View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Publications from Karolinska Institutet

From DEPARTMENT OF MICROBIOLOGY, TUMOR AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

COOPERATIVITY BETWEEN MYC AND OTHER ONCOGENIC FACTORS - IMPLICATIONS FOR TUMORIGENESIS AND TARGETING OF MYC

Siti Mariam Zakaria

Karolinska Institutet

Stockholm 2019

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Eprint AB 2019
Cover illustration: Blue with red dots are figures of MYC:MAX interactions (red) in the nuclei (blue) of cells in different cell cycle stages using *in situ* Proximity Ligation Assay (PLA) concocted in-house. The single red figure is the immunofluorescence B-actin staining of a senescent cell using Phalloidin. Figures are taken during the course of the projects in this thesis.
© Siti Mariam Zakaria, 2019
ISBN 978-91-7831-361-7

Cooperativity between MYC and other oncogenic factors - implications for tumorigenesis and targeting of MYC

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended at Karolinska Institutet

Petrén Lecture hall, Nobels väg 12B, Solna

Friday 29th March 2019 at 9:30

By

Siti Mariam Zakaria

Principal Supervisor:
Professor Lars-Gunnar Larsson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Co-supervisors:
Assistant Professor Alina Castell
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Professor Galina Selivanova
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Opponent:
Professor Anthony Wright
Karolinska Institutet
Department of Laboratory Medicine (LABMED)

Examination Board:
Professor Nico Dantuma
Karolinska Institutet
Department of Cell and Molecular Biology (CMB)

Professor Aristidis Moustakas
Uppsala University
Department of Medical Biochemistry and Microbiology (IMBIM)

Associate Professor Kaisa Lehti
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

To my family

ABSTRACT

Normal cell homeostasis in a tissue requires a delicate balance between cell growth, maintenance and death, tightly controlled by an intricate interplay between proto-oncogenes and tumor suppressor genes. When the balance is tipped due to genetic or epigenetic lesions in such genes, diseases such as cancer may arise.

MYC transcription factors has been known to regulate up to 15% of mammalian genes involved in diverse intracellular programs, including cell cycle regulation, cell growth, differentiation, apoptosis, and senescence, and is deregulated in many human cancers. MYC recruits different co-factors for activation or repression of transcription, such as MAX or Miz-1, respectively. MYC is tightly regulated at multiple levels, including transcription, post-translational modification and turnover. Ubiquitylation is one such control, and although ubiquitylation is mainly associated with proteasomal degradation, it has also been shown to be involved in non-proteolytic functions such as DNA replication and repair.

Tumorigenesis is a multistep process that involves activating or inactivating mutations or epigenetic changes in more than one gene to confer growth advantages to the cell. MYC is known to cooperate with another oncoprotein, RAS, to transform rodent cells. While RAS has been found to suppress MYC-induced apoptosis, MYC also inhibits RAS-induced senescence, thereby blocking two main anti-tumorigenic mechanisms in the cell and may, at least in part, explain the basis for the MYC/RAS cooperativity.

Inactivation of MYC in mouse tumor models demonstrated tumor regression with well-tolerated side effects, suggesting that MYC is a potential and suitable target for anti-cancer therapy. However, pharmacological targeting of transcription factors is considered difficult and no anti-MYC drugs are clinically available today.

In this thesis, we deepen our understanding on MYC biology by studying different proteins that cooperate and interact with MYC (Paper I to III), and identify small molecules that would target specific interactions involving MYC (Paper IV).

In Paper I, we found that oncogenic MYC and RAS do not cooperate to cancel out each other's intrinsic anti-tumorigenic barrier, namely apoptosis and senescence, in normal human fibroblasts as they do in murine fibroblasts, even in the absence of the tumor suppressor p53. This is in contrast to previous results from human melanocytes, where MYC was reported to suppress BRAF- and partially NRAS-induced senescence, thus suggesting that these anti-tumor barriers are orchestrated differently in different species and in different cell types.

In Papers II and III, we discovered new regulatory mechanisms for MYC. In Paper II, we found that the cyclin-dependent kinase (CDK) inhibitor $p27^{KIP1}$ (p27) binds MYC and targets it for degradation. p27 is upregulated by interferon-ɣ (IFN-ɣ) and by other growth inhibitory signals. We also found that IFN-ɣ treatment leads to the degradation of MYC, mediated by upregulation of p27. There is significant clinical relevance between high activity of nuclear

p27 levels and low MYC expression in tumor samples, and this correlates with a good prognosis and a positive clinical outcome. This may provide insights into strategies to target MYC-driven tumors, for example by finding ways to upregulate p27 expression and activity, utilizing IFN-ɣ in treatment modalities, stimulating immune cells to produce IFN-ɣ by immunotherapy and finding methods to combine these strategies to combat MYC-driven tumors.

In Paper III, we uncovered a novel F-box protein, FBXO28, that ubiquitylates MYC in a non-proteolytic manner, and enhances MYC transcriptional activity and downstream pathways. Phosphorylation of SCF^{FBXO28} by CDK1/2 during the cell cycle is required for its efficient ubiquitylation of MYC. Depletion of FBXO28 or expression of its dominant negative F-box mutant, negates this function and results in reduction of MYC-driven transcription, transformation and tumorigenesis. High MYC expression coupled with high FBXO28 expression and phosphorylation are strong and independent predictors of poor prognosis in human breast cancer. Our data suggests that the CDK-FBXO28-MYC axis is a potential molecular drug target in MYC-driven cancers, including breast cancer.

In Paper IV, we conducted a small molecule screen and found, MYCMI-6, that binds MYC, inhibits MYC/MYCN:MAX interactions, and impeded tumor cell growth in a MYC-dependent manner in a variety of tumor cell cultures and in a mouse tumor model of MYCN-amplified neuroblastoma. Importantly, this compound is highly specific and potent, has a good therapeutic window and does not have severe side effects. This discovery provides proof of principle of protein-protein targeting. MYCMI-6 can be used as a molecular tool to study MYC:MAX interactions and is a good candidate for drug development.

Altogether, the projects involved in this thesis provide insights into molecular pathways involved in MYC oncogenic activity, regulation, and transcription functions, shed light in MYC-RAS cooperativity, identified new proteins interacting with MYC and small molecules interfering with MYC function. This is of importance not only to increase the basic knowledge on mechanisms through which MYC contributes to tumor development, but will hopefully also contribute to the development of new therapeutic strategies to combat MYC-driven cancer in the future.

LIST OF SCIENTIFIC PAPERS

I. Zhang F*, **Zakaria SM***, Tabor V, Singh M, Tronnersjö S, Goodwin J, Selivanova G, Bartek J, Castell A, Larsson LG. (2018). MYC and RAS are unable to cooperate in overcoming cellular senescence and apoptosis in normal human fibroblasts, Cell cycle; 17, 2697-2715
*These authors contributed equally to this work

II. Bahram F*, Hydbring P*, Tronnersjö S*, **Zakaria SM**, Frings O, Fahlén S, Nilsson H, Goodwin J, von der Lehr N, Su Y, Lüscher B, Castell A, Larsson LG. (2016). Interferon-γ-induced $p27^{KIP1}$ binds to and targets MYC for proteasome-mediated degradation, Oncotarget; 7(3):2837-54
*These authors contributed equally to this work

III. Cepeda D, Ng H-F*, 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. (2013). CDK-mediated activation of the SCF^{FBXO28} ubiquitin ligase promotes MYC-driven transcription and tumorigenesis and predicts poor survival in breast cancer, EMBO molecular medicine;5(7):999-1018
*These authors contributed equally to this work
[‡]These authors contributed equally to this work.

IV. Castell A*, Yan Q*, Fawkner K*, Hydbring P, Zhang F, Verschut V, Franco M, **Zakaria SM**, Bazzar W, Goodwin J, Zinzalla G, Larsson LG. (2018). A selective high affinity MYC-binding compound inhibits MYC:MAX interaction and MYC-dependent tumor cell proliferation, Scientific reports 8, 10064
*These authors contributed equally to this work

CONTENTS

1 Introduction 9
1.1 Cancer 9
1.2 Oncogenes and Tumor Suppressor Genes 10
1.3 Cell Cycle 12
1.4 Senescence and Apoptosis 14
1.5 Cellular Signaling 15
1.5.1 The RAS, MAPK and PI3K signaling pathways 15
1.5.2 The Interferon-ɣ and JAK/STAT Pathway 16
1.6 Gene Expression and Regulation 17
1.6.1 Chromatin Structure and DNA Accessibility 17
1.6.2 Transcription and Transcription Factors 18
1.7 The Ubiquitin Proteasome System 19
1.8 MYC 21
1.8.1 Master Regulator 21
1.8.2 MYC Structure, Expression and Regulation 22
1.8.3 MYC and Cell Cycle 25
1.8.4 MYC and Apoptosis 26
1.8.5 MYC and Senescence 26
1.8.6 MYC in Cancer 27
1.8.7 Targeting MYC in Cancer Therapy 29
2 Aims of This Thesis 32
3 Results and Discussions 33
3.1 Paper I 33
3.2 Paper II 35
3.3 Paper III 37
3.4 Paper IV 40
4 Summary and Conclusions 43
5 Acknowledgements 45
6 References 51

LIST OF ABBREVIATIONS

Akt/PKB	Protein kinase B
ATP	Adenosine triphosphate
APC	Antigen presenting cell
BAD	Bcl-2 associated death promoter protein
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2 associated X protein
BCL-2	B cell lymphoma 2
BCL-X_L	B cell lymphoma extra large
BID	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
Caspases	Cysteine-aspartic proteases
CDC	Cell division cycle
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation assays
CKI	CDK inhibitor
Cul1	Cullin1
DSIF	DRB sensitivity-inducing factor
eIF4	Eukaryotic initiation factor-4
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
FBXW7	F-box/WD repeat-containing protein 7
FOXO	Forkhead box protein O
FRA1	Fos-related antigen 1
GDP	Guanosine diphosphate
GTFs	General transcription factors
GTP	Guanosine triphosphate
GTPase	Enzyme that cleaves GTP, usually generating GDP
Grb2	Growth factor receptor-bound protein 2
GSK-3β	Glycogen synthase kinase 3 beta
H	Histone
HATs	Histone acetyl transferases
HDACs	Histone de-acetyl transferases
HECT	Homologous to E6-AP Carboxyl Terminus
Her2	human epidermal growth factor receptor 2
HLH–LZ	Helix–loop–helix–leucine zipper
HMT	Histone methyl transferases
HNRPK	Heterogeneous Nuclear Ribonucleoprotein K
IFN	Interferon
IGFR-1	Insulin like growth factor receptor
IL	Interleukin
isPLA	in situ Proximity Ligation Assay
JAK	Janus kinases
K	Lysine residue
lncRNA	Long non-coding RNA
MAPK	Mitogen-ativated protein kinase
MAX	Myc associated factor X
MB	Myc homology boxes
Mdm2	Mouse double minute 2 homolog

Miz-1	MYC-Interacting Zinc Finger Protein 1
MEK	Mitogen-ativated protein kinase kinase
MST	Microscale thermophosis
mTOR	Mammalian target of rapamycin
MYCMI	MYC:MAX inhibitor
NELF	Negative elongation factor
NF	Nuclear fraction
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLS	Nuclear localization signal
ODC1	Ornithine decarboxylase
OIS	Oncogen induced senescence
PIC	Pre-initiation complex
PIP2	2 phosphatidylinositol 4,5-biphosphate
PI3K	Phosphatidyl Inositol-3 Kinase
Pol I, II, III	RNA polymerase I, II and III
pRB	Retinoblastoma protein
PRC2	Policomb repressive complex 2
PTEN	Phosphatase and tensin homolog
P-TEFb	Positive transcriptional elongation factor b
RAF	Rapidly accelerated fibrosarcoma kinase
Rbx	RING-box protein 1
RING	Really Interesting New Gene
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
SASP	Senescence-associated secretory phenotype
Skp	S-Phase Kinase Associated Protein
SNP	Single-nucleotide polymorphisms
SPR	Surface plasmon resonance
STAT	Signal transducer and activator of transcription proteins
SUMO	Small Ubiquitin-like Modifier
TAD	Ttranscriptional activation domain
TAFs	TBP-associated factors
TBP	TATA-binding protein
TGF-β	Transforming growth factor beta
Th1	T helper type 1 cell
TMA	Tissue microarray
TNF-α	Tumor necrosis factor-alpha
TSS	Transcription start site
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WNT	Wingless/Integrated
WRN	Werner syndrome ATP dependent helicase
YFP	Yellow fluorescent protein

1 INTRODUCTION

1.1 CANCER

In 2018, cancer cases around the world have risen to 18.1 million, leading to 9.6 million deaths (Organization, 2018). Cancer is the term used in the scientific literature to refer to a progressive and malignant tumor, which is an agglomeration of uncontrollably dividing cells, that can grow and spread (metastasize) beyond their boundaries to other organs (Greaves and Maley, 2012; Hanahan and Weinberg, 2011). It is not a single disease but rather a systemic disease. There are currently 10 hallmarks and common underlying characteristics identified that are shared in different combinations across the different cancers, making this complex disease more understandable through science (Figure 1) (Hanahan and Weinberg, 2011). Of these, sustained proliferative signaling, resistance to cell death, enabling replicative immortality, evasion of growth suppressors and genomic instability and mutation will be expounded upon later in the introduction and will be connected to the work in this thesis.

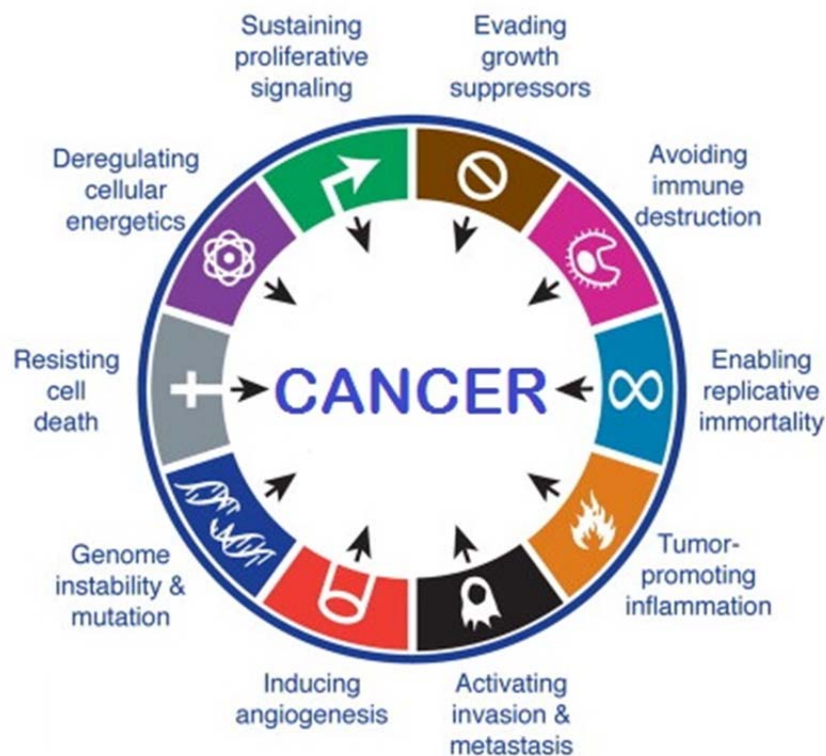

Figure 1: The eight hallmarks and two enabling characteristics (Genome instability and mutation, and tumor-promoting inflammation) of cancer (adapted from Hanahan and Weinberg (2011)) in compliance with the conditions of Elsevier user licence. Copyright © 2011 Elsevier, Inc.

Cancer can be caused by genetic or environmental factors, the former leading to cancers at a young age (Apostolou and Fostira, 2013; Sorrell et al., 2013), while the latter leads to cancer later in life and is estimated to be the cause of 90-95% of all cancers (Anand et al., 2008). Environmental factors can be chemical, physical or biological in nature. Numerous types of cancers have been caused by both natural and synthetic compounds (Poon et al., 2014; Wogan et al., 2004), X-ray and UV light (Borek, 1993), as well as viral infections, for example with human papillomavirus (HPV) (White et al., 2014).

Conventional therapeutic strategies against cancer include chemotherapy, radiotherapy and surgery. While these therapies may benefit some patients, they also have disadvantages. Chemotherapy is not specific, and can target both tumor and healthy cells, leading to side effects such as hair loss and anemia (Caley and Jones, 2012). Further, chemotherapeutic drugs can introduce mutations to healthy as well as tumor cells. This is more serious and can lead to the development of new, resistant or more aggressive tumors (Caley and Jones, 2012; Vogelstein and Kinzler, 1993). Radiotherapy, while mostly targeted to a specific site, may also similarly lead to secondary tumors (Drooger et al., 2015). Surgery may avoid these unwanted effects but is limited to tumors that are located at non-vital organs, such as breast and prostate, and may have unacceptable cosmetic outcomes (Bertozzi et al., 2017).

Novel approaches, such as immunotherapy and targeted therapies, have been developed more recently. In immunotherapy, antibodies, cytokines, transfer of cancer-specific immune cells and cancer vaccines, among other strategies, are used to activate the immune system to kill tumor cells (Saied et al., 2014; Srivastava and McDermott, 2014; Vanneman and Dranoff, 2012). Targeted therapy, on the other hand, uses small molecules to directly hit a specific protein or group of proteins that contribute to the tumor formation, thereby causing the tumor cells to die or stop growing (Widmer et al., 2014). These novel approaches show promise of being more personalized. Different therapies can also be combined to achieve a more effective treatment (Saied et al., 2014; Vanneman and Dranoff, 2012). However, the development of drug resistance, and hence relapse, is still a concern, despite discoveries of new therapies (Fong and Park, 2009; Holohan et al., 2013). Thus it is important to identify the drivers of tumor growth and optimize treatments.

1.2 ONCOGENES AND TUMOR SUPPRESSOR GENES

Tumorigenesis, i.e. the development of tumors, is a multistep process, involving at least two mutations in rodent cells and four to seven mutations in human cells (Hahn et al., 1999; Hanahan and Weinberg, 2000; Land et al., 1983; Renan, 1993). Mutations occur randomly but when hitting so called “driver genes”, which confer growth advantages to the target cell, they are selected for by a process similar to that of Darwinian evolution (Foulds, 1954; Nowell, 1976).

There are two main classes of genes that, when mutated or deregulated, would give a growth advantage that contribute to the development of tumors: proto-oncogenes and tumor suppressor genes. Both classes of genes exist as part of the normal cell genome and usually play important roles in normal cell physiology (Vogelstein and Kinzler, 2004).

Gain-of-function mutations in proto-oncogenes, resulting in their over-activation, or overexpression result in the formation of tumor-promoting so called oncogenes (Vogelstein and Kinzler, 2004). Examples of proto-oncogenes are *MYC* and *RAS*.

MYC family of proto-oncogenes, *c-MYC, MYCN* and *MYCL*, code for transcription factors that control the expression of many genes involved in distinct processes relevant for tumorigenesis, including cell growth, apoptosis, metabolism, immortalization, differentiation and stem cell function (Meyer and Penn, 2008). Deregulation of MYC expression (Pelengaris et al., 2002b) or perturbations in MYC's degradation (Bahram et al., 2000; Isobe et al., 2009; Yeh et al., 2004) have been linked to the development of many types of human tumors. As the projects in this thesis are all related to MYC, a chapter will be dedicated to discuss more on this proto-oncogene.

RAS family of proto-oncogenes, *HRAS, KRAS* and *NRAS*, encode for membrane-bound GTPases that transduces extracellular signals from growth factor receptors to activate downsteam effectors (Serrano et al., 1997). It is one of the central regulators of growth factor-induced cell proliferation and survival in normal and cancer cells. Human cancers often contain amplifications of or activating point mutations in *RAS* (Pratilas and Solit, 2010). RAS will be discussed further in later chapters.

