 2005), it is interesting to speculate that specific Cdks act to coordinate SCF^{FBXO28} and Myc function during the cell cycle. However, it is possible that SCF^{FBXO28} has other substrates as well as Myc- and/or Cdk-independent functions.
Another interesting finding in this study was that high expression and phosphorylation of FBXO28 positively correlated with several adverse clinicopathological characteristics and poor outcome in breast cancer.
Since FBXO28 phosphorylation and overall expression levels are coupled to Cdk activity, it is possible that the correlation between high FBXO28 levels and poor outcome partly reflects an increased proliferation in the tumours with high Cdk activity.

PAPER III.

Regulation of c-Myc expression and function by the von Hippel-Lindau E3 ubiquitin ligase/ Tumor Suppressor Protein

In this study we identified the tumor suppressor protein/ E3 ligase VHL as a Myc-interacting protein. VHL is part of a CBC E3 ligase complex that is known to bind HIF1α and HIF2α and target them for degradation. We show that VHL interacts with Myc through the C-terminal bHLHZip region and Myc box II, both highly conserved and essential regions required for Myc biological activities. In coimmunoprecipitation assays we show that Myc interacts with both VHL 30 and VHL 19 isoforms but prefentially interacts with the VHL19 isoform, indicating a nuclear function for the Myc–VHL interaction. The nuclear interaction of Myc and VHL is further confirmed in bimolecular fluorescence complementation assays (BiFC) assay, indicating a role for VHL in transcription or other nuclear functions.

VHL mutants that have been reported to have reduced or abolished binding to HIF1α and HIF2α, still interacted with Myc, suggesting a different mode of interaction other than of VHL and the HIFs. Further, the Myc-VHL interaction was not reduced by hypoxia suggesting that the interaction of Myc and VHL is not hydroxylation-dependent.

By using a panel of Myc deletion mutants starting from the N- and C-termini we located the binding region on Myc to VHL to the MBII and the bHLH-Zip domains. As mentioned before these are the regions through which Skp2 also interacts with Myc and play essential role in transcriptional regulation and transformation.

Similarly we utilized VHL deletion mutants to map the binding regions on VHL. In addition to our interaction assays VHL structural analysis suggested that two regions, amino acid 91-113 as well as 195-204, both located in the β-domain of VHL are important regions for VHL to interact with Myc.

Further we used a series of tumor-driven VHL point mutants belonging to different type of VHL disease to test whether they interact with Myc. VHL Y98N mutant and VHL C162F mutants that belong to type 2B and type 1 respectively did not show any reduced interaction with Myc as compared to WT VHL. VHL Y98N and VHLC162F were reported to loose their interactions with HIF and elongin C/CBC complex respectively. Hence, our data suggest that VHL binds HIF, Myc and CBC complex through different interaction sites.

Two other tumor-driven mutants S65A and S111A also did not remarkably reduce Myc interaction though S65A interaction was slightly stronger. Y98 and S111 residues have been reported among the five key HIF1α contacts on VHL in addition to W88, H115 and W117. Although Myc interaction did not significantly reduce by using Y98N and S111A we wanted to further address the question whether Myc and HIF could compete for binding to VHL. Increasing concentration of HIF1 ODD polypeptide did not abolish the interaction between VHL and WT Myc- or Myc 1-215 deletion mutant. However, in higher concentrations the total and Myc 1-215 deletion mutant protein level were reduced suggesting that free HIF might affect Myc protein level. It has been

proposed previuosly that HIF promotes degradation of Myc through the proteasome, although the mechanism was not clarified (Zhang, Gao et al. 2007).

VHL seems to participate in ubiquitylation of Myc in a non-proteolytic manner. Tumor derived VHLY98N and VHLC162F mutants dramatically decreased ubiquitylation suggesting the dominant negative nature of these mutants in repressing ubiquitylation of Myc. We examined a panel of ccRCC and MEFs cell lines with different VHL status and observed that the Myc protein level was higher in cell lines containing no or non-functional VHL. However, this was not due to increased turnover rate of Myc as no difference was observed in ^{35}S-Met pulse-chase and cycloheximide chase measurements of Myc decay in the ccRCC and MEF cell lines. The same conclusion was drawn after overexpression or RNAi-mediated depletion of VHL in a number of other cell lines.

Since several other Myc-targeting E3 ubiquitin ligases play a role in Myc-regulated transcription as described above (Paper II), we addressed whether VHL might have a similar function. Indeed, we found that overexpression of VHL enhanced Myc-driven transcription of in promoter/luciferase reporter assays.

By using ChIP assays we found that VHL associates with MYC at several, but not all Myc binding target genes. The association of VHL to these promoters seems to be Myc-dependent as indicated by loss of VHL association to the PGC1-B gene promoter region upon turning off Myc expression in P493-6 tetracycline Myc regulatble cell line. Our ChIP assays using ccRCC WT7 ($Vhl^{+/+}$) and PRC3 ($Vhl^{-/-}$) cell line suggest an interdependent relation between Myc and VHL for target gene interaction. Myc association to PGK1, CDS1, miR17 and DUSP11 gene promoter regions were dramatically attenuated in the PRC3 cell line.

Further, the association of Myc to the PGK1 HIF target gene was reduced during hypoxia despite increasing VHL association with this gene promoter. Since HIF1 is liberated from VHL interaction and degradation during hypoxia leading to increased association of HIF with the gene, it is possible that free HIF1 counteract Myc from chromatin binding. It has been reported that HIFα counteracts Myc from Sp1 at the gene promoter (Koshiji, To et al. 2005). Another possibility is Myc binding is reduced because of increased turnover during hypoxia. We have no clear indications that this caused by VHL, but could involve HIFs, as discussed above.

