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IDENTIFICATION AND CHARACTERIZATION OF SMALL MOLECULES TARGETING MYC FUNCTION

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Identification and characterization of small molecules targeting MYC function

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To My Dear Mother

献给我亲爱的妈妈

ABSTRACT

The *MYC* family genes (c-, N- and L-MYC) encode potent oncoproteins/transcription factors regulating fundamental cellular processes involved in cell proliferation, metabolism and survival, and they play an important role in tumor development.

Overexpression of MYC often induce apoptosis as a failsafe mechanism to prevent tumor development and it is known to sensitize cells to genotoxic agents that induce DNA damage by triggered apoptosis. However, the MYC-regulated effectors acting upstream of the mitochondrial apoptotic pathway in response to DNA damage are still obscure. We focused on apoptosis activated by DNA damage responses in this study by comparing cell death induced upon ionizing radiation (IR), bacterial cytolethal distending toxin (CDT) and UV irradiation. We could demonstrate that phosphorylation of the ATM kinase and its downstream effectors, such as histone H2AX, were impaired in the MYC null cell line HO15.19, in comparison to the wild type parental cell line TGR-1 and MYC reconstituted HOMYC3 cells in response to IR or CDT. We also found that nuclear foci formation of the Nijmegen Breakage Syndrome (Nbs) 1 protein, which is essential for efficient ATM activation, was also reduced in the absence of MYC. Knocking down of endogenous MYC by siRNA in the HCT116 human colon cancer cell line resulted in decreased ATM and CHK2 phosphorylation in response to ionizing irradiation. However, the response to UV irradiation, which is known to activate the ATR dependent checkpoint, was functional in all of these cell lines, indicating MYC status did not play an important role in ATR signaling. In summary, we found that MYC is required for the activation of ATM-dependent checkpoint in response to IR and CDT; it contributes to DNA damage response by stimulating ATM phosphorylation and promoting NBS1 expression and nuclear translocation, thereby enhancing the apoptotic response, but potentially also stimulating DNA repair.

Deregulated MYC expression is implicated in the development of a wide variety of cancers and is often strongly correlated with poor prognosis, underscoring the importance of finding ways to counteract MYC function. To exert its oncogenic activity, MYC must be able to interact with a number of cofactors that are essential for MYC function. For instance, dimerization with the partner Max enables the MYC to bind target gene promoters. This requirement for cofactors may allow for control of MYC activity with small molecules that interfere with interactions with these factors. We used bimolecular fluorescence complementation (BiFC) assay to visualize interactions between MYC and cofactors in living cells. Using BiFC we screened a 2000 compound library for molecules inhibiting the interaction between MYC and MAX, and found several interesting compounds. MYCMI-6 emerged among the top hits, and was further validated by split Gaussia luciferase (Gluc), in situ proximity ligation (isPLA), microscale thermophoresis (MST) and surface plasmon resonance (SPR) assays and was found to be a strong selective inhibitor of MYC:MAX interaction in cells and *in vitro* at single-digit micromolar concentrations without affecting MYC expression. SPR showed that

MYCMI-6 binds to the recombinant MYC bHLHZip domain with a K_D of $1.6 \pm 0.5 \mu\text{M}$. MYCMI-6 downregulated MYC-driven transcription and inhibited tumor cell proliferation and viability in a MYC-dependent manner in the low micromolar range, but was not cytotoxic to normal cells. In vivo studies using a xenograft mouse model of MYCN-amplified neuroblastoma revealed that daily intraperitoneal injections of MYCMI-6 led to reduced tumor cell proliferation, reduced microvascular density and induced massive apoptosis in tumor tissue without causing severe side effects for the mice.

MYCMI-7 is another of the top hits identified in the BiFC screening. The efficacy and selectivity of MYCMI-7 was further validated with respect to inhibition MYC:MAX interaction, binding to MYC and effects MYC-driven tumor cell growth. Using a number of protein interactions assays which could demonstrate that MYCMI-7 efficiently blocks MYC:MAX interaction both in cells and in vitro. Using MST and SPR we showed that MYCMI-7 binds to recombinant MYC with an affinity of approximately $4 \mu\text{M}$. In contrast to MYCMI-6, MYCMI-7 downregulated the steady state levels of MYC protein subsequent to the inhibition of MYC:MAX interaction, suggesting that it could inhibit MYC in both direct and indirect ways. MYCMI-7 strongly inhibited tumor cell growth and induced apoptosis in a MYC dependent manner in a number of different tumor cell lines such as neuroblastoma, glioblastoma, Burkitt's lymphoma, AML, lung cancer and several other epithelial tumors as well as patient-derived AML and glioblastoma tumor samples, while it only causing G1 arrest with cytotoxicity in normal cells. Moreover, MYCMI-7 blocked transformation of primary rat embryo fibroblasts by MYC together with activated RAS. Importantly, treatment with MYCMI-7 in vivo inhibited tumor growth and prolonged survival in mouse models of MYC-driven acute leukemia, triple negative breast cancer and MYCN-amplified neuroblastoma.