Tumor development also requires inactivating or loss-of-function mutations of tumor suppressor genes, such as genes encoding for p53 and retinoblastoma protein (pRB), which are the main brakes in the cell cycle, and control cell survival and genome integrity (Vogelstein and Kinzler, 2004).

The p53 protein is a transcription factor that functions in cell growth inhibitory pathways and is able to promote cell death, senescence or cell cycle arrest under conditions which exert cellular stress. Point mutations disrupting its DNA-binding capacity or increase in factors that binds and inactivate its function, like MDM2, are among the common ways to disrupt the p53 pathway (Oren, 2003; Prives and Hall, 1999; Vogelstein et al., 2000)

pRB is also a transcription factor and it is one of the proteins that directly control the transition from G1 to S phase of the cell cycle, and its activation causes cells to undergo arrest in the G1 phase. The *RB* gene can be inactivated by mutation and the pRB protein is an important target of DNA tumor viruses (Classon and Harlow, 2002).

A delicate balance between cell growth, maintenance and death, tightly controlled by an intricate interplay between proto-oncogenes and tumor suppressor genes, governs normal cell homeostasis. Genetic or epigenetic lesions in these genes tip the balance, hence leading to the development of tumors.

1.3 CELL CYCLE

The cell cycle is a precise and regulated process through which cells duplicate and segregate their genome and organelles to produce daughter cells and proliferate. The eukaryotic cell cycle is divided into four discrete phases: gap1 (G1), DNA synthesis (S), gap2 (G2) and mitosis (M). Cells that are resting and non-proliferating exit the cell cycle and enter a quiescence state (G0). Cell cycle durations differ between different organisms and even between different cell types in an organism. In human, cell cycle of the somatic cells takes about 24 hours to complete. The G1, S and G2 phases together make up the cell cycle interphase and typically take between 18-22 hours, with the G1 phase being the most variable and often the longest. The M phase takes only one hour and is the shortest phase.

G1 is the phase where the cells monitor if the environmental factors are favorable for replication before it decides to proceed with the cell cycle or go into G0. In the presence of mitogenic signals and favorable conditions, it will pass a checkpoint, called the restriction point (R point), and proceed into the S phase where the DNA is replicated. After replication of the genome is completed, the cells go into G2 phase, which is a phase where the cell prepares for M phase. G2 contains an important checkpoint to ensure that the DNA is correctly duplicated and structurally intact before entering into the M phase. This last phase, mitosis, is where the cell divides into two daughter cells, and the process is divided into four sub-phases: prophase (condensation of the DNA), metaphase (alignment of duplicated chromosomes), anaphase (separation of chromosomes) and telophase (decondensation of the DNA). There is another checkpoint mid-mitosis to ensure that chromosomes are properly attached to the mitotic spindle before separating. Extracellular signals are able to affect the process only in the early G1 phase. After the cell cycle passes the R point, it will proceed autonomously according to schedule until the end point, mitosis (reviewed in Nurse (2011)).

Cell cycle progression is regulated by cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKI). Cyclins bind and activate CDKs, which are serine/threonine kinases that phosphorylate substrates of relevance for cell cycle progression. There are four families of cyclins, namely Cyclin A, B, D and E, and four CDKs, namely CDK1, 2, 4 and 6, that have direct participation in the mammalian cell cycle, although there are more members of the family. In general, the levels of S and M phase CDKs are rather constant but their activities depend on the availability and levels of cyclins, which fluctuates in a tightly regulated manner, in synchronization with the progression through the different phases of the cell cycle. The cell cycle is kept in check by CKIs, which negatively regulate the process by inhibiting the CDKs. Inhibitors of CDK4/6 (INK4) comprise of $p16^{INK4A}$ (p16), $p15^{INK4B}$ (p15), $p18^{INK4C}$ (p18) and $p19^{INK4D}$ (p19), while the CIP/KIP family $p21^{CIP1}$ (p21), $p27^{KIP1}$ (p27) and $p57^{KIP2}$ (p57) inhibit CDK2 and CDK1 complexes (Weinberg, 2014). These CKIs are also involved in other cellular processes besides cell cycle progression and will be brought up later in this thesis.

In the G1 phase of the cell cycle, growth factor signaling upregulates the levels of cyclin D1, D2 and D3. Cyclin D associates with and activates CDK4 or CDK6, which phosphorylates pRB. pRB is a cell cycle brake that controls the R point by interacting with and sequestering the transcription factor E2F. At the G1/S border, increased expression of cyclin E, which binds and activates CDK2, occurs. Upon hyper-phosphorylation of pRB, which involves cyclin D/CDK4/6 and cyclin E/CDK2, E2F is released and will upregulate the expression of several proteins required for DNA replication and metabolism (Trimarchi and Lees, 2002). On top of this, Cyclin D/CDK4/6 sequesters p27, which inhibits CDK2, and in doing so, further increases cyclin E/CDK2 activity and promotes the G1 progression (Polyak et al., 1994; Reynisdottir et al., 1995). Cyclin E/CDK2, drives the G1 to S phase transition by targeting pRB and inducing degradation of p27 via the E3 ligase SKP2 (Elledge and Harper, 1998; Sheaff et al., 1997; Vlach et al., 1997). Cyclin A, which is one of the proteins induced by E2F, starts to pair with CDK2 in the early S phase, where cyclin E is degraded via a CDK2-dependant pathway (Welcker et al., 2004). Cyclin A/CDK2 participates in the initiation of DNA replication. During the late S phase and into the G2 phase, cyclin A binds CDK1, but as the cell cycle proceeds from the G2 to M phase, CDK1 will form complexes with cyclin B, and this complex trigger M-phase entry. After mitosis, the level of cyclin B/CDK1 drops and the cell waits for a signal to start another cell cycle (Arellano and Moreno, 1997) (reviewed in Hydbring et al. (2016)).

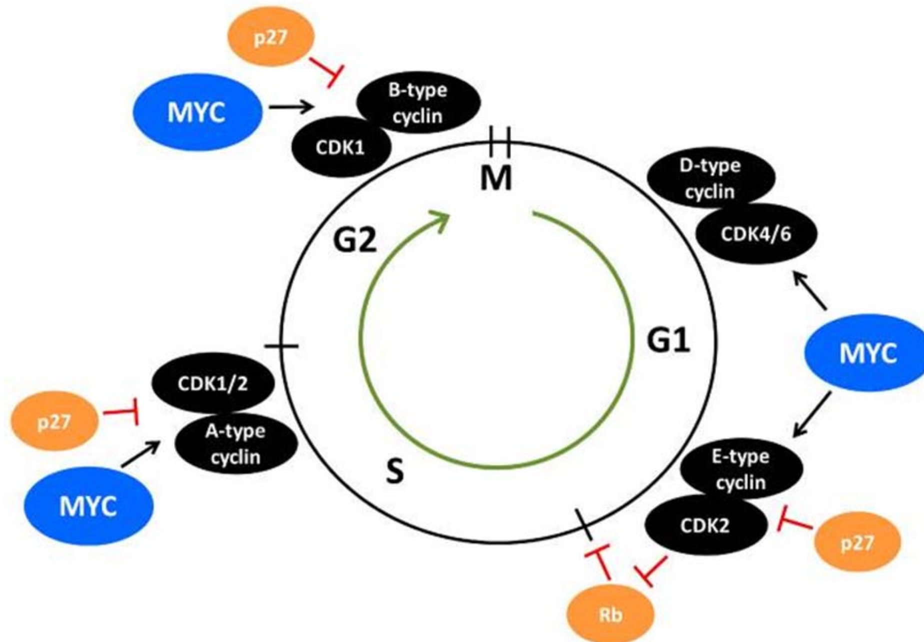

Figure 2: The mammalian cell cycle. The illustration depicts the cell cycle phases, the cyclin/cyclin-dependent kinase (CDK) complexes (black) and growth/tumor-inhibitors (orange) involved, and their periods of activity during the cell cycle. MYC's (blue) points of intervention in the cell cycle are also depicted (picture from Hydbring et al. (2017), open access).

1.4 SENESCENCE AND APOPTOSIS

Cancers do not arise from a single gene defect. In a normal mammalian cell, the activation of a single oncogene triggers intrinsic safeguard mechanisms via tumor suppressor genes, which limit its tumorigenic potential and protect the cell. Thus, each mutated or deregulated cancer-critical genes would only be a contributing factor, and it is only when several genes are defective does an invasive cancer develop (Vogelstein and Kinzler, 2004).

Apoptosis is a "programmed" cell death that dictates the controlled destruction of cellular constituents and their ultimate engulfment by other cells. Generally, there are several pathways through which apoptosis occur, all leading to the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that ends with the final demise of the cell. Activated initiator caspases 2, 8, 9 and 10 starts the caspase cascade which acts on downstream effector caspases 3, 6, 7 and 14. In the extrinsic pathway, cell surface receptors such as FAS induce the apoptosis signal, leading to the activation of caspases 8, after which caspase 3 and 7 get activated, leading to apoptosis. The intrinsic pathway is induced by cellular stress or via crosstalk with the extrinsic pathway and is controlled by the pro-apoptotic factors of the BCL-2 family such as BAX, BAK and BID, which target mitochondrial membrane and induces cytosolic cytochrome c release. The anti-apoptotic family members of the BCL-2, namely BCL-2 and BCL-X_L, balances the apoptotic pathway. The apoptosis program is normally a homeostatic mechanism to maintain cell populations, and occurs during development and aging. It may also be employed as a defense mechanism when cells are damaged or in immune reactions (reviewed in Elmore (2007)).

Senescence is a state of irreversible growth arrest and normally occurs in normal cells as a result of telomere erosion through the aging process. The difference between a senescent and a quiescent cell is that the former remains metabolically active in the senescent state while the latter lies dormant in the G0 phase of the cell cycle. Some senescence cells have been shown to be able to secrete factors, including cytokines and chemokines, which can induce responses such as inflammation. The secretome is profoundly different from that of a normal cell and is called the senescence-associated secretory phenotype (SASP). Senescence can be induced prematurely by acute stress signals such as deregulation of oncogenes, which results in replicative stress and generation of reactive oxygen species (ROS), and is termed Oncogene-Induced Senescence (OIS). This often triggers a DNA damage response and is associated with increased levels of the tumor suppressor p53, which activates downstream effectors, such as the expression of the CKI p21. The p16/pRB pathway has also been shown to be important in senescence induction in several cell types (reviewed in Kuilman et al. (2010)). As outlined above, p16 in an inhibitor of CDK4/6, and thus prevents the cyclin D/CDK4/6 complex from hyperphosphorylating pRB. Thus pRB remains bound to the E2F transcription factor and inhibits the transcription of the other factors involved in the cell cycle progression, hence bringing the cell cycle to a halt (Dublin et al., 1998; Li et al., 2011; Muirhead et al., 2006; Parry et al., 1995).

Both apoptosis and premature senescence can be employed as extreme responses to cellular stress and are important tumor-suppressive mechanisms, by quickly eliminating, or preventing the growth of damaged or stressed cells, respectively.

1.5 CELLULAR SIGNALING

Cells receive signals from their surrounding environment that dictate the cell fate; if they should proliferate, differentiate, die, or go through some other process. These signals can come in the form of growth factors, growth inhibitory factors, cytokines and hormones, amongst others. Ligands from extracellular space bind and activate their cognate cell surface receptors. The receptors in turn transduce the information through the plasma membrane into the cytoplasmic intracellular environment to induce signaling cascades that eventually reach the cell nucleus to regulate gene expression (Shaw and Cantley, 2006). There are multiple signaling pathways and among the major ones are receptor tyrosine kinases (RTK), Wnt, Hedgehog (Hh), Notch, nuclear receptors, mTOR, TGF-β, NFkB and JAK/STAT pathways. Although the signaling pathways have distinct intermediate players, and there is extensive crosstalk between them. The basic mechanism of some signaling pathways that are often deregulated in cancer and are of relevance to this thesis will be briefly outlined below.

1.5.1 The RAS, MAPK and PI3K signaling pathways

Receptor tyrosine kinases (RTKs) are similarly structured receptors that transmit signals from growth factors. Binding of the ligand leads to dimerization and activation of the receptors, which transphosphorylate each other on tyrosine residues. Through the function of adaptor proteins such as Grb2, which recognize phospho-tyrosine through its SH2 domain, inactive GDP-bound RAS is recruited and converted to its active GTP-bound form and remains activated until the GTP is hydrolyzed to GDP. GTP-bound RAS activates the RAF protein kinase family, triggering the Mitogen-Activated Protein Kinase (MAPK) pathway. RAF phosphorylates and activates MEK, which in turn phosphorylates and activates Extracellular signal Regulated Kinase (ERK1/2) protein kinases. ERK functions in activating factors involved in protein synthesis in the cytoplasm, and regulating transcription by phosphorylating several transcription factors in the nucleus that stimulate cell proliferation and growth (Downward, 2003; Finocchiaro et al., 2008; Katz et al., 2007).

RAS can also activate the Phosphatidyl Inositol-3 Kinase (PI3K) pathway. This pathway is also activated for example by Insulin-like Growth Factor (IGF)-1/IGF1-receptor (IGFR) RTK, which is involved in cell growth and survival. PI3K is a lipid kinase that phosphorylates 2 phosphatidylinositol 4,5-biphosphate (PIP2) to generate PIP3, which is then

recognized by and activates the AKT/PKB kinase. Activated AKT signaling inhibits apoptosis and promotes cell growth and survival by phosphorylating a number of substrates, including caspase 9, Bad, MDM2 (Cantley, 2002; Engelman et al., 2006), and suppressing proteins that negatively regulate MYC-mediated cell proliferation such as GSK-3β (Frame and Cohen, 2001) and FOXO proteins (Bouchard et al., 2004). PI3K also activates the mTOR pathway leading to increased translation. The components of the MAPK and the PI3K signaling pathways can interact and crosstalk with each other to promote the growth and survival of transformed cells.

Single activating mutations on *RAS* are prevalent in some types of human cancers such as pancreatic, colon and lung cancers where the frequency of *RAS* mutations are being as high as 91%, 42% and 33%, respectively. When this happens, the mutated RAS proteins produced become constitutively active and are no longer require upstream signaling for activity. Such deregulation in the signaling pathways contributes to one hit on the way to transformation, by allowing the cell to acquire self-sufficiency to growth signals, one of the hallmarks of cancer (Hanahan and Weinberg, 2011; Vogelstein and Kinzler, 2004).

1.5.2 The Interferon-ɣ and JAK/STAT Pathway

One family of cytokines relevant to this thesis is the Interferons (IFN), which are involved in regulating cell growth, antiviral defense and immune response. It comprises of three classes, namely IFN-α, −β and-γ, and are further subdivided into two types, type I and II, based on their structure, function and stimuli that induce their expression. Type I consists of IFN-α and -β, while type II consist only of IFN-γ. IFN-γ has been found to be secreted by several components of the immune system, including T helper type 1 (Th1) lymphocytes and natural killer (NK) cells, B cells, natural killer T cells and professional antigen-presenting cells (APCs) (Stark et al., 1998). This type II interferon plays a role in many biological functions, such as in anti-proliferative and anti-viral pathways, activation of macrophages and regulation of cell differentiation and apoptosis (Boehm et al., 1997; Hu et al., 2002). IFN-γ possesses anti-tumor properties and has in fact been used in some cancer treatment (Gleave et al., 1998).

There are two ways of regulating IFN-γ activity, either by controlling production of IFN- γ or modulating the IFN-γ signaling. IFN-γ production is controlled by interleukin 12 (IL-12,) and 18 (IL-18) in natural killer cells, and by T-cells receptor engagement in T-helper cells. IFN-ɣ binds and activates the receptor tyrosine kinases Janus kinases (JAKs), which then phosphorylate transcription factors in the cytoplasm called signal transducer and activator of transcription (STATs). Upon phosphorylation, STATs translocate into the nucleus, bind specific DNA sites and direct the transcription of IFN target genes. There are four known mammalian JAKs (JAK1, JAK2, JAK3 and Tyk2) and seven STATs (STAT1, 2, 3, 4, 5a, 5b and 6) (Aaronson and Horvath, 2002; Decker and Kovarik, 2000; Stark et al., 1998).

STAT1 has shown to be necessary for the anti-proliferative effects of IFNs. For example, expression of the CDK inhibitor p21, which is a negative regulator of the cell cycle, is upregulated by IFN-γ. On the other hand, the expression of positive cell cycle regulators, like MYC, cyclin D3 and CDC25a, is reduced in response to IFN-α (Chin et al., 1996; Kominsky et al., 1998; Ramana et al., 2000; Tiefenbrun et al., 1996). In this thesis, we showed that IFN-γ also mediates MYC degradation (Bahram et al., 2016) (Paper II).

Other than the JAK/STAT pathway, IFN-γ receptors can also mediate biological responses through the RAS/RAF (MAPK) and PI3K pathways (Kalvakolanu, 2003; Stark et al., 1998).

1.6 GENE EXPRESSION AND REGULATION

Most signaling through different pathways is transmitted to the nucleus where it regulates gene expression. Many of the targets of mutation or deregulation in cancer are transcription factors, such as MYC, p53 and pRB, as have been outlined above. Gene expression can be regulated at multiple steps and involves numerous components. In this chapter, only certain aspects of the process will be briefly discussed, all in relation to this thesis work.

1.6.1 Chromatin Structure and DNA Accessibility

Genetic information is stored in the DNA, which is nearly 2 meters long for a human cell and is tightly packed in a highly organized structure of chromosomes to fit in the cell nucleus. The strand of DNA is wrapped around an octamer core consisting of histone H2A, H2B, H3 and H4 proteins, forming the basic unit of chromatin, the nucleosome, which is then further organized to form the chromatin structure. The chromatin structure is highly dynamic and can be modulated to make it accessible for proteins involved in transcription, replication and DNA repair (Woodcock and Ghosh, 2010). The histone-DNA contact interface can be altered by covalent histone modification and chromatin-remodeling complexes, thus regulating gene expression (Peterson and Laniel, 2004; Wang et al., 2007a, b).

Histone modifying enzymes, called writers and erasers, are recruited by transcription regulators to covalently add or remove groups of histones marks at histone tails, respectively. These groups include acetyl (ac) and methyl (me) of lysine (K) and arginine (R), phospho, ubiquitin, SUMO and ribosyl groups. Different combinations of the histone marks code for different biological outcomes. Examples of active or repressed histone marks are given below (Nicholson et al., 2015; Peterson and Laniel, 2004).

- Active chromatin/ transcription: H3K4me3, H3K27ac, H3K9ac, H3K79me2
- Repressed chromatin/ transcription: H3K9me2/3, H3K27me2/3, H4K20me3

Histone acetyl transferases (HATs) and histone de-acetyl transferases (HDACs) are examples of writers and erasers, for the addition (acetylation) or removal (deacetylation) of acetyl groups at histones tails, respectively (Allfrey et al., 1964; Nicholson et al., 2015). GCN5 was already known to be a transcriptional co-activator when it was discovered as the first HAT (Brownell et al., 1996), thus it became clear that histone modifications can regulate transcription directly. Later, other transcriptional co-activators like CBP/p300 were shown to have HAT activities while transcriptional co-repressors like SIN3-RPD3 were linked to HDAC activities (reviewed in Peterson and Laniel (2004)).

The different histone modifications can change the charge of the histone tail, and can thereby affect the affinity between DNA and nucleosomes, but they also constitute a "histone code" controlling the binding of specific non-histone proteins to chromatin. These histone codes can be recognized by another group of proteins, called readers, which bind chromatin modifications and decipher the chromatin state of the locus. For instance, bromodomain-containing proteins are readers that bind acetylated histones, while methylated histones recruit chromodomain-containing proteins (Nicholson et al., 2015; Yun et al., 2011).