An interesting observation is that the expression of the genes that are bound by Myc/VHL as shown by ChIP assays was regulated in response to Myc activation and knock down of VHL. By using U2OS-MycER system and shRNA mediated knock down of VHL we examined the expression of cyclin D2, DUSP11, CDS1 and PGK1 genes that were bound by Myc/VHL.

Upon activation of MycER by addition of 4-OHT, expression of cyclin D2 increased, as previously reported (Bouchard, Marquardt et al. 2004). Interstingly, shRNA knock down of VHL led to a complete reversion of the induction and instead led to decreased expression of cyclin D2. Without Myc activation VHL knockdown had no effect on this gene. In contrast, the expression of CDS1 that was strongly repressed upon Myc activation. Also here knock down of VHL led to a complete reversal of the Myc effect, and the expression of CDS1 was markedly increased. when combined

activation of Myc and knock down of VHL was applied. In the absence of Myc activation VHL knockdown rather decreased the expression of this gene. Similar but not as pronounced effects were observed for two other genes bound by Myc and VHL, DUSP11 and PGK1. DUSP11 was strongly repressed by Myc and a slight derepression was observed upon knock down of VHL. Also here VHL knockdown alone reduced the expression of the gene. PGK1 gene expression was somewhat reduced by Myc activation, but was reversed by VHL depletion leading to a strong increased expression. The observation here is reminiscence of gene specific regulation of Myc through interaction with cofactors, in this case VHL. Myc and VHL may together regulate a subset of genes leading to either activation or repression of gene expression. Global analysis of Myc/VHL chromatin association such as ChIP-Seq combined with global expression analysis is required to get a comprehensive overview of this subset of genes and what pathways/biological functions are coregulated by Myc and VHL in different contexts.

VHL also associated with exonic and intronic sequences as well as upstream promoter region of *myc* gene. Many factors have been reported to play a role in regulating the *myc* gene. The association of Myc and VHL to *myc* gene locus suggested that VHL might contribute in regulating *myc* gene expression. While this manuscript was under preparation Hwang et al reported that association of VHL to Myc gene promoter region through interacting with Myc was associated with recruitment of HDAC1/2 that led to repression of Myc gene (Hwang I, Roe J, 2012). The authors did not provide the mechanism behind this but suggested that might be part of the well-known Myc auto-repression discussed in section “The Myc promoter and auto-repression” section.
. Our ChIP assays show that Myc and VHL co-localize to not only promoter region but also upstream and exonic/intronic regions as well as 3’UTR region of Myc gene. These suggest that different level of regulation may exist possibly affecting both transcription initiation and elongation. The Myc/VHL association to 3’UTR region of the Myc gene suggest that there might be an additional post-transcriptional level of regulation through interacting with 3’UTR regulatory sequences. In line with this in luciferase reporter assay we observed that c-Myc but not N-Myc 3’UTR luciferase reporter activity was enhanced in VHL deficient cell line indicating that VHL may also regulates Myc mRNA expression post-transcriptionally.
Analysis of Myc gene expression in the “TCGA RCC project “ revealed that the relative expression of Myc was elevated in ccRCC whereas the chromophobe RCC subtype display downregulated Myc expression. The expression of Myc in the papillary RCC subtype was slightly increased. The relation between VHL status or hot spot VHL mutations with respect to Myc expression remains to be further studied.

VHL has previously been reported to be involved in HIF-independent regulation of RNA pol II activity through non-proteolytic ubiquitylation of Rpb1 subunit associated with Ser5 phosphorylation upon oxidative stress. These studies provide evidence of a HIF-independent role of VHL in gene transcription/expression. Interestingly upon different stress stimuli the consequence of ubiquitylation by VHL with respect to proteolytic or non-proteolyitc ubiquitylation varies, as has been observed for other E3

ligases. Our expression analysis of Myc/VHL-bound genes indicate that some genes are upregulated whereas others are repressed upon Myc/VHL gene promoter interaction. Together with the reported role of Myc in promoter clearance (Rahl, Lin et al. 2010) it is tempting to speculate that under stress condition such as DNA damage or oxidative stress VHL may interact with Myc or as part of a complex together with Myc regulate RNA pol II transcription. Our results show that Myc and VHL continue to interact under UV- and hypoxia-induced stress and the interaction seems to be enhanced under hypoxic conditions. Further studies are required to investigate the observed co-localization of Myc/VHL at chromatin plays any role in regulating RNA pol II transcription under stress conditions.

The proposed opposing and synergistic functional relation of Myc with HIF1α and HIF2α, respectively, that was discussed before in section "Myc and hypoxia" requires careful investigation in the context of Myc/VHL interaction. VHL's role in hypoxia and different reported roles of VHL in normoxia needs an elaborate assessment of its functional and biological properties with respect to Myc function. A Myc-dependent stabilization of HIF1α during normoxia that enhanced Myc-induced anchorage-independent growth and Myc-induced proliferation was reported by Doe et al. The authors showed that overexpressed Myc in non-transformed epithelial and fibroblasts induced accumulation of HIF1α in normoxia resulting in upregulation of HIF1α target genes. This was associated with reduced HIF1α/VHL interaction upon overexpression of Myc. Although we have not been able to demonstrate any clear cut competition between Myc and HIF for VHL binding, we cannot exclude that this may occur under certain conditions, and this should be explored further.

Taken together these data suggests a mechanism where VHL interacts with and ubiquitylates Myc at certain target promoters in order to regulate Myc's transcriptional activity, including regulation of the MYC gene itself. This regulation may also engage the 3'UTR region of MYC mRNA thus regulating MYC expression at multiple levels.

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