Histone modifications, chromatin remodeling complexes and other recruited non-histone proteins form part of the "epigenetic landscape" , which regulates chromatin structure, and in turn affects gene expression in a reversible and highly dynamic manner, without changing the DNA sequence ((Jakopovic et al., 2013; Wolffe and Hayes, 1999) reviewed in Peterson and Laniel (2004)). DNA methylation is another factor affecting the epigenetic landscape. Hypomethylation of promoter DNA is an active mark, which leads to transcriptional activation, while hypermethylation is a repressive mark that leads to transcriptional repression, in part due to methyl-CpG-binding proteins bound at the methylated DNA, and the recruitment of HDACs and chromatin remodeling factors, all leading to a repressive transcription mark and repressed chromatin (Ballestar and Wolffe, 2001; Zwart et al., 2001).

1.6.2 Transcription and Transcription Factors

Information encoded in the DNA molecules is transcribed into RNA molecules, after which protein-encoding mRNAs are translated into functional proteins (Cramer et al., 2008). The transcription is driven by RNA polymerases, comprising of RNA polymerase I, II and III (Pol I, II and III), which synthesizes rRNA, mRNA and tRNA, respectively. Protein-encoding genes are only transcribed by Pol II, which also transcribes non-coding RNAs like miRNAs. Transcriptional regulation occurs mostly during transcription initiation. There are various control regions positioned on a protein-encoding gene, namely the core promoter, the proximal and distal promoters, enhancers and silencers (Delgado and León, 2006).

The initiation of Pol II-mediated transcription requires the recruitment of general transcription factors (GTFs), such as TFIIB, TFIID, TFIIA, TFIIE, TFIIF and TFIIH, which

forms the pre-initiation complex (PIC) and directs the polymerase to the transcription start site (TSS). The PIC containing RNA pol II recognizes promoters containing a TA-rich DNA sequence called TATA box. TFIID is able to recognize TATA boxes through its subunits TATA-binding protein (TBP) and TBP-associated factors (TAFs). Another important element called initiator (Inr) sequence is also located within the transcription start site (Kornberg, 2007; Moqtaderi et al., 1996).

DNA-binding transcription factors bind to specific promoter or enhancer DNA sequences in target genes that are upstream of the transcription initiation site. When bound to enhancer elements, the transcription factor is able to bring a specific promoter to the PIC by looping the DNA (Delgado and León, 2006). These two components are then connected by a multiprotein complex called the Mediator (Kornberg, 2005; Malik et al., 2005). RNA Pol II then leaves the PIC and start synthesizing RNA. Binding of the transcription factor to silencers, though, would result in repression of the gene expression (reviewed in Delgado and León (2006)).

Transcription machinery in higher eukaryotes would pause after the initiation steps and transcribing few nucleotides. This is known as promoter-proximal pausing and is an important mechanism of transcription regulation. In RNA pol II-driven transcription, pausing is mediated by DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) (Nechaev and Adelman, 2011). CyclinH/CDK7 which are subunits of TFHII contributes to clearance of the promoter by phosphorylating the Ser5 residue of the C-terminal domain (CTD) of RBP1, the largest subunit of RNA polymerase II, thus allowing pause release and transcription elongation to proceed (Espinosa, 2010; Hengartner et al., 1998). Ser 5 phosphorylation by TFIIH also contributes to the recruitment of capping enzyme to the 5' end of nascent mRNA.

Processive transcription elongation is mediated by positive transcriptional elongation factor b (P-TEFb) complex, consisting of CDK9 and cyclin T. It phosphorylates Ser2 of the CTD tail and elongation factors DSIF and NELF, leading to the dissociation of DSIF and NELF from RNA pol II. Ser-2 phosphorylation also promotes recruitment of the mRNA splicing complex 3' as well as processing and termination factors (Ahn et al., 2004; Meinhart and Cramer, 2004).

1.7 THE UBIQUITIN PROTEASOME SYSTEM

The ubiquitin-proteasome system was discovered as a regulated protein degradation mechanism involved in a wide range of cellular processes including the transcription, protein quality control, signal transduction, cell cycle, apoptosis, receptor mediated endocytosis and metabolic pathways (Ciechanover, 2005). Quite a portion of the human genome is found to

be devoted to the ubiquitin pathway, taking into account both proteolytic and non-proteolytic functions (Semple et al., 2003).

The ubiquitin-proteasome system involves two steps. The first step is ubiquitylation, where ubiquitin, a 76-amino acid polypeptide (Goldstein, 1974; Goldstein, 1975; Schlesinger et al., 1975), is attached to the substrate. This is followed by degradation of the ubiquitylated substrate by the 26S-proteasome. Ubiquitylation proceeds through three enzymatic steps involving at least three types of enzymes. Firstly, ubiquitin is activated by E1, a ubiquitin activating enzyme, which forms a thiol-ester bond with the C-terminal glycine on the ubiquitin protein an ATP-dependent process. Secondly, ubiquitin is transferred to E2, a ubiquitin conjugating enzyme that accepts ubiquitin from the E1 by a transesterification reaction. Finally, the ubiquitin is conjugated to its substrate either directly by the E2 or through E3 ubiquitin-ligases. E3-ligases are enzymes that can catalyze the conjugation process, which results in covalent attachment of ubiquitin to a specific substrate (for reviews see (Ardley and Robinson, 2005; Fang and Weissman, 2004; Pickart and Eddins, 2004; Weissman, 2001). Upon attachment of one ubiquitin molecule to the target protein, more ubiquitins can be attached to a lysine residue on the surface of the first ubiquitin molecule to build a poly-ubiquitin chain, or the target protein can remain mono-ubiquitylated (Kim et al., 2007). Recent studies have indicated that the polyubiquitin chain may already be formed on the E2 and E3 enzymes before conjugation to the substrate (Ben-Saadon et al., 2006; Li et al., 2007).

There are seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) on ubiquitin, all of which have been found to participate in ubiquitin-ubiquitin interactions. There are several different formations of the polyubiquitin chains. Similar lysine residues can be used for conjugating the ubiquitin molecule, creating a homotypic chain, or different lysines can be used to form a mixed-linkage chain. Other ubiquitin-like molecules such as SUMO can also be conjugated, thus forming a heterologous polyubiquitin chain (for review see (Ikeda and Dikic, 2008)). The length of the ubiquitin chain and specific lysine residues involved will determine the substrate fate and function, whether to be targeted for degradation or other non-proteolytic functions like DNA-repair (Spence et al., 1995), transcriptional activation (Adhikary et al., 2005a) or many other processes.

The E3 ubiquitin ligases

Ubiquitin-E3-ligases are classified into four classes based on their structure motifs: RING (Really Interesting New Gene) finger, HECT (Homologous to E6-AP Carboxyl Terminus), U-box E3 and PHD (Plant Homeo-Domain)-finger E3 ligases (Deshaies and Joazeiro, 2009; Hatakeyama et al., 2001; Rotin and Kumar, 2009; Scheffner and Kumar, 2014). For the purpose of this thesis, the RING finger will be briefly discussed on.

One of the extensively studied subclass of the RING-finger E3 ligase family is the SCF (Skp1-Cullin1-F-box protein) E3 ligase. The SCF complex consists of the Cul1, Rbx1, Skp1, and F-box protein subunits. The Cul1 and Rbx1 subunits form a catalytic core that recruits the upstream E2 enzyme while the variable F-box subunit mediates interaction with Skp1 and also confers substrate specificity to the whole complex (Deshaies, 1999; Jackson and Eldridge, 2002; Zheng et al., 2002).

There are about seventy F-box proteins that have been identified in human genome. They are categorized based on their interaction domain, namely F-box proteins containing WD40 repeats (FBXW), leucin-rich repeats (FBXL) or other domain (FBXO) (Skaar and Pagano, 2009).

Some SCF E3 ligases have been shown to play important role during the cell cycle, such as the S-phase associated kinase associated protein 2 (SKP2/FBXL1) and FBW7 (FBXW7) that will be mentioned in later chapters in this thesis. We have also discovered a novel E3 ligase, FBXO28, which activates transcription via MYC. This will be discussed more in the paper III results and discussion.

1.8 MYC

1.8.1 Master Regulator

MYC is the human homologue of the avian myelocytomatosis retroviral oncogene, *v-myc*, first described more than 30 years ago (Hayward et al., 1981; Sheiness and Bishop, 1979; Vennstrom et al., 1982). The *MYC* family of proto-oncogenes comprises of 3 members, *MYC* (c-*MYC*), *MYCN* and *MYCL*, which encode nuclear oncoproteins/transcription factors of the basic helix-loop-helix-leucine zipper (bHLHZip) family. MYC has been estimated to regulate up to 15% of all genes in humans and coordinate the expression of genes involved in diverse intracellular programs, including, but not limited to, cell cycle, proliferation and growth, energy metabolism, DNA replication, global RNA production and many biosynthetic pathways, as well as differentiation, apoptosis and senescence (Adhikary and Eilers, 2005; Dang et al., 2006; Eilers and Eisenman, 2008; Kress et al., 2015; Larsson and Henriksson, 2010; Meyer and Penn, 2008). MYC proteins also play important roles in normal development and cell physiology. In mice studies, loss of c- and N-*MYC* had been found to be embryonic lethal (Baudino et al., 2002; Charron et al., 1992; Davis et al., 1993; Knoepfler et al., 2002; Stanton and Parada, 1992; Stanton et al., 1992), but L-*MYC* knockout do not seem to lead to any phenotypic abnormalities (Pirity et al., 2006).

MYC controls expression of its target genes for the many different functions by recruiting different co-factors for activation or repression of transcription. For its transcriptional and

oncogenic activity, MYC must dimerize with MAX, another bHLHZip protein that is ubiquitously expressed. The MYC:MAX complex binds to DNA recognition elements at target gene promoters, primarily E-box sequence CACGTG or its variants, and activates transcription. MYC represses transcription at alternative DNA sites through association with other cofactors such as the transcription factor Miz-1 (Adhikary and Eilers, 2005; Eilers and Eisenman, 2008; Meyer and Penn, 2008). MYC also recruits a number of other cofactors, such as histone acetyl transferases (HATs), histone methyl transferases (HMTs), ATP-dependent chromatin remodeling complexes, E3 ubiquitin ligases and kinases, amongst others, to up- or downregulate its target genes and execute the different cellular processes (Adhikary et al., 2005b; Hydbring et al., 2017; Kress et al., 2015).

In 2012, studies emerged to propose MYC as a global amplifier of transcription. MYC is proposed to interact with all active promoters and enhancers and, upon its upregulation, MYC would invade them and amplify the active transcriptional program further. This model provides an explanation for why high MYC levels often increase the total RNA content in cells (Lin et al., 2012; Nie et al., 2012). This view was opposed by others who stand by the idea that MYC regulates specific sets of genes, be it for coding genes, miRNA regulation or long non-coding RNAs (lncRNAs), which, however, in turn regulates global gene expression (Kress et al., 2015; Sabo et al., 2014).

Given its central role in transcription and regulation of many cellular processes, it comes as no surprise that the MYC family possesses potent oncogenic capabilities and contributes to a large number and variety of human tumors (See section: MYC in Cancer). Thus MYC expression is tightly regulated and is usually kept at low levels, only to be induced by specific cues such as growth factor signaling (Marcu et al., 1992; Wierstra and Alves, 2008).

1.8.2 MYC Structure, Expression and Regulation

The *MYC* gene is found in all major metazoan lineages and possibly as far back as unicellular progenitors (Kress et al., 2015; Young et al., 2011), but is absent in the nematode C. elegans (Young et al., 2011). The *MYC* family of genes consists of three exons. Exon 1 is the target site for transcription factors and is noncoding, while exons 2 and 3 contain the coding regions. Two promoters at the 5' end of exon 1 accounts for 90-95% of transcription of *MYC* mRNAs, which are about 2.2 and 2.4 kb, from which MYC proteins of about 64 and 67 kDa are translated (DePinho et al., 1987; Katoh et al., 1988; Sawai et al., 1990; Watson et al., 1983).

MYC, MYCN and MYCL1 proteins are highly conserved in most of the regions termed the MYC homology boxes (MB). The N-terminus of MYC contains MBI (amino acids (aa) 44–63) and MBII (aa 128–143), which form part of the transactivation domain (TAD; aa 1–143). In the central region lies MBIIIa (aa 188–199), which is conserved in MYC and MYCN but

not in MYCL, MBIIIb (aa 259–270), and MBIV (aa 304–324), the latter containing the nuclear localization signal (NLS; aa 320–328). The C-terminus of MYC contains the basic region (b; aa 355–369) and the helix–loop–helix–leucine zipper (HLH–LZ; aa 370–439). The bHLHZIP component is required for MYC binding to its transcription partner protein, MAX, and for full transformation of primary and immortal cells. The basic region confers the specificity of binding to canonical and non-canonical E-boxes DNA sequences (Figure 3) (reviewed in Meyer and Penn (2008)).

MYC is regulated at multiple levels, including transcription, translation, post-translational modification and turnover.

The *MYC* proto-oncogene is a direct target and effector downstream of growth-regulatory and oncogenic signaling pathways, such as, but not limited to, RTKs, Notch, WNT, Hedgehog and Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) signaling, which induce MYC transcription. On the other hand, *MYC* transcription is repressed by transforming growth factor‑β (TGFβ) signaling (reviewed in (Kress et al., 2015; Wierstra and Alves, 2008)). Many of these signaling pathways act on proximal promoter elements and distal enhancers, including super-enhancers, regulating transcription of the *MYC* loci (reviewed in Dang (2012) and Hydbring et al. (2017)).

MYC translation can be affected by a number of signaling cascades, such as mTOR signaling including mTOR complex 1 (mTORC1)–S6K1, eIF4A and eIF4F, MAPK–HNRPK and MAPK–FOXO3A signaling cascades (reviewed in (Hydbring et al., 2017; Kress et al., 2015)).

Post-translationally, MYC activity and/or turnover are regulated via a number of modifications, such as phosphorylation, acetylation, glycosylation, proteolytic or non-proteolytic ubiquitylation, and small ubiquitin-related modifier (SUMO)-ylation (Kamemura et al., 2002; Vervoorts et al., 2003)(reviewed in Hydbring et al. (2017)). Of interest to this thesis is the phosphorylation and ubiquitylation of MYC and will be expanded on later in this chapter.

The MYC protein is very short-lived, with a half-life of about 15–30 min, depending on cell type (Gregory and Hann, 2000; Gregory et al., 2003). PI3K and RAS signaling pathways have been found to cooperate to increase MYC stability (Sears et al., 1999; Sears et al., 2000). MYC is then regulated and degraded mainly by the ubiquitin/proteasome system.

Several E3 ubiquitin ligases have been shown to ubiquitylate MYC with different consequences for MYC´s function and stability, including SCF^{FBXW7}, SCF^{SKP2}, HUWE1/HECTH9, $SCF^{\beta TRCP}$, TRUSS, PIRH2, CHIP, SCF^{FBXL14}, SCF^{FBXO28} and SCF^{FBX29} (reviewed in Hydbring et al. (2017)). SCF^{SKP2}, for example, has been shown both to induce degradation of MYC and to work as a transcriptional cofactor (von der Lehr et al., 2003). FBXW7 is a classical E3 ligase for MYC degradation (Welcker et al., 2004; Yada et al., 2004; Yeh et al., 2004).

Studies on MYC transformation and regulation of its stability and activity has very much been focused on the MYC phosphorylation sites Thr58 and Ser62 in the MBI region, which has been associated with degradation of MYC by via the E3 ligase FBXW7 (Bahram et al., 2000; Hann, 2006; Henriksson et al., 1993; Pulverer et al., 1994). A variety of proliferative stimuli, for example cytokines, mitogenic signals, UV exposure and DNA damage, activates specific kinases to phosphorylate MYC at Ser62 (reviewed in Hann (2006)). Phosphorylation at Ser62 stabilizes and activates MYC, and also serves as a platform for Thr58 phosphorylation by glycogen synthase kinase 3β (GSK3β) (Sears et al., 2000), which in turn recruits phosphatase PP2A to dephosphorylate Ser62 and then the E3 ligase FBXW7 to degrade MYC (Welcker et al., 2004; Yada et al., 2004; Yeh et al., 2004).

Our contribution from this thesis work has been the discovery of a novel E3 ligase, SCF^{FBXO28}, which non-proteolytically ubiquitylates MYC in a cell cycle dependent manner and activates MYC activity of relevance to tumorigenesis (paper III). We have also found a new pathway for MYC degradation mediated by IFN-ɣ and p27 pathway (paper II), with the involvement of an unknown E3 ligase. These will be further discussed in the results and discussion section.

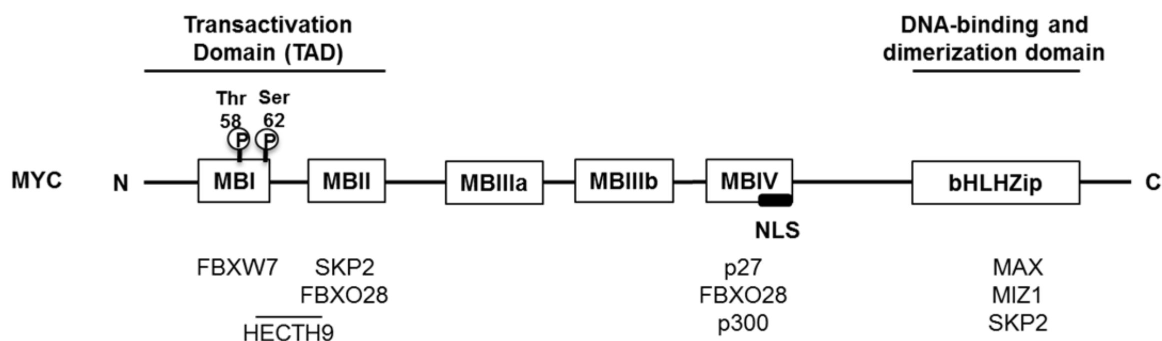

Figure 3: MYC structure and interaction partners. Upper part: MYC protein structure with MBI-IV, the conserved MYC homology boxes I–IV; Thr-58 and Ser-62, regulatory phosphorylation sites; TAD, transcriptional activation domain; NLS, nuclear localization signal; bHLHZip, basic region/helix–loop–helix/leucine zipper. Lower part: Interacting and regulatory proteins that are of interest for this thesis interacting with respective regions.

Negative feedback loops also keep MYC level in check. MYC has been found to represses its own promoter in a concentration-dependent manner as a form of homeostatic control mechanism (reviewed in Meyer and Penn (2008)). In this complex regulatory mechanism MYC target genes loops to control the transcription. For example, PTEN tumor suppressor that is activated by MYC, enhances PRC2 activity, which in turn represses many genes, including *MYC* itself (Kaur and Cole, 2012). In another scenario, *MYC* expression is positively regulated by E2F1. MYC, in turn, induces expression of microRNAs miR-17 and

miR-20, which downregulate E2F1 transcription factors, hence autorepressing *MYC* expression (O'Donnell et al., 2005).

Intrinsic anti-proliferative programs are also elicited to counter MYC activity. MYC activation under conditions of limited survival factors induces apoptosis by both p53-dependent and -independent mechanisms (reviewed in Green and Evan (2002); Nilsson and Cleveland (2003)). In the absence of CDK2, activation of MYC induces senescence (Campaner et al., 2010). The induction of these two phenomena by MYC will be further explained in later chapters.

1.8.3 MYC and Cell Cycle

MYC plays an important role in cell cycle progression. In quiescent G0 cells, MYC expression is very low. Upon increase in mitogenic signaling, MYC expression is rapidly induced and drives the transition from G0 to G1 to from G1 to S phase. It has been shown that quiescent cells re-enter the cell cycle and proceed through G1 and S phase upon expression of MYC alone, even in the absence of serum factors. Also, it was found that G1 phase in the cell cycle is often shortened in cells with activated MYC (Bouchard et al., 2004) (reviewed in Meyer and Penn (2008)).

MYC has been shown to directly or indirectly activate most of the cell cycle players, including cyclin D/CDK4, cyclin E/CDK2, cyclin A/CDK2, cyclin B/CDK1, E2F1, E2F2, and cell division cycle 25A (CDC25A) ((Amati et al., 1998; Bouchard et al., 1999; Coller et al., 2000; Galaktionov et al., 1996; Hermeking et al., 2000; Leone et al., 1997; Luscher, 2001; Mateyak et al., 1999), also reviewed in Meyer and Penn (2008) and Pelengaris et al. (2002a)). These factors are important both for the G1-S phase transition, to drive the cell cycle through the R point, and also for G2-M phase transition. Additionally, MYC directly or indirectly downregulates or inhibits several cell cycle checkpoint genes, such as CKIs, like $p15^{INK4B}$, $p21^{CIP1}$, $p27^{KIP1}$ and GADD45 and GADD153 (Chandramohan et al., 2008; Obaya et al., 1999; Oster et al., 2002; Seoane et al., 2002; Staller et al., 2001; Wu et al., 2003).

The complexity of the cell cycle regulation increases with regulatory loops to provide a tight control over cell proliferation. One example is that MYC also upregulates miR-17-5p and miR-20a, which downregulates E2F translation (O'Donnell et al., 2005). Another example is our finding that p27 targets MYC for degradation, as will be discussed in paper II.

1.8.4 MYC and Apoptosis

In order to maintain homeostasis, activation or ectopic expression of MYC will induce the cell to undergo apoptosis under the conditions where survival factors are limiting. This sensitizes cells to stimuli such as death receptor signaling (Fas/TNF-α), DNA damage and cancer drugs (reviewed in Nilsson and Cleveland (2003)). MYC-induced apoptosis is mediated through two known pathways: p53 upregulation *via* p19ARF, and cytochrome release via BAX activation and/or repression of BCL-X_L and BCL-2 (Zindy et al., 1998).

In tumorigenesis, oncogenic events will hinder the pro-apoptotic function of MYC by inducing anti-apoptotic factors, like BCL-2, BCL-X_L or BIM, or repressing/ablating pro-apoptotic factors such as p53 and p19ARF ((Eischen et al., 1999), reviewed in (Meyer and Penn, 2008)). It was found that MYC-induced apoptosis is dependent on the level and intensity of MYC expression. Low level of activated MYC would lead to proliferation and is better at initiating tumorigenesis, while high level of MYC overexpression would lead to apoptosis. However, in the presence of anti-apoptotic factors, MYC apoptotic function is inhibited and MYC overexpression leads to tumorigenesis (Evan et al., 1992; Murphy et al., 2008). Consequently, apoptosis can be induced in MYC-driven tumors by reviving the abrogated pathways, leading to destruction of the tumor cells (Goga et al., 2007; Meyer et al., 2006).

1.8.5 MYC and Senescence

Senescence is another anti-proliferative program elicited by MYC overexpression. High levels of MYC lead to replication stress and genomic instability. This can lead to premature cellular senescence that is independent of the telomere, a condition termed oncogene-induced senescence (OIS). Studies have showed that overexpressed MYC can provoke OIS when the anti-senescence and DNA repair gene, WRN, is ablated (Grandori et al., 2003).

Another study showed that *CDK2* knockout mouse embryonic fibroblasts would initially enter a proliferative state when exposed to MYC activation and then undergo senescence, without induction of replicative stress as in the WRN model. This was accompanied by upregulation of $p21^{Cip1}$ and $p16^{INK4a}$, and thus dependent on the ARF–p53–$p21^{Cip1}$ and $p16^{INK4a}$–pRb pathways. Moreover, in the Eμ-myc transgenic mouse lymphoma model, *CDK2* ablation sensitizes cell to MYC-induced senescence, with delayed onset of lymphoma (Campaner et al., 2010).

Paradoxically, MYC plays a dual role with regards to senescence and has been shown to suppress senescence induced by other oncogenes. Our group and others have shown that MYC suppresses activated RAS/BRAF-induced (BRAF is a downstream effector of RAS)

senescence in rat embryonic fibroblast (REF) cells and in a BRAF^{V600E}/MYC mouse lung tumor model. Reciprocally, MYC inactivation in this system restores BRAF^{V600E}– and NRAS^{Q61R}–induced senescence (Hydbring et al., 2010; Juan et al., 2014; Tabor et al., 2014). Phosphorylation of MYC at Ser62 has been shown to be required for this suppressive function. It was also shown that cyclin E/CDK2 phosphorylates this site, and that the CDK-inhibitor p27^{Kip1} and CDK2-selective pharmacological compounds inhibit phosphorylation and abrogates this function of MYC (Hydbring et al., 2010). Further, as mentioned above, MYC also promotes senescence in the absence of CDK2 (Campaner et al., 2010). Thus in this scenario, CDK2 acts as a switch between MYC function in repressing or inducing senescence (reviewed in Hydbring et al. (2017)).

Human melanoma cells expressing mutant BRAF^{V600E} or NRAS^{Q61R} has also been shown to undergo p16INK4A- or p53-independent senescence upon depletion of MYC. Conversely, overexpression of MYC suppresses BRAF^{V600E}– and NRAS^{Q61R}–induced senescence in melanocytes, albeit more efficiently with the former. This suggests that in melanoma with BRAF^{V600E} or NRAS^{Q61R} mutations, MYC overexpression functions to sustain tumorigenesis by suppressing the innate senescence program that would have otherwise been elicited (Zhuang et al., 2008) (see also chapter MYC in Cancer -*Oncogene Cooperation*).

1.8.6 MYC in Cancer

Despite being subjected to stringent regulation in the cell at many levels, MYC deregulation has been found in a wide variety of human malignancies, tallying to more than half of all human tumors (Nilsson and Cleveland, 2003; Vita and Henriksson, 2006). The central role of MYC in growth control and orchestrating a large variety of genes and pathways, endows MYC with strong oncogenic potential. This allows MYC activation to contribute directly to and being a driver of malignant transformation (Adams et al., 1985; Gabay et al., 2014; Land et al., 1983; Leder et al., 1986; Meyer and Penn, 2008), often correlating strongly with tumor progression to advanced stages and poor prognosis (Adhikary and Eilers, 2005; Eilers and Eisenman, 2008; Larsson and Henriksson, 2010; Meyer and Penn, 2008; Nilsson and Cleveland, 2003). Apart from driving tumor initiation and progression, MYC is also needed to maintain the transformed state, even in tumors driven by other oncogenes (Felsher and Bishop, 1999; Gabay et al., 2014; Jain et al., 2002; Pelengaris et al., 2002b; Sodir et al., 2011; Soucek et al., 2008; Zhuang et al., 2008).

Among the mechanisms leading to MYC overexpression discovered early in the history of MYC research includes insertional mutagenesis, chromosomal translocation and gene amplification. Insertional mutagenesis is where a non-mutated cellular gene is activated by the insertion of a foreign promoter or enhancer, in this case from a retrovirus. In chromosomal translocation, *MYC* oncogene is juxtaposed to the immunoglobin (Ig) heavy chain loci by consistent recombination, which often gives rise to lymphomas (reviewed in

Meyer and Penn (2008)). Amplifications of *MYC* (Alitalo et al., 1983; Dalla-Favera et al., 1982) and *MYCN* (which usually is expressed during development and in different immature cell types) have been detected across many human neuroblastoma cell lines and tumor samples (Kohl et al., 1983; Schwab et al., 1983) and were quickly associated with poor patient prognosis (Brodeur et al., 1984; Schwab et al., 1984). In contrast to chromosomal translocations of the *MYC* genes which lead to haematopoietic cancers, activation by amplification is often involved in solid human tumors (Meyer and Penn, 2008; Vita and Henriksson, 2006).

There are several other known mechanisms of indirect activation of MYC to date. Physiological pathways that activate either MYC or MYCN in normal conditions may themselves undergo oncogenic mutations (reviewed in Kress et al. (2015)). Apart from activation, enhanced stability of *MYC* mRNA and protein may also occur as a result of deregulation in the various pathways involved (reviewed in Meyer and Penn (2008); (Nilsson and Cleveland, 2003). There are also aberrations in other pathways that can prevent removal of cells containing activated MYC. One example is p53 or ARF loss of function, which disables the cell protective programs like apoptosis, senescence, or cell cycle arrest in the event of MYC deregulation, leading to MYC-induced lymphomagenesis (Eischen et al., 1999). Single-nucleotide polymorphisms (SNPs) affecting proximal promoter-elements, distal enhancers and super-enhancers that controls MYC transcription also can lead to deregulated MYC expression (Dang, 2012; Hydbring et al., 2017).

Direct mutations in the coding sequence leading to activation, increased stability or reduced degradation, are uncommon for *MYC*, unlike many other oncogenes like *RAS*. One such occurrence in *MYC* is mutations of the Thr58 and Ser62 residues, leading to stabilized mutant proteins. These mutations have been found mostly in Burkitt's and other lymphomas (Dang, 2012; Nilsson and Cleveland, 2003).

Deregulation or activation of MYC alone may not be sufficient to form a tumor as complete transformation requires at least two mutations in rodent cells and four to seven mutations in human cells (Hahn et al., 1999; Hanahan and Weinberg, 2000; Land et al., 1983; Renan, 1993). However, MYC overexpression or stabilization, coupled with alterations in the apoptotic pathway, results in a pool of cells proliferating uncontrollably with an increased risk of acquiring secondary mutations leading to transformation and tumor development (Vita and Henriksson, 2006).

Oncogene cooperation

Studies with MYC not only pioneered the field of oncogene-induced apoptosis. Prior to that, experimentations with overexpressed *MYC* and activated *RAS* helped established the concept of oncogene cooperation (Land et al., 1983). Further work by others increases our understanding of the concept better when more cooperating oncogenes partners were found,

like *MYC* and *BCL2*. The concept of cooperating oncogenes holds true for both human and murine systems, albeit with different number of participating aberrations involved (reviewed in (Meyer and Penn, 2008)).

As mentioned in earlier in the MYC and senescence chapter, our group and others had found that MYC suppresses activated RAS-induced senescence (Hydbring et al., 2010; Juan et al., 2014; Tabor et al., 2014). In the same light, RAS has been found to suppress oncogenic MYC-induced apoptosis (Kauffmann-Zeh et al., 1997). This explains, at least in part, the basis for the MYC/RAS cooperativity that occurs in the rodent system, which is blocking the two main anti-tumorigenic mechanisms in the cell.

One of our thesis projects is to recapitulate the MYC and RAS system in normal human fibroblast and examine if these two oncogenes cooperate in the same way in the human cells as they do in the rat embryonic fibroblast. The results are discussed later in paper I in the Results and Discussions chapter.

1.8.7 Targeting MYC in cancer therapy

The engagement of MYC in many fundamental cellular functions makes it predictable that its deregulation has an important role in tumor formation and maintenance. Indeed, MYC has been shown to play a role in each of the hallmarks of cancer outlined by Hanahan and Weinberg (2011). Within the scope of this thesis, we have only discussed how MYC could give rise to cancer through sustained proliferative signaling, resistance of cell death, enabling replicative immortality, evasion of growth suppressors and genomic instability and mutation.

Among the intrinsic tumor suppressor mechanisms triggered by deregulated MYC are induction of apoptosis and senescence, and suppression of these programs is vital to tumorigenesis. Many experiments have shown that re-enabling the cell suicide pathway can force tumor cells to self-destruct (reviewed in Kress et al. (2015); Larsson and Henriksson (2010)).

In mouse tumor models with regulatable MYC expression systems, shut down of *MYC* often leads to rapid and sustained tumor regression, also in tumors driven by RAS, loss of APC etc, suggesting that these tumors are “addicted” to MYC (Arvanitis and Felsher, 2006; Jain et al., 2002). Various tumor models also exhibited regression mediated by apoptosis, senescence and/or differentiation upon withdrawal of ectopic *MYC* expression (reviewed in Dang (2012); Kress et al. (2015); Meyer and Penn (2008)). The degenerative phenotypes of normal tissues induced by systemic MYC inhibition were rapidly and completely reversible on restoration of MYC function, and were shown to be tolerable for the animals (Sodir et al., 2011; Soucek et al., 2008). These observations suggest that inhibition of MYC is a possible way of eradicating not only MYC-driven tumors, but also those initiated by other oncogenes, making MYC a

tempting target for therapy (Alderton, 2011; Castell and Larsson, 2015; Larsson and Henriksson, 2010; Prochownik and Vogt, 2010; Vita and Henriksson, 2006).

Insights into MYC biology and cofactor-interactions have been useful to strategize different ways of therapeutically targeting MYC and developing new MYC inhibitors. However, no specific anti-MYC drugs are clinically available today (Castell et al., 2018).

Pharmacological targeting of MYC has proven to be challenging. Several groups have attempted to target MYC indirectly through targeting the different pathways regulating MYC at different levels, including transcription, translation or stability of the mRNA or proteins, but many have limited success due to the multiple ways whereby the tumors can escape (Castell and Larsson, 2015; McKeown and Bradner, 2014; Whitfield et al., 2017). One strategy that has gained interest recently is the targeting of the bromo and extra terminal (BET) domain-containing transcription regulator BRD4, which is important in transcription of *MYC* gene in many cells. BRD4 binds acetylated lysines in histones on the chromatin and contributes to the transcription process. A small molecule inhibitor, JQ1, was found to interfere this interaction by binding to the domain of BRD4 important for this interaction (Fletcher and Prochownik, 2014; McKeown and Bradner, 2014). Pre-clinical models, including of hematopoietic cancers and neuroblastoma, indicated that JQ1 is able to inhibit tumorigenesis. However, it is found to work only in some tumor types but not others (Alderton, 2011; Delmore et al., 2011; Loven et al., 2013; Mertz et al., 2011; Puissant et al., 2013).

Another way of devising strategies is to target MYC directly, but that turns out to be not very straightforward, due to it lacking enzymatic active sites (Prochownik and Vogt, 2010) and being an intrinsically disordered protein (IDP) (Dyson and Wright, 2005).

Studies into targeting protein-protein interactions (PPIs) of IDP proteins, including of MYC, have given insight that it is possible to target limited binding surfaces (Fletcher and Prochownik, 2015; Follis et al., 2008; Hammoudeh et al., 2009; Michel and Cuchillo, 2012; Prochownik and Vogt, 2010). Since heterodimerization with MAX is crucial for MYC to bind to E-boxes for its transcriptional and oncogenic activity (Meyer and Penn, 2008), it is a conceivable approach to target MYC:MAX interaction.

Small molecules inhibitors used successfully to inhibit PPIs (Filippakopoulos et al., 2010; Fletcher and Prochownik, 2014; Lane et al., 2010; Saha et al., 2013; Tse et al., 2008; Vassilev et al., 2004) and leading to clinical trials (Arkin et al., 2014; Nero et al., 2014), have spurred interest of MYC researchers to dive into the search for small molecule inhibitors of MYC:MAX interaction. Screening of small-molecule libraries have led to the identification of several compounds by different groups. Unfortunately, these compounds have a number of limitations including relatively low potency in vitro or in cells, poor specificity or insufficient bioavailability in vivo, and thus have not made their way for clinical studies (Fletcher and Prochownik, 2015; McKeown and Bradner, 2014; Prochownik and Vogt, 2010; Whitfield et al., 2017).

In our work, we have identified a compound MYCMI-6 that is able to bind MYC directly with high affinity, and inhibit the MYC:MAX interaction efficiently and selectively in vitro and in cells. Moreover, this compound is active in vivo and inhibits MYC-dependent tumor cell growth with high efficacy without affecting MYC expression (Castell et al., 2018). The work will be discussed in more detail in the result and discussion section (paper IV).

2 AIMS OF THIS THESIS

Interaction and cooperation between MYC and different cofactors/ proteins are absolutely essential for the different functions of MYC and its stability. The overall aim of this thesis is to deepen our understanding of several different proteins that cooperate and interact with MYC (Paper I to III), and identify small molecules that would target specific interactions involving MYC (Paper IV). The outcome is hopefully to identify possible strategies to suppress the tumorigenic function of MYC or, alternatively, enhance its anti-tumorigenic function, such as induction of apoptosis and senescence, as a way of counteracting MYC in cancer cells driven by MYC or other cooperating oncogenes, particularly, RAS.

More specifically, the aims are:

I. To investigate the oncogenic cooperativity between MYC and RAS in normal human fibroblasts with regards to senescence and apoptosis regulation

II. To elucidate the mechanism by which IFN-ɣ inhibits MYC function, in particular via p27, and the involvement of ubiquitin-proteosome system

III. Identify novel F-box proteins that has implications in cancer, and characterize the role of the novel SCF^{FBXO28} E3 ubiquitin ligase complex in regulating MYC's function

IV. To identify and validate small molecules inhibitors of MYC:MAX interaction and to evaluate their biological activity and selectivity towards MYC-driven tumor cells *vs.* normal cells

3 RESULTS AND DISCUSSIONS

3.1 PAPER I

MYC and RAS are unable to cooperate in overcoming cellular senescence and apoptosis in normal human fibroblasts

For several decades, co-expression of MYC and RAS had been known to be sufficient to transform cells in rodent based models (Hydbring et al., 2010; Land et al., 1983; Larsson and Henriksson, 2010; Meyer and Penn, 2008; Pulverer et al., 1994). The mechanism can be partly explained by the capability of RAS to override MYC-induced apoptosis (Kauffmann-Zeh et al., 1997) and MYC to override RAS-induced senescence (Hydbring et al., 2010). In this study, we sought to understand if MYC and RAS cooperate to suppress these fail-safe mechanisms in human cells in a similar way as in rodent cells.

We first aimed to create a stable dual inducible system, so as to avoid transfection inefficiencies and instabilities. Such a system would also allow us to easily control the expression of the vectors and remove the stress of transient transfection in each setting. To this end, we used normal human BJ fibroblasts that were stably transduced with tetracycline-regulated H-RAS^{V12} as previously described in Evangelou et al. (2013) and Maya-Mendoza et al. (2015), and stably introduced 4-hydroxytamoxifen (OHT)-controlled MycER expression/activation system. In this system, MYC activation alone led to apoptosis, whereas RAS activation alone led to premature cellular senescence, in line with well-known reports of the intrinsic fail-safe programs elicited by these two oncogenes (Evan et al., 1992; Serrano et al., 1997). These were accompanied by DNA damage and upregulation of p53 (Figure 4A).

When the two oncogenes were activated concurrently, the cells undergo apoptosis, DNA damage (ɣH2AX) and p53 induction, similar to the cell culture with MYC activation alone. The cells that did not undergo apoptosis seemed to be pushed into senescence like the cells activated by RAS alone, as shown by β-Gal staining and induction of p16^{INK4A}. However, there is a reduced staining in one senescence marker, the histone H3 lysine 9 trimethylation (H3K9me3) in these dual-induced cells compared to RAS induction alone (Figure 4, all panels). H3K9me3 is associated with cellular senescence in some cells, including BJs, depending on the stimulus, and often follow the expression of p16 (Kosar et al., 2011).

Since p53 plays an important role in oncogene-induced apoptosis and senescence, and we observed induction of p53 upon MYC activation, we then stably knocked down p53 using short hairpin RNA (shRNA). We found that p53 depletion only rescued cells from senescence, both induced by RAS and MYC, but not MYC-induced apoptosis whether with or without RAS co-activation. Additionally, the triple insult caused the cell culture to undergo

apoptosis after a few days (Figure 5, all panels). Hence, our study shows that MYC and RAS do not cooperate to bypass senescence and apoptosis in human BJ cells, even when p53 is removed. Additional mutational events must occur, not just for the transformation of normal human fibroblasts (Vogelstein and Kinzler, 1993), also for suppression of senescence and apoptosis. Unlike the rodent model, coexpression of MYC and RAS in the human BJ model is insufficient for the cross suppression of these two anti-tumorigenic processes.

One possible explanation for the lack of cooperation between these two oncogenes is that MYC enhanced, instead of suppressed p16 expression in RAS-induced BJ cells (Figure 4A). p16 is known to maintain pRB activity, and thus can lead to reinforcement of the RAS-induced senescence in fibroblasts. Previous reports had shown that rodent and human cells with reduced or loss of p16 expression can overcome RAS-induced senescence (Drayton et al., 2003; Huot et al., 2002; Lin et al., 1998; Serrano et al., 1997).

In contrast to our results, previous studies using human normal melanocytes had shown that MYC is able to overcome either BRAF- or NRAS-induced senescence (Zhuang et al., 2008). Further, melanomas with activated BRAF/NRAS undergo senescence upon MYC knockdown, in a p16- and p53-independent manner. This shows that senescence is regulated differently in different cell types.

We conclude that MYC and RAS do not cooperate by cancelling out each other's fail safe mechanisms in normal human fibroblasts as they do in the rodent fibroblasts, even with the removal of the tumor suppressor p53. This indicates that tumorigenesis in human cells takes a different route and require other or additional mutations to activate oncogenic pathways and/or deactivate tumor suppressor pathways (including senescence and apoptosis) that lead to cell transformation and development of cancer. Further, other reports showing that these two oncogenes cooperate in normal human melanocytes indicate that senescence regulation in different human cell types may work differently. This finding also impact the way we study animal models and translate the outcomes into the human system, in this case with regards to the transformation process in human cells and extrapolating results into future clinical studies.

3.2 PAPER II

Interferon-γ-induced p27^{KIP1} binds to and targets MYC for proteasome-mediated degradation

Earlier, our group had shown that CDK2-mediated phosphorylation of MYC at Ser-62 is important for MYC's role in suppressing two barriers of transformation: senescence and differentiation. Further, pharmacological and physiological inhibition of CDK2 by CVT313 or p27 respectively, restored senescence and differentiation (Campaner et al., 2010; Hydbring et al., 2010).

In this paper, we show that p27, via mechanisms independent of CDK2, can also induce senescence and degrade MYC through the ubiquitin-proteosome system via its C-terminus. Additionally, there is a significant correlation between high expression of active p27 protein with low MYC protein level in human breast cancer, with implications in prognosis.

Firstly, we demonstrated here that p27 is able to override MYC's suppression of activated RAS-induced senescence regardless of MYC Ser-62 status. We then showed that p27 affects MYC protein expression levels by inducing degradation of the MYC protein via the ubiquitin-proteosome system.

Expression of p27 protein can be stimulated by grown inhibitory cytokines, such as IFN-ɣ and TGF-β. Here, we showed that IFN-ɣ treatment leads to increased degradation of MYC in several cell lines; U-937 cells, Colo-320 colon carcinoma cells with amplified *MYC* and human 2fTGH fibrosarcoma cells. This is accompanied by increased MYC ubiquitylation and upregulation of p27. A p27 null cell line, p27-/- mouse embryonic fibroblast (MEF), does not have reduced MYC protein level upon IFN-ɣ treatment, even though the wildtype MEFs does. Thus, IFN-ɣ induces degradation of MYC via the ubiquitin-proteosome system, mediated by p27. By using fluorescent reporter protein and BiFC, we localized the IFN-ɣ-induced degradation of MYC to the nucleus, predominantly in the nucleoli, as previously suggested as the site of rapid MYC turnover (Arabi et al., 2005), and in complex with Max. The mechanism is dependent on the Jak/Stat pathway but is independent of MYC Thr-58 phosphorylation site, which is pivotal in MYC's degradation via Fbxw7. Using dominant-negative SKP2ΔF mutant, we also showed that SKP2 is not involved in p27-mediated degradation of MYC. Hence a new unknown E3 ligase is implicated in p27 degradation of MYC.

We further addressed the mechanism through which p27 promotes degradation of MYC. We found that p27 interacts directly with MYC in the nucleus. After mapping the domains, we found that MYC binding to p27 requires amino acids 294 – 366 of MYC, which contains the MYC Box 4 (MB4), the nuclear localization signal (NLS) and the basic DNA binding region of MYC. Binding of p27 to MYC requires the C-terminal part of p27 (amino acids 82 – 198),

which contains several regulatory phosphorylation sites such as T187, T157 and T198, and the part important for p27 nuclear export. The p27 C-terminus is sufficient to induce MYC degradation without involving CDK2 nor affecting *MYC* mRNA levels.

We investigated further the correlation between p27 and MYC in human tumors, to tumor grade and patient outcome. Selecting the breast invasive carcinoma (BRCA) data set from The Cancer Genome Atlas (TCGA) (2012) data portal (Zhu et al., 2009) for sufficient statistical power, we found no significant correlation between MYC and p27 expression levels as a whole. However, a subpopulation with high p27 expression but low level of phosphorylation of p27 at the T157 Akt/Rsk/Pim1 phosphorylation site, which is the signature of cytoplasmic p27 that will not affect CDK2 or the nuclear functions of MYC, has a strong inverse correlation with MYC protein levels. Correlating this selected group to clinical parameters showed a positive correlation with good prognostic markers, namely grade 1 tumors, luminal A subtype, estrogen receptor (ER) positivity and Her2 negativity, and a negative correlation with poor prognostic markers, namely grade III and IV tumors, basal and Her2 subtypes, ER negativity and Her2 positivity. A second subpopulation with high p27 and low MYC protein levels, and low phosphorylation of the retinoblastoma protein protein (pRb) at CDK-sites (a functional readout of p27 activity) correlated significantly with relapse-free patient survival and overall patient survival.

Taken together, in this paper, we showed that IFN-ɣ-induced p27 induces senescence and degrades MYC independently of CDK2 and MYC Thr-58 phosphorylation. This occurred through the ubiquitin-proteosome system via an unknown E3 ligase. Additionally, there is a significant correlation between high expression of active nuclear p27 protein with low MYC protein level in human breast cancer, and this correlates positively with favourable prognostic markers, relapse-free patient survival and overall patient survival. These results may support immunotherapeutic approaches of targeting MYC-driven tumors in future.

3.3 PAPER III

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

This project was aimed at identifying F-box genes involved in cell proliferation that may have a role in tumor growth. Two screens were employed, the first using an siRNA library targeting all human F-box genes and the second was on the whole genome, which included 53 F-box genes. In both screens, FBXO28, a novel uncharacterized F-box protein, is among the top candidate that, upon knock-down, led to a highly significant reduction in cell proliferation in several tumor-derived cell lines.

Microarray expression analyses showed that FBXO28 depletion resulted in significant downregulation of genes involved in important biological processes including rRNA processing, ribosome biogenesis, cell cycle and metabolism. These set of processes are reminiscent of those regulated by MYC. Further Gene Set Enrichment Analysis (GSEA) confirmed that MYC target genes were downregulated upon FBXO28 depletion and this was confirmed by qRTPCR. When FBXO28 and MYC were both knocked down at the same time, the proliferation rate was similar to the single knock down of FBXO28. This indicated that MYC and FBXO28 are possibly involved in similar pathways, and hence we proceeded to characterize FBXO28 and elucidate the relationship between FBXO28 and MYC.

Mass spectrometric analysis of purified FBXO28 showed serine 344 (S344) to be a specific phosphorylation site of the protein. Phosphorylation stabilized the protein and localized it to the nucleus. We also found that cyclin A-CDK2 and cyclin B-CDK1 phosphorylate this site, but not cyclin E-CDK2, and that phosphorylation of FBXO28 was at the maximum at S-G2/M phase and minimum at early G1 phase.

We discovered that FBXO28 forms a SCF^{FBXO28} ubiquitin ligase with SKP1 and CUL1, independently of phosphorylation status. Phosphorylation of the protein at serine 344 enables the ubiquitin ligase complex to target MYC for ubiquitylation. Further, we also showed that the phospho-mimetic form of FBXO28, S344E-FBXO28, is able to ubiquitylate MYC, but not the phospho-deficient S344A-FBXO28. However, cycloheximide chase experiments indicated that MYC was not degraded upon ubiquitylation by FBXO28.

Interaction mapping analysis indicated that FBXO28 binds MYC at the MYC Box II (MBII) and possibly the helix-loop-helix leucine zipper (HLH-LZ) domain of MYC. By using in situ Proximity Ligation Assay (isPLA), we also showed that FBXO28 co-localizes with MYC endogenously in the nucleus, and by chromatin immunoprecipitation assays (ChIP), we showed that it binds with MYC in the E-box region. Thus FBXO28 interacts with and binds MYC at the promoters. We also found that upon ubiquitylation by SCF^{FBXO28} the cofactor p300 was recruited to MYC target gene promoters.

Knock down of FBXO28 was found to attenuate MYC ubiquitylation. We also found that an F-box deletion mutant, ΔF-FBXO28, can bind MYC, but as it cannot form the SCF complex, ΔF-FBXO28 failed to ubiquitylate MYC. This suggests that ΔF-FBXO28 acts in a dominant negative way. Interestingly, ΔF-FBXO28 overexpression also reduced the interaction of p300 and histone H4 acetylation at MYC target gene promoters, though MYC-MAX binding was not significantly affected. Unsurprisingly, overexpression of ΔF-FBXO28 or FBXO28 knock down led to reduced MYC-dependent luciferase reporter gene activity. Taken together, this indicated that FBXO28 binds and ubiquitylates MYC in a non-proteolytic manner and regulate MYC transcriptional activity of genes important in proliferation.

We further investigated the role of FBXO28 in tumorigenesis. Interestingly, expression of ΔF-FBXO28 or FBXO28 knock down led to reduction in both colony growth in 2D cultures, and MYC-induced transformation in a 3D soft agar assay and tumor growth *in vivo* in an immunodeficient mouse tumor model system. When expressing mutant FBXO28 that is incapable of being phosphorylated by CDK1/2 (S344A-FBXO28), the growth of tumor cells slowed down and the ability of MYC to transform p53-/- MEFs was strikingly reduced. This suggests that FBXO28 activates MYC in a CDK1/2-dependant manner, possibly in the cell cycle.

Next, we searched through the GeneSapiens System transcriptomics database (www. genesapiens.org) and the Oncomine database, and found that FBXO28 is highly expressed in various tumor types, such as breast cancer. We further investigated FBXO28 expression in gene expression data of 327 primary breast tumor specimens, and found correlation between expression of FBXO28 with more than 100 genes, most of which are positive correlation when analyzed through ENCODE (http://genome.ucsc.edu/ENCODE/ analyses). There is also a significantly high representation of MYC and p300 association at the promoters.

In order to establish a possible clinical significance of FBXO28 in human breast cancer, we analyzed a panel of 144 primary breast cancers for FBXO28 phosphorylation using immunohistochemistry on tissue microarray (TMA). We found that most of the tumors showed high nuclear intensity, though there was a significant difference in the nuclear fraction (NF) of pS344-FBXO28 between the different tumors. Interestingly, we found a correlation between a high NF of pS344-FBXO28 in samples with other markers of poor patient outcome, such as tumor size, high grade and estrogen receptor (ER) -negative status. Multivariate analysis also indicated that FBXO28 expression and phosphorylation could independently predict poor survival.

In summary, we have identified a novel F-box protein, FBXO28, which is a substrate of CDK1/2 and tightly regulated during cell cycle progression. FBXO28 assembles an SCF^{FBXO28} ubiquitin ligase and, upon phosphorylation, ubiquitylates MYC, thereby enabling MYC to recruit p300 to promoters of MYC target genes and activate MYC transcription of genes important for proliferation. Silencing or aberrant expression of FBXO28 leads to attenuation of MYC-driven transcription, proliferation, transformation and tumor growth. We

also found that phosphorylated FBXO28 is an independent prognostic marker of poor overall survival in breast cancer patients. Hence, the CDK-FBXO28-MYC axis may be a potential target for drug discovery for MYC-driven cancers, particularly breast cancer.

3.4 PAPER IV

A selective high affinity MYC-binding compound inhibits MYC:MAX interaction and MYC-dependent tumor cell proliferation

MYC is involved in many important cell processes and is a key player in tumorigenesis. However, to date, no specific anti-MYC drugs have reached clinical development. Previous efforts targeting other drivers like EGFR and BRAF have led to resistance via activation of redundant pathways, which all activate MYC. It is therefore important to target MYC. The best strategy would be to target MYC directly since there is a risk of escape with indirect targeting (Castell and Larsson, 2015; McKeown and Bradner, 2014; Whitfield et al., 2017). One Achilles' heel of MYC is its dependency on interacting with MAX protein for its transcriptional function, and hence tumorigenesis (Meyer and Penn, 2008). Thus, targeting this interaction is a plausible way of crippling MYC function. Attempts by others to target other protein-protein interactions had proven to be fruitful and are now in clinical trials but yet, there had been limited success in targeting MYC:MAX interaction, namely due to low potency, specificity or bioavailability (Fletcher and Prochownik, 2015; McKeown and Bradner, 2014; Prochownik and Vogt, 2010; Whitfield et al., 2017).

In this project, we used a cell-based Bimolecular Fluorescence Complementation (BiFC) assay (Kerppola, 2006) in a protein interaction screen that is specifically designed to identify small molecules that inhibit MYC:MAX interaction. The main advantage of this assay is its use of life cells, and hence it already screens out small molecules that are not able to enter cells or that are generally cytotoxic. From a diversity set library of NCI/DTP Open Chemical Repository (http://dtp.cancer.gov), we screened 1990 compounds at 25µM concentration and identified six molecules for further investigation that we termed MYCMIs (**MYC**:**M**AX **I**nhibitors): MYCMI 2, 6, 7, 9, 11 and 14. These compounds reduced the BiFC/CFP readout by more than 40%, normalized to DMSO (vehicle), the most potent being MYCMI 6 and 7, and do not affect the control pair of bZip transcription factors, namely FOS and JUN.

These hits were then validated using other techniques. Split Gaussia luciferase (GLuc) assay (Remy and Michnick, 2006) is a high/mid-throughput method used to validate that the inhibitory effects are on MYC:MAX, and not the fluorescence molecule in BiFC itself or other bZip transcription factors (GCN4 homodimer was used for this control). By western blot analysis, we also sieved out compounds that reduced MYC protein expression (MYCMI 7 and 9), as that was not the mechanism of action we were looking for in this case. Our aim is to identify compounds that only inhibit the interaction between MYC and MAX, without affecting MYC protein levels or other MYC activities. This is due to the experience of others who attempted to target MYC indirectly via different pathways that regulate MYC, but have met with limited success as there are multiple escape routes for the tumors through other

redundant pathways (Castell and Larsson, 2015; McKeown and Bradner, 2014; Whitfield et al., 2017). Thus, from this point, we focused on three hits, MYCMI 6, 11 and 14.

We next applied *in situ* proximity ligation assay (isPLA) to confirm that the selected compounds inhibits endogenous MYC:MAX interactions in cells (Soderberg et al., 2006). The readout is nuclear fluorescent dots, and is evaluated by fluorescence microscopy. We also performed a titration and found that compound MYCMI-6 has an IC_{50} of less than 1.5 μM and MYCMI 11 and 14 has IC_{50} of about 6 μM for MYC:MAX inhibition. Furthermore, MYCMI-6 affected endogenous MYC:MAX interaction already 3 hours after the treatment, as shown by coimmunoprecipitation. All the three compounds shows the selectivity for MYC:MAX interaction as they do not affect other bZip protein partners FRA1:JUN and MAX:MXD1(MAD1). Investigation on the effect of these compounds on transcription of MYC target genes ODC1, RSG16 and CR2 revealed that MYCMI-6 reduced the expression of all these genes significantly, while MYCMI 11 and 14 only significantly reduced RSG16 expression. Taken together, these results revealed MYCMI-6 to be the most selective and potent inhibitor of MYC:MAX interactions and MYC-driven transcription *in vivo*.

Further, we evaluated MYCMI-6 in *in vitro* assays based on recombinant MYC and MAX proteins, microscale thermophosis (MST) and surface plasmon resonance (SPR) (manuscript in prep.). In MST assay, a shift in the reading compared to DMSO indicates an effect on the conformation of the protein pair. We found MYCMI-6 to cause a thermophoresis shift with a K_d of 4.3 +/– 2.9 μM in a mixture with MYC and labeled MAX, but causing minimal change in a labeled MAX:MAX mixture. This indicates that MYCMI-6 differentially inhibits MYC:MAX interactions.

SPR analysis can determine the affinity between protein and ligand, and measure the interaction kinetics with high sensitivity. In this analysis, MAXbHLHZip that was covalently bound to the sensor chip, and MYCMI-6 premixed with MYCbHLHZip was added. MYCMI-6 inhibited MYCbHLHZip from binding to MAXbHLHZip with an IC50 of 3.8 +/– 1.2 μM, which is more efficient than that of other experimental MYC inhibitors 10058-F4 (Yin et al., 2003) and KJ-Pyr-9 (Hart et al., 2014) we tested here. Further investigations indicated that MYCMI-6 binds the bHLHZip domain of Myc directly, and more efficiently than other MYC:MAX inhibitors 10074-G5, #474 (10058-F4 analouge) (Wang et al., 2007c) and KJ-Pyr-9. MYCMI-6 binding to MAX, MXD1 (MAD1), p53, BSA, YFP or BCL-X_L is negligible or minimal. All these results point to MYCMI-6 being a direct, selective and potent inhibitor of MYC protein.

Next, we tested if MYCMI 6 (6.25 μM), 11 (25 μM) and 14 (25 μM), with 10058-F4 (64 μM) as reference, would inhibit MYCN-driven tumor growth in neuroblastoma cell lines with or without *MYCN*-amplification. The growth of *MYCN*-amplified cell lines were more significantly reduced than that of the *MYCN*-non-amplified cell lines by all four compounds. Titration of MYCMI-6 gives an average growth inhibition (GI_{50}) values of 2.5-6 μM for the former and 20 μM or higher for the latter group of cell lines. In a cell line where MYCN or MYC was hardly detectable (SK-N-F1), there was essentially no response with MYCMI-6.

Further, anchorage-independent growth of *MYCN*-amplified neuroblastoma cell lines were inhibited by MYCMI 6, 11 and 14 with GI_{50} values of less than 0.4, 5 and 0.75 µM, respectively. Altogether, these results showed that MYCMI-6 is more potent and selective among the MYCMIs and is selected to be the focus of the rest of our study.

Taking into account the MYC mRNA/protein levels of different cell lines, we analyzed available data on the growth inhibitory effects of MYCMI-6 from the NCI-60 diverse human tumor cell line panel. We found that MYCMI-6 has a significantly higher growth inhibitory effect in human cancer cell lines that have higher MYC mRNA/protein expression, although there was no significance based on *MYC* mRNA data alone. We tested the MYC dependence further using Tgr1 rat fibroblast (parental cells), and its *MYC*-null (H015.19) and *MYC*-reconstituted variants (H0Myc3), and found that, similarly, MYCMI-6 treatment has a MYC-dependent effect. In addition, comparison between the effects of MYCMI-6 on *MYCN*-amplified SK-N-DZ neuroblastoma and normal lung (IMR-90) and foreskin (BJ) human fibroblasts showed that the compound can be well tolerated by normal human cells at the same concentration (12.5 µM) that was highly toxic to a tumor cell with high MYC. Thus, this indicates that MYCMI-6 functions in a MYC-dependent manner and has a good therapeutic window.

To evaluate the tumor physiopathology effects of MYCMI-6 *in vivo*, we used a mouse xenograft tumor model where we injected *MYCN*-amplified SK-N-DZ neuroblastoma cells into the flanks of athymic nude mice, and after tumor formation MYCMI-6 or vehicle were administered. 1-2 weeks later the mice were sacrificed and evaluated for several parameters. We found that there is reduction in MYCN:MAX interaction (by isPLA), tumor cell proliferation (by Ki67 staining) and microvascular density (MVD) (by CD31 staining of endothelial cells) in MYCMI-6-treated mouse compared to vehicle treatment. On the other hand, there is increase in apoptosis (by TUNEL staining), signs of necrosis, hemorrhage and scar tissue formation in the compound-treated cells, with only a slight and temporary effect on body weight, indicating that the side effects are well-tolerable.

Taken together, we have found a small molecule, MYCMI-6 that inhibits MYC/MYCN:MAX interactions in human and rat cells, *in vitro* and *in vivo* (in mouse tumor model), and reduced tumor growth or kill tumor cells in a MYC-dependent manner, without severe side effects.

4 SUMMARY AND CONCLUSIONS

MYC is a master regulator of transcription but its function and regulation is dependent on interaction with other proteins and it also works in cooperation with other oncogenes. Overexpression of MYC alone leads to apoptosis but not to tumor development. Nonetheless, deregulated expression of MYC plays an important role in tumorigenesis. It has even been suggested that a low level of MYC expression contributes more to tumorigenesis since a higher level of overexpression engages the apoptotic pathway (Murphy et al., 2008). However, by cooperating with other oncogenes like RAS, accompanied by deregulation of tumor suppressor pathways, high levels of MYC can be tolerated and leads to aggressive tumor progression. MYC-driven tumors are often strongly correlated with poor prognosis.

Inactivation of MYC had been shown to lead to tumor regression with well-tolerated side effects (Soucek et al., 2008), often accompanied by senescence (Wu et al., 2007) or apoptosis (Soucek et al., 2008). This suggests that MYC is a potential and suitable target for anti-cancer therapy. MYC itself is very difficult to target as it is unstructured and does not have an enzymatic activity. MYC:MAX interaction is known to be important for the DNA binding function of MYC, however, targeting this protein-protein interaction is a challenge because of the lack of any enzymatic active site and the intrinsic disordered nature of the proteins. The purpose of this thesis was to elucidate the pathway of interaction between overexpressed MYC and activated RAS in normal human fibroblasts, and determine if they interact the same way in these cell as in their rodent counterpart. We also wanted to identify new MYC cofactors and dissect the mechanism by which they interact and may contribute to tumorigenesis. Finally, our aim was to also find new strategies to target MYC by identifying potent and selective inhibitors of the MYC:MAX interaction.

In Paper I, we showed that oncogenic MYC and RAS do not cooperate in normal human fibroblasts as they do in rat embryonic fibroblasts, to cancel out each other's intrinsic anti-tumorigenic barrier, namely apoptosis and senescence, even in the absence of tumor suppressor p53. Hence, additional mutations are required for the development of MYC- and RAS-driven tumors in human, and to override the intrinsic tumor suppressor pathways of these oncogenes. This sheds light on how oncogenic transformation proceeds in human cells.

In Paper II, we discovered a new pathway and interactors that regulate MYC turnover. We found that p27 targets Myc both indirectly through CDK2 and directly by binding MYC, resulting in MYC degradation, removal of MYC binding to target gene promoters. This resulted in lowered expression of MYC target genes, and senescence and differentiation of MYC-driven tumor cells. Since p27 can be stimulated by growth inhibitory cytokines, such as IFN-ɣ, we also investigated if IFN-ɣ treatment leads to the degradation of MYC, and it does, via the upregulation of p27. Interestingly, we found that this degradation process occurs via an E3 ligase that is not currently known in the MYC regulation repertoire. It would be of future scientific interest to identify this E3 ligase. We have also found that there is significant

clinical relevance between high activity of nuclear p27 levels and low MYC expression in tumor samples, and this correlates with a good prognosis and a positive clinical outcome. Thus a new strategy to target MYC-driven tumors is by finding ways to upregulate p27 expression and activity. One of the possible ways is by utilizing IFN-ɣ, which is one of the cytokines produced by activated T-lymphocytes. This may provide support to develop immunotherapy methods to combat MYC-driven tumors, in particular enhancing IFN-ɣ-producing T-cells, as a complementary treatment along with molecular therapies targeting CDK2 or signaling pathways that enhances p27 expression/activity.

In Paper III, we uncovered a novel F-box protein, FBXO28, that ubiquitylates MYC in a non-proteolytic manner, and enhances MYC transcriptional activity and downstream pathways. SCF^{FBXO28} is phosphorylated by CDK1/2 during the cell cycle, which regulates its activity and stability, and is required for its efficient ubiquitylation of MYC. When FBXO28 is depleted or a dominant negative F-box mutant is overexpressed, it cannot support MYC ubiquitylation, and results in reduction of MYC-driven transcription, transformation and tumorigenesis. We also found that high MYC expression coupled with high FBXO28 expression and phosphorylation are strong and independent predictors of poor prognosis in human breast cancer. In conclusion, our data suggest that the CDK-FBXO28-MYC axis is a potential molecular drug target in MYC-driven cancers, including breast cancer.

In Paper IV, despite the difficulty in targeting protein-protein interactions, especially that of unstructured proteins such as MYC, we found a small molecule that binds MYC and inhibits MYC:MAX interaction. We found MYCMI-6 to inhibit MYC/MYCN:MAX interactions in human and rat cells, *in vitro* and *in vivo* (in mouse tumor model). Further, this compound impeded tumor growth and killed tumor cells in a MYC-dependent manner, both in cells and *in vivo*. Importantly, this is achieved with specificity to tumor cells expressing high levels of MYC, high potency (low concentration needed), a good therapeutic window and without severe side effects. Apart from being a prototype and proof of principle of protein-protein targeting, we discovered MYCMI-6 as a unique molecular tool to target MYC:MAX pharmacologically with high specificity and efficacy, and it is a good candidate for drug development.

Altogether, the projects involved in this thesis provide insights into molecular pathways involved in MYC oncogenic activity, regulation, and transcription functions. We identified a difference in pathways of cooperation between oncogenic MYC and RAS in rats and human (Paper I), found new cooperating protein partners and possible therapeutic targets (Paper II and III), and provided proof of principle/ concept of targeting non-enzymatic protein-protein interaction (Paper IV). This will be of importance not only to increase the basic knowledge on mechanisms through which MYC contributes to tumor development, but will hopefully also contribute to the development of new therapeutic strategies to combat MYC-driven cancer in the future.

5 ACKNOWLEDGEMENTS

The work in this thesis was performed at the department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet. I would like to thank the various organizations that provided financial support for the research and conferences, especially KID-funding, KI Foundations & Funds, Robert Lundberg Memory Foundation, Konung Gustaf V:s Jubileumsfond (Radiumhemmets Forskningsfonder), Barncancerfonden and Lembaga Biasiswa Kenangan Maulud (LBKM) Singapura.

PhD is not just a qualification destination. It is a journey, and one that I would not have completed without the support from so many people around me.

Lars-Gunnar Larsson, thank you from the bottom of my heart for accepting me to learn some techniques in your lab at first, then to applying for the KID-funding together and finally for this whole PhD journey till the end. We did it! I am sincerely grateful for your patience and kindness, and tough measures when called for. You have taught me so much in science, with your expertise and experiences. It has been challenging for me to be in a foreign country across the continent for this journey, alone at first till having kids now, but if I have to choose all over again, I would not have chosen any differently, albeit with much better preparation and faster ending! 😊 As a saying goes, hindsight is always 20/20.

Alina Castell, I really would like to thank you for guiding and teaching me a lot. There are a lot of takeaways I have gotten from you on good general research principals and lab safety, especially when I became pregnant the first time! And thank you for doing your best to assist in my projects, especially the senescence project, despite feeling it was not within your expertise then. All of us learnt a lot together during the journey, didn’t we? Your kindness, enthusiasm and passion for research motivate me a lot, and I admire you pushing through all the challenges (especially during your pregnancy and later on!). You rock! ;)

Galina Selivanova, thank you for accepting to be my co-supervisor. I am amazed with your work on p53 and am so glad that we are able to tap your expertise and work together in the BJ paper. We did not get to meet much but I really appreciate your time and insights, when we did. I always appreciate our small talks and smiles when we bump into each other in the hallways, coffee room and even Petite France! Your smile is so heart-warming. Thank you!

Sven Pettersson, you are my first link to Sweden and KI when we met in Singapore. I am sincerely grateful to you for the interview, brainstorming the opportunities that might suit my interest and later opening the doors to KI for me. Had you not recommended Lars-Gunnar and be my reference, my PhD (and life!) journey would have probably taken a different turn. You have also been a great mentor. I remember some of our more serious discussions and am very grateful for your very real advices.

Ingemar Ernberg, thank you for agreeing to be my unofficial mentor, when I am nearing the end of my PhD and pondering about my long-time interest in alternative and complementary medicine. You also do such a great job organizing all the interesting and mind-opening courses, seminars and discussions! Thank you!

Eva Klein, no words can describe how grateful I am to have met you. You became my friend and then the closest I have to a family in Sweden. You are my rock during the storm. I always thank God for letting our path crossed. We talked about everything, from our families and scientific work to religion and the Holocaust. I would like you to know that you have a permanent place in my and my family's hearts and minds. Late Georg (God bless his soul) once thank me for being your friend, but really, I am the one who should thank you. You raised me up from my lowest point. Both of you and George are the epitome of what a scientist should be and are inspirations to me and I believe every researcher who knows you!

I really want to thank all the current and former members of **LGL's group** for the great group atmosphere and camaraderie. The fika is always so interesting with all of your stories and more so when someone brings home-baked goodies ☺ **Per**, **Karin**, **Helén**, **Sanna**, **Hamid**, **Qinzi**, **Eduar** and **Aishe**, thank you for welcoming me into the group when I first came and know nothing about Sweden and the culture. **Karin**, thank you for picking me up at the airport. **Aishe**, I appreciate your help with all the admin and governmental work to make my settling down in Sweden a smoother process. Thank you **Eduar**, **Qinzi**, **Hamid**, **Sara**, and especially **Helén**, **Karin** and **Sanna**, for teaching me techniques, more importantly the tricks, and background to get started in the projects. **Per**, you visited and have been in touch often for the senescence project that it felt you never left the group. **Hamid**, you are always kind to everyone, we all had fun and *beautiful* memories at the dinner at your place and defence party, didn't we? 😊 **Vedrana**, I am indebted for the skills and techniques you taught me for the senescence project, I learnt so much from you. **Fan**, **Marcela** and **Wesam**, I really appreciate working with all of you and for all the help, especially while I was on parental leave (till now!) **Fan**, I am happy you are carrying on the senescence torch and have enjoyed developing the protocols together, thank you for bringing the BJ project I started to completion! **Linda**, **Janina**, **Nyosha**, **Jacob** and **Ara**, although we did not work so long or much together, I appreciate our interactions and all help with techniques or other matters in or outside the lab. It has been a pleasure meeting and getting to know previous members of the LGL group at various occasions and through your still needed contributions to the projects! **Fuad** and **Cihan**, I was amazed that you still remembered how you labeled the IFN-ɣ tubes. **Natalie**, it is always nice to meet you at the seminars you conducted. Students, **Matteo**, **Bas**, **James**, **Najoua**, **Samudra** and **Tamer**, you all add color each in your own way when you are in the group! **Matteo**, there is laughter wherever you are. **Bas**, thank you for being my pillar, even for a while! **Najoua**, I am happy you kept my name as it is, and not change it to something else ;)

I would like to thank the **PIs** and the **past and present members** of:

Marie Arsenian Henriksson's group, especially Ulrica, Diogo, Johanna, Anna F. and Ganna for teaching methods, sharing tips, and at times lending me reagents when I ran out of them in the middle of an experiment. Also to Inga, Hanna, Therese, Karin, Marjon, Jakob, Roel and Nikolay, as well as **Margareta Wilhem** and her group members, Evelyn, Habib, Marina and Ana, thank you all of you for the shared lunches, fika breaks, information exchanges, journal clubs, outside activities and some very interesting conversations!

Sonia Lain's group, Ingeborg, Cath, Marijke, Marcus, Chloe, Tanzina, and Melina, **Mats Wahlgren's "Malaria" group**, Jeap, Hodan, Pilar, Hon, Chim, Sherwin, Zul and Mattias, **Martin Rottenberg's group**, Carina O., Suman and Chaniya, **Cao Yihai's group**, Samantha, Sharon, Lasse, Patrik, Carina F, Oliver, Ceylan and Kayoko, **Rolf Ohlsson's group**, Samer, Alejandro, Chengxi, Nori, Farzaneh, Marta, Xingqi, Moumita, I just love the camaraderie in your groups. Thank you for sharing with me a piece of it and letting me join in some of your group events, dinners or outings, and just being so nice to me all the time! Samer, I am so grateful that you are there when I needed in-house expertise and reagents for isPLA. Rolf and Anita, thank you very much for letting me use your group's massage chair :D

Georg and Eva's group, Noemi, Harsha, Eahsan, Tatiana and Hayrettin, **Laszlo Szekely's group**, Emilie, Orsi and Andrei, **Pontus group**, Vishal, friends from **CMB**, Tiago and Shahul, **Ola Söderberg** from Uppsala University: I sincerely appreciate you for teaching me methods, sharing reagents and discussing my projects, as well as lunches, smiles, friendly exchanges everytime we meet and also activities and friendship outside lab with some of you!

Many thanks to **Olle Sangfelt's group**, Diana and Charis, and **Galina Selivanova's group**, Madhurendra, for working together and discussing our co-authored paper. Thank you also to **all collaborators** for sharing your expertise, we all made it happen together!

A big thank you also goes to everyone in the **MTC administration and service group**, in particular, Studierektor Gesan, past and present administrators for PhD students, Anita, Anna, Åsa and Lena, Helen, Kristina and John, Alex, Torbjörn, Magnus, David, and Per. Thank you for your kind and helpful assistance, and for keeping the department running.

To all the committee members of the **MTC student Association (MSA)** during the time I was serving, Samer, Maria Lisa, Arnika, Soazig, Paola, Agata, Habib, Marijke, Sunita, Afrouz and Carina F. It was great my pleasure working with all of you on issues to improve our student conditions and all the cool networking activities!

Administrators in Career service, **Annette**, and International Researcher Unit, **Ulla Tunkara**, Thank you for all the discussions and support! **Library Services**, Thank you for all the great workshops and seminars! Everyone should attend these to learn to manage better all the other aspects of research that is not the research itself! ☺

Boris Zhivotovsky, **Beston** Nore, **Klas** Wiman, and all the PIs and researchers aforementioned, thank you for all the great seminars, courses and discussions!

Everyone that I worked with in Medicinska Föreningen, particularly the International Committee, Social Dance@KI, Climbing@KI and Global Friends, and MenDansa, especially Ferdi, Astrid, Kai, Elisa, Lisa, Ester, Johan, Jorge, Jörgen, Annie, Fredrika, Shervin, Kalle, Amanda, Marie, Monika, Nadilly, Cynthia, Dan, Solmaz, Rubin, Devy, Annisa, Martin, I really appreciate the experience working together and also the fun (after the hard work!) we have together. Anja, Inge, Lynsky, Daniela, Liana, João, Gaëlle, Patrik, Carina and Tony, Thomas, Mikko, Martin, Shu Mei, Hodan and Su, thank you for all the great climbing sessions and facilitating! These are my outlets to keep sane through the failed experiments ;)

Rozina, Lotta, Liudmila, Santtu and Sofia, Sabrina and Florian, Mattias and Noomi, Elodie, Him, Miriam and Andreas, Sini, Anna V and Erno, Emma, Qiang and Mei, Anuj, Suman, Damian, Michalina, Sadia, Changhan, Li-Sophie, Franziska, Srisuda, Joanna Packau, Marc, Petrykk, Mustafa, Robban, Krissi, Ben and Gerry, and the NIH people, Lynsky (Kelly), Stal (Saurav), Tracey, Miriam and Ray, I feel so privileged to have met and gotten to know all of you and some of your families too.

There are so many more people I have met in and outside of MTC and KI that have made my PhD journey better, more colorful and memorable. I can't thank you all enough for accepting me into your various inner circles, discussing life and science or fika and activities! I am sorry I can't name everyone but please know that I appreciate you!

Pak Wan and Dr Yaacob Ibrahim, thank you for all the support from undergraduate till now.

SG/MY friends, esp Nurzian and Andreas, Faezzah, Samantha, Rachel, Jingmei, Xiaohui, Yinghui, Delina, Farhana, Sharon, Jinny and Sam, Alvin, Lena's family, Diane, Michelle, Aida's Family, Normah's family, Justina, the rest of the Makcik group (Hana, Didi, Tina, Yaty, Nadia), thank you for welcoming me to your homes and events. Of course, the Se Lovelies in SG group (all of you!) for all the online laughs and keeping me company online during the final lapse of my PhD. The chat spam is real! I would also like to thank my friends in Singapore, particularly the Al Widad, NUS study group, Adel and Asyikin, Aida R, Ryan and Lee Lee, and Sisters in Ukhwah, Thank you for always keeping base with me on my trips home. Even though we have been far in distance, we are always close at heart.

People whom I have shared accommodation with or who passed over accommodation to me, Samantha, Hien, Thu, Rachel, Elisabeth R, Olga, Sofia, and Gera. It was really a pleasure getting to know all of you.

Everyone who helped and supported during my thesis writing, particularly Lars-Gunnar, Adam Quek, Nur Ridayu Bates, Marijke, Samer, Aqbar, Noemi, Fan, Hamid, Latifahaida, teachers and staff at Imagination International Preschool Solna and also Pia and staff from E-print. Thank you so so much for helping me make this thesis print a reality.

To my family,

The ones who always support me throughout this whole journey (and my whole life!) I am so grateful to have two sets of parents who are always supporting my endeavors and being there to give advises, Rose Abdullah and Razak Salleh, Zakaria Daud and Asmah Hussin. Especially to my mom, Rose, for always being there for me, coming at the drop of a pin to wherever you are needed, in Singapore, Malaysia and even Sweden, cheering me on and inspiring me to brave through all odds. My siblings are my partners in crime for having fun or for having arguments just for the sake of it sometimes. Abang Taufiq, Hidhir, Aqbar, Yusran and Tsuraiya, thank you for tolerating my antics and giving help when needed (which is very often) especially now with the kids! :D

To my husband, Zakaria Ahmad, thank you for your patience and your love. You came (back) into my life exactly when I needed you. Thank you for the journey we went through together and the sacrifices you made for me to finish up the PhD. Our beautiful kids, Zulaikha and Maisarah, you are the light of my life. You can drive me up the wall one moment and then in the next moment make the world the most beautiful place. Be kind and loving to each other, as you always have been, and take care of the third little one coming <3

To the extended families and in-laws on all sides, thank you for being a great big family. It is always wonderful to be around the family be it at the (not so) quiet meals to the big occasions.

Special acknowledgement for my late sister Hidayah. You were gone too soon. You were the one who taught me to be adventurous, taking me along to camping trips. You were so intelligent, creative and energetic, and I always looked up to you, not to mention you always have such nice things that I loved borrowing, sometimes behind your back. I missed you and wished we could have still been challenging each other to achieve in life. You will always be in our hearts and we miss you.

I can’t express enough how much I appreciate all my family members. I love you all and this thesis is dedicated to you.

6 REFERENCES

(2012). Comprehensive molecular portraits of human breast tumours. Nature *490*, 61-70.

Aaronson, D.S., and Horvath, C.M. (2002). A road map for those who don't know JAK-STAT. Science; Washington *296*, 1653-1655.

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533-538.

Adhikary, S., and Eilers, M. (2005). Transcriptional regulation and transformation by Myc proteins. Nature reviews Molecular cell biology *6*, 635-645.

Adhikary, S., Marinoni, F., Hock, A., Hulleman, E., Popov, N., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., *et al.* (2005a). The Ubiquitin Ligase HectH9 Regulates Transcriptional Activation by Myc and Is Essential for Tumor Cell Proliferation. Cell *123*, 409-421.

Adhikary, S., Marinoni, F., Hock, A., Hulleman, E., Popov, N., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., *et al.* (2005b). The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell *123*, 409-421.

Ahn, S.H., Kim, M., and Buratowski, S. (2004). Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. Molecular cell *13*, 67-76.

Alderton, G.K. (2011). Targeting MYC? You BET. Nature reviews Drug discovery *10*, 732-733.

Alitalo, K., Schwab, M., Lin, C.C., Varmus, H.E., and Bishop, J.M. (1983). Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proceedings of the National Academy of Sciences of the United States of America *80*, 1707-1711.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). ACETYLATION AND METHYLATION OF HISTONES AND THEIR POSSIBLE ROLE IN THE REGULATION OF RNA SYNTHESIS. Proceedings of the National Academy of Sciences of the United States of America *51*, 786-794.

Amati, B., Alevizopoulos, K., and Vlach, J. (1998). Myc and the cell cycle. Front Biosci *3*, d250-268.

Anand, P., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B., and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. Pharmaceutical research *25*, 2097-2116.

Apostolou, P., and Fostira, F. (2013). Hereditary breast cancer: the era of new susceptibility genes. BioMed research international *2013*, 747318.

Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fatyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I., *et al.* (2005). c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nature cell biology *7*, 303-310.

Ardley, H.C., and Robinson, P.A. (2005). E3 ubiquitin ligases. Essays Biochem *41*, 15.

Arellano, M., and Moreno, S. (1997). Regulation of CDK/cyclin complexes during the cell cycle. Int J Biochem Cell Biol *29*, 559-573.

Arkin, M.R., Tang, Y., and Wells, J.A. (2014). Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. Chemistry & biology *21*, 1102-1114.

Arvanitis, C., and Felsher, D.W. (2006). Conditional transgenic models define how MYC initiates and maintains tumorigenesis. Semin Cancer Biol *16*, 313-317.

Bahram, F., Hydbring, P., Tronnersjo, S., Zakaria, S.M., Frings, O., Fahlen, S., Nilsson, H., Goodwin, J., von der Lehr, N., Su, Y., *et al.* (2016). Interferon-gamma-induced p27KIP1 binds to and targets MYC for proteasome-mediated degradation. Oncotarget *7*, 2837-2854.

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

Ballestar, E., and Wolffe, A.P. (2001). Methyl-CpG-binding proteins. Targeting specific gene repression. European journal of biochemistry *268*, 1-6.

Baudino, T.A., McKay, C., Pendeville-Samain, H., Nilsson, J.A., Maclean, K.H., White, E.L., Davis, A.C., Ihle, J.N., and Cleveland, J.L. (2002). c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes & development *16*, 2530-2543.

Ben-Saadon, R., Zaaroor, D., Ziv, T., and Ciechanover, A. (2006). The Polycomb Protein Ring1B Generates Self Atypical Mixed Ubiquitin Chains Required for Its In Vitro Histone H2A Ligase Activity. Molecular cell *24*, 701-711.

Bertozzi, N., Pesce, M., Santi, P.L., and Raposio, E. (2017). Oncoplastic breast surgery: comprehensive review. European review for medical and pharmacological sciences *21*, 2572-2585.

Boehm, U., Klamp, T., Groot, M., and Howard, J.C. (1997). CELLULAR RESPONSES TO INTERFERON-γ. Annual Review of Immunology *15*, 749-795.

Borek, C. (1993). Molecular mechanisms in cancer induction and prevention. Environmental health perspectives *101 Suppl 3*, 237-245.

Bouchard, C., Marquardt, J., Brás, A., Medema, R.H., and Eilers, M. (2004). Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. The EMBO journal *23*, 2830.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. The EMBO journal *18*, 5321-5333.

Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E., and Bishop, J.M. (1984). Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science (New York, NY) *224*, 1121-1124.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell *84*, 843-851.

Caley, A., and Jones, R. (2012). The principles of cancer treatment by chemotherapy. Surgery (Oxford) *30*, 186-190.

Campaner, S., Doni, M., Hydbring, P., Verrecchia, A., Bianchi, L., Sardella, D., Schleker, T., Perna, D., Tronnersjo, S., Murga, M.*, et al.* (2010). Cdk2 suppresses cellular senescence induced by the c-myc oncogene. Nature cell biology *12*, 54-59; sup pp 51-14.

Cantley, L.C. (2002). The Phosphoinositide 3-Kinase Pathway. Science (New York, NY) *296*, 1655.

Castell, A., and Larsson, L.G. (2015). Targeting MYC Translation in Colorectal Cancer. Cancer Discov *5*, 701-703.

Castell, A., Yan, Q., Fawkner, K., Hydbring, P., Zhang, F., Verschut, V., Franco, M., Zakaria, S.M., Bazzar, W., Goodwin, J.*, et al.* (2018). A selective high affinity MYC-binding compound inhibits MYC:MAX interaction and MYC-dependent tumor cell proliferation. Scientific reports *8*, 10064.

Chandramohan, V., Mineva, N.D., Burke, B., Jeay, S., Wu, M., Shen, J., Yang, W., Hann, S.R., and Sonenshein, G.E. (2008). c-Myc represses FOXO3a-mediated transcription of the gene encoding the p27(Kip1) cyclin dependent kinase inhibitor. J Cell Biochem *104*, 2091-2106.

Charron, J., Malynn, B.A., Fisher, P., Stewart, V., Jeannotte, L., Goff, S.P., Robertson, E.J., and Alt, F.W. (1992). Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene. Genes & development *6*, 2248-2257.

Chin, Y.E., Kitagawa, M., Su, W.-C.S., You, Z.-H., Iwamoto, Y., and Fu, X.-Y. (1996). Cell Growth Arrest and Induction of Cyclin-Dependent Kinase Inhibitor p21^{WAF1/CIP1} Mediated by STAT1. Science (New York, NY) *272*, 719.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. Nature Reviews Molecular Cell Biology *6*, 79.

Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. Nature reviews Cancer *2*, 910-917.

Coller, H.A., Grandori, C., Tamayo, P., Colbert, T., Lander, E.S., Eisenman, R.N., and Golub, T.R. (2000). Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proceedings of the National Academy of Sciences of the United States of America *97*, 3260-3265.

Cramer, P., Armache, K.J., Baumli, S., Benkert, S., Brueckner, F., Buchen, C., Damsma, G.E., Dengl, S., Geiger, S.R., Jasiak, A.J.*, et al.* (2008). Structure of Eukaryotic RNA Polymerases. Annu Rev Biophys *37*, 337-352.

Dalla-Favera, R., Wong-Staal, F., and Gallo, R.C. (1982). Onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. Nature *299*, 61-63.

Dang, Chi v. (2012). MYC on the Path to Cancer. Cell *149*, 22-35.

Dang, C.V., O'Donnell, K.A., Zeller, K.I., Nguyen, T., Osthus, R.C., and Li, F. (2006). The c-Myc target gene network. Semin Cancer Biol *16*, 253-264.

Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R., and Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes & development *7*, 671-682.

Decker, T., and Kovarik, P. (2000). Serine phosphorylation of STATs. Oncogene *19*, 2628-2637.

Delgado, D.M., and León, J. (2006). Gene expression regulation and cancer. Clinical and Translational Oncology *8*, 780-787.

Delmore, J.E., Issa, G.C., Lemieux, M.E., Rahl, P.B., Shi, J., Jacobs, H.M., Kastritis, E., Gilpatrick, T., Paranal, R.M., Qi, J.*, et al.* (2011). BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell *146*, 904-917.

DePinho, R.A., Hatton, K.S., Tesfaye, A., Yancopoulos, G.D., and Alt, F.W. (1987). The human myc gene family: structure and activity of L-myc and an L-myc pseudogene. Genes & development *1*, 1311-1326.

Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. Annual review of cell and developmental biology *15*, 435-467.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem *78*, 399-434.

Downward, J. (2003). Role of receptor tyrosine kinases in G-protein-coupled receptor regulation of Ras: transactivation or parallel pathways? Biochem J *376*, e9.

Drayton, S., Rowe, J., Jones, R., Vatcheva, R., Cuthbert-Heavens, D., Marshall, J., Fried, M., and Peters, G. (2003). Tumor suppressor p16INK4a determines sensitivity of human cells to transformation by cooperating cellular oncogenes. Cancer cell *4*, 301-310.

Drooger, J.C., Hooning, M.J., Seynaeve, C.M., Baaijens, M.H., Obdeijn, I.M., Sleijfer, S., and Jager, A. (2015). Diagnostic and therapeutic ionizing radiation and the risk of a first and second primary breast cancer, with special attention for BRCA1 and BRCA2 mutation carriers: a critical review of the literature. Cancer treatment reviews *41*, 187-196.

Dublin, E.A., Patel, N.K., Gillett, C.E., Smith, P., Peters, G., and Barnes, D.M. (1998). Retinoblastoma and p16 proteins in mammary carcinoma: their relationship to cyclin D1 and histopathological parameters. International journal of cancer *79*, 71-75.

Dyson, H.J., and Wright, P.E. (2005). Intrinsically unstructured proteins and their functions. Nature reviews Molecular cell biology *6*, 197-208.

Eilers, M., and Eisenman, R.N. (2008). Myc's broad reach. Genes & development *22*, 2755-2766.

Eischen, C.M., Weber, J.D., Roussel, M.F., Sherr, C.J., and Cleveland, J.L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes & development *13*, 2658-2669.

Elledge, S.J., and Harper, J.W. (1998). The role of protein stability in the cell cycle and cancer. Biochimica et biophysica acta *1377*, M61-70.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicologic pathology *35*, 495-516.

Engelman, J.A., Luo, J., and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nature Reviews Genetics *7*, 606.

Espinosa, J.M. (2010). The meaning of pausing. Molecular cell *40*, 507-508.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119-128.

Evangelou, K., Bartkova, J., Kotsinas, A., Pateras, I.S., Liontos, M., Velimezi, G., Kosar, M., Liloglou, T., Trougakos, I.P., Dyrskjot, L., *et al.* (2013). The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis. Cell Death Differ *20*, 1485-1497.

Fang, S., and Weissman, A.M. (2004). A field guide to ubiquitylation. Cellular and molecular life sciences : CMLS *61*, 1546-1561.

Felsher, D.W., and Bishop, J.M. (1999). Reversible tumorigenesis by MYC in hematopoietic lineages. Molecular cell *4*, 199-207.

Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., *et al.* (2010). Selective inhibition of BET bromodomains. Nature *468*, 1067-1073.

Finocchiaro, R., Hayes, B.J., Siwek, M., Spelman, R.J., Van Kaam, J.B.C.H.M., Ådnøy, T., and Portolano, B. (2008). Comparison of casein haplotypes between two geographically distant European dairy goat breeds. Journal of Animal Breeding and Genetics *125*, 68-72.

Fletcher, S., and Prochownik, E.V. (2014). Small-molecule inhibitors of the Myc oncoprotein. Biochimica et biophysica acta *1849*, 525-543.

Fletcher, S., and Prochownik, E.V. (2015). Small-molecule inhibitors of the Myc oncoprotein. Biochimica et biophysica acta *1849*, 525-543.

Follis, A.V., Hammoudeh, D.I., Wang, H., Prochownik, E.V., and Metallo, S.J. (2008). Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by small molecules. Chemistry & biology *15*, 1149-1155.

Fong, A., and Park, J.R. (2009). High-risk neuroblastoma: a therapy in evolution. Pediatric hematology and oncology *26*, 539-548.

Foulds, L. (1954). The experimental study of tumor progression: a review. Cancer research *14*, 327-339.

Frame, S., and Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. Biochem J *359*, 1.

Gabay, M., Li, Y., and Felsher, D.W. (2014). MYC activation is a hallmark of cancer initiation and maintenance. Cold Spring Harbor perspectives in medicine *4*.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc. Nature *382*, 511-517.

Gleave, M.E., Elhilali, M., Fradet, Y., Davis, I., Venner, P., Saad, F., Klotz, L.H., Moore, M.J., Paton, V., Bajamonde, A., *et al.* (1998). Interferon gamma-1b compared with placebo in metastatic renal-cell carcinoma. The New England Journal of Medicine; Boston *338*, 1265-1271.

Goga, A., Yang, D., Tward, A.D., Morgan, D.O., and Bishop, J.M. (2007). Inhibition of CDK1 as a potential therapy for tumors over-expressing MYC. Nature medicine *13*, 820-827.

Grandori, C., Wu, K.J., Fernandez, P., Ngouenet, C., Grim, J., Clurman, B.E., Moser, M.J., Oshima, J., Russell, D.W., Swisshelm, K., *et al.* (2003). Werner syndrome protein limits MYC-induced cellular senescence. Genes & development *17*, 1569-1574.

Greaves, M., and Maley, C.C. (2012). Clonal evolution in cancer. Nature *481*, 306-313.

Green, D.R., and Evan, G.I. (2002). A matter of life and death. Cancer cell *1*, 19-30.

Gregory, M.A., and Hann, S.R. (2000). c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Molecular and cellular biology *20*, 2423-2435.

Gregory, M.A., Qi, Y., and Hann, S.R. (2003). Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. The Journal of biological chemistry *278*, 51606-51612.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hammoudeh, D.I., Follis, A.V., Prochownik, E.V., and Metallo, S.J. (2009). Multiple independent binding sites for small-molecule inhibitors on the oncoprotein c-Myc. Journal of the American Chemical Society *131*, 7390-7401.

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

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hann, S.R. (2006). Role of post-translational modifications in regulating c-Myc proteolysis, transcriptional activity and biological function. Semin Cancer Biol *16*, 288-302.

Hart, J.R., Garner, A.L., Yu, J., Ito, Y., Sun, M., Ueno, L., Rhee, J.K., Baksh, M.M., Stefan, E., Hartl, M.*, et al.* (2014). Inhibitor of MYC identified in a Krohnke pyridine library. Proceedings of the National Academy of Sciences of the United States of America *111*, 12556-12561.

Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K.I. (2001). U box proteins as a new family of ubiquitin-protein ligases. The Journal of biological chemistry *276*, 33111-33120.

Hayward, W.S., Neel, B.G., and Astrin, S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature *290*, 475-480.

Hengartner, C.J., Myer, V.E., Liao, S.M., Wilson, C.J., Koh, S.S., and Young, R.A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Molecular cell *2*, 43-53.

Henriksson, M., Bakardjiev, A., Klein, G., and Luscher, B. (1993). Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. Oncogene *8*, 3199-3209.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F.*, et al.* (2000). Identification of CDK4 as a target of c-MYC. Proceedings of the National Academy of Sciences of the United States of America *97*, 2229-2234.

Holohan, C., Van Schaeybroeck, S., Longley, D.B., and Johnston, P.G. (2013). Cancer drug resistance: an evolving paradigm. Nature reviews Cancer *13*, 714-726.

Hu, X., Herrero, C., Li, W.-P., Antoniv, T.T., Falck-Pedersen, E., Koch, A.E., Woods, J.M., Haines, G.K., and Ivashkiv, L.B. (2002). Sensitization of IFN-? Jak-STAT signaling during macrophage activation. Nature Immunology *3*, 859.

Huot, T.J., Rowe, J., Harland, M., Drayton, S., Brookes, S., Gooptu, C., Purkis, P., Fried, M., Bataille, V., Hara, E., *et al.* (2002). Biallelic mutations in p16(INK4a) confer resistance to Ras- and Ets-induced senescence in human diploid fibroblasts. Molecular and cellular biology *22*, 8135-8143.

Hydbring, P., Bahram, F., Su, Y., Tronnersjo, S., Hogstrand, K., von der Lehr, N., Sharifi, H.R., Lilischkis, R., Hein, N., Wu, S., *et al.* (2010). Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation. Proceedings of the National Academy of Sciences of the United States of America *107*, 58-63.

Hydbring, P., Castell, A., and Larsson, L.G. (2017). MYC Modulation around the CDK2/p27/SKP2 Axis. Genes *8*.

Hydbring, P., Malumbres, M., and Sicinski, P. (2016). Non-canonical functions of cell cycle cyclins and cyclin-dependent kinases. Nature Reviews Molecular Cell Biology *17*, 280.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. EMBO reports *9*, 536-542.

Isobe, T., Hattori, T., Kitagawa, K., Uchida, C., Kotake, Y., Kosugi, I., Oda, T., and Kitagawa, M. (2009). Adenovirus E1A inhibits SCF(Fbw7) ubiquitin ligase. The Journal of biological chemistry *284*, 27766-27779.

Jackson, P.K., and Eldridge, A.G. (2002). The SCF ubiquitin ligase: an extended look. Molecular cell *9*, 923-925.

Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., Sundberg, C.D., Bishop, J.M., and Felsher, D.W. (2002). Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science (New York, NY) *297*, 102-104.

Jakopovic, M., Thomas, A., Balasubramaniam, S., Schrump, D., Giaccone, G., and Bates, S.E. (2013). Targeting the epigenome in lung cancer: expanding approaches to epigenetic therapy. Frontiers in oncology *3*, 261.

Juan, J., Muraguchi, T., Iezza, G., Sears, R.C., and McMahon, M. (2014). Diminished WNT -> beta-catenin -> c-MYC signaling is a barrier for malignant progression of BRAFV600E-induced lung tumors. Genes & development *28*, 561-575.

Kalvakolanu, D.V. (2003). Alternate interferon signaling pathways. Pharmacology & Therapeutics *100*, 1-29.

Kamemura, K., Hayes, B.K., Comer, F.I., and Hart, G.W. (2002). Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. The Journal of biological chemistry *277*, 19229-19235.

Katoh, K., Sawai, S., Ueno, K., and Kondoh, H. (1988). Complete nucleotide sequence and exon-intron boundaries of the 5' non-coding region of the mouse N-myc gene. Nucleic Acids Res *16*, 3589.

Katz, M., Amit, I., and Yarden, Y. (2007). Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1773*, 1161-1176.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997). Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. Nature *385*, 544-548.

Kaur, M., and Cole, M.D. (2012). MYC acts via the PTEN tumor suppressor to elicit autoregulation and genome-wide gene repression by activation of the Ezh2 methyltransferase. Cancer research *73*, 695-705.

Kerppola, T.K. (2006). Complementary methods for studies of protein interactions in living cells. Nature methods *3*, 969-971.

Kim, H.T., Kim, K.P., Lledias, F., Kisselev, A.F., Scaglione, K.M., Skowyra, D., Gygi, S.P., and Goldberg, A.L. (2007). Certain Pairs of Ubiquitin-conjugating Enzymes (E2s) and Ubiquitin-Protein Ligases (E3s) Synthesize Nondegradable Forked Ubiquitin Chains Containing All Possible Isopeptide Linkages. J Biol Chem *282*, 17375-17386.

Knoepfler, P.S., Cheng, P.F., and Eisenman, R.N. (2002). N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes & development *16*, 2699-2712.

Kohl, N.E., Kanda, N., Schreck, R.R., Bruns, G., Latt, S.A., Gilbert, F., and Alt, F.W. (1983). Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell *35*, 359-367.

Kominsky, S., Johnson, H.M., Bryan, G., Tanabe, T., Hobeika, A.C., Subramaniam, P.S., and Torres, B. (1998). IFNγ inhibition of cell growth in glioblastomas correlates with increased levels of the cyclin dependent kinase inhibitor p21^{WAF1/CIP1}. Oncogene *17*, 2973-2979.

Kornberg, R.D. (2005). Mediator and the mechanism of transcriptional activation. Trends Biochem Sci *30*, 235-239.

Kornberg, R.D. (2007). The molecular basis of eukaryotic transcription. Proceedings of the National Academy of Sciences of the United States of America *104*, 12955-12961.

Kosar, M., Bartkova, J., Hubackova, S., Hodny, Z., Lukas, J., and Bartek, J. (2011). Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16(ink4a). Cell cycle (Georgetown, Tex) *10*, 457-468.

Kress, T.R., Sabo, A., and Amati, B. (2015). MYC: connecting selective transcriptional control to global RNA production. Nature reviews Cancer *15*, 593-607.

Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. Genes & development *24*, 2463-2479.

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

Lane, D.P., Cheok, C.F., and Lain, S. (2010). p53-based cancer therapy. Cold Spring Harb Perspect Biol *2*, a001222.

Larsson, L.G., and Henriksson, M.A. (2010). The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. Experimental cell research *316*, 1429-1437.

Leder, A., Pattengale, P.K., Kuo, A., Stewart, T.A., and Leder, P. (1986). Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development. Cell *45*, 485-495.

Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J.R. (1997). Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. Nature *387*, 422-426.

Li, J., Poi, M.J., and Tsai, M.D. (2011). Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. Biochemistry *50*, 5566-5582.

Li, W., Tu, D., Brunger, A.T., and Ye, Y. (2007). A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. Nature *446*, 333.

Lin, A.W., Barradas, M., Stone, J.C., van Aelst, L., Serrano, M., and Lowe, S.W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes & development *12*, 3008-3019.

Lin, C.Y., Loven, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I., and Young, R.A. (2012). Transcriptional amplification in tumor cells with elevated c-Myc. Cell *151*, 56-67.

Loven, J., Hoke, H.A., Lin, C.Y., Lau, A., Orlando, D.A., Vakoc, C.R., Bradner, J.E., Lee, T.I., and Young, R.A. (2013). Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell *153*, 320-334.

Luscher, B. (2001). Function and regulation of the transcription factors of the Myc/Max/Mad network. Gene *277*, 1-14.

Malik, S., Baek, H.J., Wu, W., and Roeder, R.G. (2005). Structural and functional characterization of PC2 and RNA polymerase II-associated subpopulations of metazoan Mediator. Molecular and cellular biology *25*, 2117-2129.

Marcu, K.B., Bossone, S.A., and Patel, A.J. (1992). myc function and regulation. Annu Rev Biochem *61*, 809-860.

Mateyak, M.K., Obaya, A.J., and Sedivy, J.M. (1999). c-Myc regulates cyclin D-Cdk4 and -Cdk6 activity but affects cell cycle progression at multiple independent points. Molecular and cellular biology *19*, 4672-4683.

Maya-Mendoza, A., Ostrakova, J., Kosar, M., Hall, A., Duskova, P., Mistrik, M., Merchut-Maya, J.M., Hodny, Z., Bartkova, J., Christensen, C., *et al.* (2015). Myc and Ras oncogenes engage different energy metabolism programs and evoke distinct patterns of oxidative and DNA replication stress. Molecular oncology *9*, 601-616.

McKeown, M.R., and Bradner, J.E. (2014). Therapeutic strategies to inhibit MYC. Cold Spring Harbor perspectives in medicine *4*.

Meinhart, A., and Cramer, P. (2004). Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors. Nature *430*, 223-226.

Mertz, J.A., Conery, A.R., Bryant, B.M., Sandy, P., Balasubramanian, S., Mele, D.A., Bergeron, L., and Sims, R.J., 3rd (2011). Targeting MYC dependence in cancer by inhibiting BET bromodomains. Proceedings of the National Academy of Sciences of the United States of America *108*, 16669-16674.

Meyer, N., Kim, S.S., and Penn, L.Z. (2006). The Oscar-worthy role of Myc in apoptosis. Semin Cancer Biol *16*, 275-287.

Meyer, N., and Penn, L.Z. (2008). Reflecting on 25 years with MYC. Nature reviews Cancer *8*, 976-990.

Michel, J., and Cuchillo, R. (2012). The impact of small molecule binding on the energy landscape of the intrinsically disordered protein C-myc. PloS one *7*, e41070.

Moqtaderi, Z., Bai, Y., Poon, D., Weil, P.A., and Struhl, K. (1996). TBP-associated factors are not generally required for transcriptional activation in yeast. Nature *383*, 188-191.

Muirhead, D.M., Hoffman, H.T., and Robinson, R.A. (2006). Correlation of clinicopathological features with immunohistochemical expression of cell cycle regulatory proteins p16 and retinoblastoma: distinct association with keratinisation and differentiation in oral cavity squamous cell carcinoma. J Clin Pathol *59*, 711-715.

Murphy, D.J., Junttila, M.R., Pouyet, L., Karnezis, A., Shchors, K., Bui, D.A., Brown-Swigart, L., Johnson, L., and Evan, G.I. (2008). Distinct thresholds govern Myc's biological output in vivo. Cancer cell *14*, 447-457.

Nechaev, S., and Adelman, K. (2011). Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. Biochimica et biophysica acta *1809*, 34-45.

Nero, T.L., Morton, C.J., Holien, J.K., Wielens, J., and Parker, M.W. (2014). Oncogenic protein interfaces: small molecules, big challenges. Nature reviews Cancer *14*, 248-262.

Nicholson, T.B., Veland, N., and Chen, T. (2015). Chapter 3 - Writers, Readers, and Erasers of Epigenetic Marks. In Epigenetic Cancer Therapy, S.G. Gray, ed. (Boston: Academic Press), pp. 31-66.

Nie, Z., Hu, G., Wei, G., Cui, K., Yamane, A., Resch, W., Wang, R., Green, D.R., Tessarollo, L., Casellas, R.*, et al.* (2012). c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. Cell *151*, 68-79.

Nilsson, J.A., and Cleveland, J.L. (2003). Myc pathways provoking cell suicide and cancer. Oncogene *22*, 9007-9021.

Nowell, P.C. (1976). The clonal evolution of tumor cell populations. Science (New York, NY) *194*, 23-28.

Nurse, P. (2011). The cell cycle. Preface. Philosophical transactions of the Royal Society of London Series B, Biological sciences *366*, 3493.

O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature *435*, 839-843.

Obaya, A.J., Mateyak, M.K., and Sedivy, J.M. (1999). Mysterious liaisons: the relationship between c-Myc and the cell cycle. Oncogene *18*, 2934-2941.

Oren, M. (2003). Decision making by p53: life, death and cancer. Cell Death Differ *10*, 431-442.

Organization, W.H. (2018). World Cancer Report.

Oster, S.K., Ho, C.S., Soucie, E.L., and Penn, L.Z. (2002). The myc oncogene: MarvelouslY Complex. Advances in cancer research *84*, 81-154.

Parry, D., Bates, S., Mann, D.J., and Peters, G. (1995). Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. The EMBO journal *14*, 503-511.

Pelengaris, S., Khan, M., and Evan, G. (2002a). c-MYC: more than just a matter of life and death. Nature reviews Cancer *2*, 764-776.

Pelengaris, S., Khan, M., and Evan, G.I. (2002b). Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. Cell *109*, 321-334.

Peterson, C.L., and Laniel, M.-A. (2004). Histones and histone modifications. Current Biology *14*, R546-R551.

Pickart, C.M., and Eddins, M.J. (2004). Ubiquitin: structures, functions, mechanisms. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research *1695*, 55-72.

Pirity, M., Blanck, J.K., and Schreiber-Agus, N. (2006). Lessons learned from Myc/Max/Mad knockout mice. Current topics in microbiology and immunology *302*, 205-234.

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

Poon, S.L., McPherson, J.R., Tan, P., Teh, B.T., and Rozen, S.G. (2014). Mutation signatures of carcinogen exposure: genome-wide detection and new opportunities for cancer prevention. Genome medicine *6*, 24.

Pratilas, C.A., and Solit, D.B. (2010). Targeting the mitogen-activated protein kinase pathway: physiological feedback and drug response. Clinical cancer research : an official journal of the American Association for Cancer Research *16*, 3329-3334.

Prives, C., and Hall, P.A. (1999). The p53 pathway. The Journal of pathology *187*, 112-126.

Prochownik, E.V., and Vogt, P.K. (2010). Therapeutic Targeting of Myc. Genes & cancer *1*, 650-659.

Puissant, A., Frumm, S.M., Alexe, G., Bassil, C.F., Qi, J., Chanthery, Y.H., Nekritz, E.A., Zeid, R., Gustafson, W.C., Greninger, P.*, et al.* (2013). Targeting MYCN in neuroblastoma by BET bromodomain inhibition. Cancer Discov *3*, 308-323.

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

Ramana, C.V., Grammatikakis, N., Chernov, M., Nguyen, H., Goh, K.C., Williams, B.R.G., and Stark, G.R. (2000). Regulation of c-myc expression by IFN-γ through Stat1-dependent and -independent pathways. The EMBO journal *19*, 263-272.

Remy, I., and Michnick, S.W. (2006). A highly sensitive protein-protein interaction assay based on Gaussia luciferase. Nature methods *3*, 977-979.

Renan, M.J. (1993). How many mutations are required for tumorigenesis? Implications from human cancer data. Molecular carcinogenesis *7*, 139-146.

Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes & development *9*, 1831-1845.

Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nature reviews Molecular cell biology *10*, 398-409.

Sabo, A., Kress, T.R., Pelizzola, M., de Pretis, S., Gorski, M.M., Tesi, A., Morelli, M.J., Bora, P., Doni, M., Verrecchia, A.*, et al.* (2014). Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. Nature *511*, 488-492.

Saha, M.N., Qiu, L., and Chang, H. (2013). Targeting p53 by small molecules in hematological malignancies. Journal of hematology & oncology *6*, 23.

Saied, A., Pillarisetty, V.G., and Katz, S.C. (2014). Immunotherapy for solid tumors--a review for surgeons. The Journal of surgical research *187*, 525-535.

Sawai, S., Kato, K., Wakamatsu, Y., and Kondoh, H. (1990). Organization and expression of the chicken N-myc gene. Molecular and cellular biology *10*, 2017-2026.

Scheffner, M., and Kumar, S. (2014). Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects. Biochimica et biophysica acta *1843*, 61-74.

Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. (1983). Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature *305*, 245-248.

Schwab, M., Ellison, J., Busch, M., Rosenau, W., Varmus, H.E., and Bishop, J.M. (1984). Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proceedings of the National Academy of Sciences of the United States of America *81*, 4940-4944.

Sears, R., Leone, G., DeGregori, J., and Nevins, J.R. (1999). Ras enhances Myc protein stability. Molecular cell *3*, 169-179.

Sears, R., Nuckolls, F., Haura, E., Taya, Y., Tamai, K., and Nevins, J.R. (2000). Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes & development *14*, 2501-2514.

Semple, C.A.M., Group, R.G., and Members, G.S.L. (2003). The Comparative Proteomics of Ubiquitination in Mouse. Genome Res *13*, 1389-1394.

Seoane, J., Le, H.V., and Massague, J. (2002). Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature *419*, 729-734.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Shaw, R.J., and Cantley, L.C. (2006). Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature *441*, 424-430.

Sheaff, R.J., Groudine, M., Gordon, M., Roberts, J.M., and Clurman, B.E. (1997). Cyclin E-CDK2 is a regulator of p27Kip1. Genes & development *11*, 1464-1478.

Sheiness, D., and Bishop, J.M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. Journal of virology *31*, 514-521.

Skaar, J.R., and Pagano, M. (2009). Control of cell growth by the SCF and APC/C ubiquitin ligases. Curr Opin Cell Biol *21*, 816-824.

Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K., Leuchowius, K.J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L.G.*, et al.* (2006). Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nature methods *3*, 995-1000.

Sodir, N.M., Swigart, L.B., Karnezis, A.N., Hanahan, D., Evan, G.I., and Soucek, L. (2011). Endogenous Myc maintains the tumor microenvironment. Genes & development *25*, 907-916.

Sorrell, A.D., Espenschied, C.R., Culver, J.O., and Weitzel, J.N. (2013). Tumor protein p53 (TP53) testing and Li-Fraumeni syndrome : current status of clinical applications and future directions. Molecular diagnosis & therapy *17*, 31-47.

Soucek, L., Whitfield, J., Martins, C.P., Finch, A.J., Murphy, D.J., Sodir, N.M., Karnezis, A.N., Swigart, L.B., Nasi, S., and Evan, G.I. (2008). Modelling Myc inhibition as a cancer therapy. Nature *455*, 679-683.

Spence, J., Sadis, S., Haas, A.L., and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Molecular and cellular biology *15*, 1265.

Srivastava, N., and McDermott, D. (2014). Update on benefit of immunotherapy and targeted therapy in melanoma: the changing landscape. Cancer management and research *6*, 279-289.

Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F.*, et al.* (2001). Repression of p15(INK4b) expression by Myc through association with Miz-1. Nature cell biology *3*, 392-399.

Stanton, B.R., and Parada, L.F. (1992). The N-myc proto-oncogene: developmental expression and in vivo site-directed mutagenesis. Brain pathology (Zurich, Switzerland) *2*, 71-83.

Stanton, B.R., Perkins, A.S., Tessarollo, L., Sassoon, D.A., and Parada, L.F. (1992). Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. Genes & development *6*, 2235-2247.

Stark, G.R., Kerr, I.M., Williams, B.R.G., Silverman, R.H., and Schreiber, R.D. (1998). How Cells Respond to Interferons. Annual Review of Biochemistry *67*, 227.

Tabor, V., Bocci, M., Alikhani, N., Kuiper, R., and Larsson, L.G. (2014). MYC synergizes with activated BRAFV600E in mouse lung tumor development by suppressing senescence. Cancer research *74*, 4222-4229.

Tiefenbrun, N., Melamed, D., Levy, N., Resnitzky, D., Hoffman, I., Reed, S.I., and Kimchi, A. (1996). Alpha interferon suppresses the cyclin D3 and cdc25A genes, leading to a reversible G0-like arrest. Molecular and cellular biology *16*, 3934-3944.

Trimarchi, J.M., and Lees, J.A. (2002). Sibling rivalry in the E2F family. Nature reviews Molecular cell biology *3*, 11-20.

Tse, C., Shoemaker, A.R., Adickes, J., Anderson, M.G., Chen, J., Jin, S., Johnson, E.F., Marsh, K.C., Mitten, M.J., Nimmer, P.*, et al.* (2008). ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer research *68*, 3421-3428.

Wang, G.G., Allis, C.D., and Chi, P. (2007a). Chromatin remodeling and cancer, part I: covalent histone modifications. Trends in Molecular Medicine *13*, 363-372.

Wang, G.G., Allis, C.D., and Chi, P. (2007b). Chromatin remodeling and cancer, part II: ATP-dependent chromatin remodeling. Trends in Molecular Medicine *13*, 373-380.

Wang, H., Hammoudeh, D.I., Follis, A.V., Reese, B.E., Lazo, J.S., Metallo, S.J., and Prochownik, E.V. (2007c). Improved low molecular weight Myc-Max inhibitors. Mol Cancer Ther *6*, 2399-2408.

Vanneman, M., and Dranoff, G. (2012). Combining immunotherapy and targeted therapies in cancer treatment. Nature reviews Cancer *12*, 237-251.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., *et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science (New York, NY) *303*, 844-848.

Watson, D.K., Reddy, E.P., Duesberg, P.H., and Papas, T.S. (1983). Nucleotide sequence analysis of the chicken c-myc gene reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, delta gag-myc. Proceedings of the National Academy of Sciences of the United States of America *80*, 2146-2150.

Weinberg, R.A. (2014). The biology of cancer (New York: Garland Science).

Weissman, A.M. (2001). Themes and variations on ubiquitylation. Nature Reviews Molecular Cell Biology *2*, 169.

Welcker, M., Orian, A., Jin, J., Grim, J.E., Harper, J.W., Eisenman, R.N., and Clurman, B.E. (2004). The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proceedings of the National Academy of Sciences of the United States of America *101*, 9085-9090.

Vennstrom, B., Sheiness, D., Zabielski, J., and Bishop, J.M. (1982). Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. Journal of virology *42*, 773-779.

Vervoorts, J., Luscher-Firzlaff, J.M., Rottmann, S., Lilischkis, R., Walsemann, G., Dohmann, K., Austen, M., and Luscher, B. (2003). Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP. EMBO reports *4*, 484-490.

White, M.K., Pagano, J.S., and Khalili, K. (2014). Viruses and human cancers: a long road of discovery of molecular paradigms. Clinical microbiology reviews *27*, 463-481.

Whitfield, J.R., Beaulieu, M.E., and Soucek, L. (2017). Strategies to Inhibit Myc and Their Clinical Applicability. Front Cell Dev Biol *5*, 10.

Widmer, N., Bardin, C., Chatelut, E., Paci, A., Beijnen, J., Leveque, D., Veal, G., and Astier, A. (2014). Review of therapeutic drug monitoring of anticancer drugs part two--targeted therapies. European journal of cancer (Oxford, England : 1990) *50*, 2020-2036.

Wierstra, I., and Alves, J. (2008). The c-myc promoter: still MysterY and challenge. Advances in cancer research *99*, 113-333.

Vita, M., and Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol *16*, 318-330.

Vlach, J., Hennecke, S., and Amati, B. (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. The EMBO journal *16*, 5334-5344.

Wogan, G.N., Hecht, S.S., Felton, J.S., Conney, A.H., and Loeb, L.A. (2004). Environmental and chemical carcinogenesis. Semin Cancer Biol *14*, 473-486.

Vogelstein, B., and Kinzler, K.W. (1993). The multistep nature of cancer. Trends in genetics : TIG *9*, 138-141.

Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nature medicine *10*, 789-799.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Wolffe, A.P., and Hayes, J.J. (1999). Chromatin disruption and modification. Nucleic Acids Res *27*, 711-720.

von der Lehr, N., Johansson, S., Wu, S., Bahram, F., Castell, A., Cetinkaya, C., Hydbring, P., Weidung, I., Nakayama, K., Nakayama, K.I.*, et al.* (2003). The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Molecular cell *11*, 1189-1200.

Woodcock, C.L., and Ghosh, R.P. (2010). Chromatin Higher-order Structure and Dynamics. Cold Spring Harb Perspect Biol *2*, a000596.

Wu, C.H., van Riggelen, J., Yetil, A., Fan, A.C., Bachireddy, P., and Felsher, D.W. (2007). Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation. Proceedings of the National Academy of Sciences of the United States of America *104*, 13028-13033.

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

Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., and Nakayama, K.I. (2004). Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. The EMBO journal *23*, 2116-2125.

Yeh, E., Cunningham, M., Arnold, H., Chasse, D., Monteith, T., Ivaldi, G., Hahn, W.C., Stukenberg, P.T., Shenolikar, S., Uchida, T.*, et al.* (2004). A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nature cell biology *6*, 308-318.

Yin, X., Giap, C., Lazo, J.S., and Prochownik, E.V. (2003). Low molecular weight inhibitors of Myc-Max interaction and function. Oncogene *22*, 6151-6159.

Young, S.L., Diolaiti, D., Conacci-Sorrell, M., Ruiz-Trillo, I., Eisenman, R.N., and King, N. (2011). Premetazoan ancestry of the Myc-Max network. Mol Biol Evol *28*, 2961-2971.

Yun, M., Wu, J., Workman, J.L., and Li, B. (2011). Readers of histone modifications. Cell Res *21*, 564-578.

Zheng, N., Schulman, B.A., Song, L., Miller, J.J., Jeffrey, P.D., Wang, P., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M.*, et al.* (2002). Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature *416*, 703-709.

Zhu, J., Sanborn, J.Z., Benz, S., Szeto, C., Hsu, F., Kuhn, R.M., Karolchik, D., Archie, J., Lenburg, M.E., Esserman, L.J.*, et al.* (2009). The UCSC Cancer Genomics Browser. Nature methods *6*, 239-240.

Zhuang, D., Mannava, S., Grachtchouk, V., Tang, W.H., Patil, S., Wawrzyniak, J.A., Berman, A.E., Giordano, T.J., Prochownik, E.V., Soengas, M.S.*, et al.* (2008). C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. Oncogene *27*, 6623-6634.

Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J., and Roussel, M.F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes & development *12*, 2424-2433.

Zwart, R., Sleutels, F., Wutz, A., Schinkel, A.H., and Barlow, D.P. (2001). Bidirectional action of the Igf2r imprint control element on upstream and downstream imprinted genes. Genes & development *15*, 2361-2366.