Besides MYCMI-6 and MYCMI-7, yet another compound MYCMI-2 was identified from the BiFC screening. MYCMI-2 exhibited outstanding specific inhibition of heterodimerization of in vitro translated or recombinant MYC and MAX in vitro as determined by split GLuc, SPR and FRET assays, the latter showing an IC_{50} of 150 nM. Further, MYCMI-2 bound to MYC with extraordinary high affinity (K_D 1.3 nM) as determined by SPR. We utilized cell based Gluc and *is*PLA to validate MYCMI-2's MYC:MAX inhibitory efficacy in cells. The latter assay demonstrated an IC_{50} of about $5 \mu\text{M}$ in MCF7 cells. Further, MYCMI-2 inhibited MYC-driven tumor cell growth and viability in a MYC-dependent manner, in a number of tumor cell lines with an IC_{50} of 1.5-6 μM , while viability of normal cells was not affected. Due to the difference between MYCMI-2's extraordinary activity in vitro and limited efficacy in cell cultures, we attempted to identify analogues with improved efficacy in cells with maintained activity in vitro. The analogue molecule MYCMI-2:7 showed lower but acceptable potency and maintained selectively towards MYC:MAX heterodimerization *in vitro* compared with MYCMI-2, but demonstrated slightly better MYC:MAX inhibitory effect in the cell based Gluc and the *is*PLA assays with inhibition down to about 40% of DMSO treatment. Unlike MYCMI-2, MYCMI-2:7 downregulated the endogenous MYC protein level in

MCF7 cells, which indicated that MYCMI-2 and MYCMI-2:7 work via different mechanism. In conclusion, we have demonstrated that MYCMI-2 has an extraordinary potency in vitro binding at a KD of 1 nM, and an activity in cells in the lower μ M range, while the analogue MYCMI-2:7 was less active in vitro and only marginally better in cells. Both molecules could potentially contribute to the development of bioactive MYC inhibitors of therapeutic interest in cancer therapy in the future.

We now try to improve the bioactivity of these compounds by modifications, evaluate their efficacies in vivo, and to further elucidate their mode of action and selectivity. Our protein-protein interaction platforms could potentially be used for high-throughput screens of larger chemical libraries for inhibitors of interactions between MYC and MAX as well as other cofactors of therapeutic and biological interest. We hope this project will lead to better understanding of the biological functions of the MYC network during tumorigenesis and provide new therapeutic tools to combat cancer in the future.

LIST OF SCIENTIFIC PAPERS

- I. Lina Guerra, Ami Albiñ, Susanna Tronnersjö, QINZI YAN, Riccardo Guidi, Bo Stenerlöv, Torsten Sterzenbach, Christine Josenhans, James G. Fox, David B. Schauer, Monica Thelestam, Lars-Gunnar Larsson, Marie Henriksson and Teresa Frisan. MYC Is Required for Activation of the ATM-Dependent Checkpoints in Response to DNA Damage. PLoS One. 2010 Jan 7;5(1):e8924
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- III. Alina Castell*, QINZI YAN*, Karin Fawcner*, Fan Zhang, Malin Wickström, Wesam Bazzar, Marcela Franco, Cecilia Dyberg, Per Hydbring, Vasiliki Verschut, Linnéa Schmidt, Suman Vodnala, Mårten Fryknäs, Lars Johansson, Sören Lehmann, Cecilia Krona, Sven Nelander, John Inge Johnsen and Lars-Gunnar Larsson, MYCMI-7 - a small MYC-binding compound that inhibits MYC:MAX interaction and tumor cell growth in culture and in vivo in a MYC-dependent manner. Manuscript.
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- IV. QINZI YAN, Karin Ridderstråle, Fan Zhang, Jacob Goodwin, Giovanna Zinzalla, Lars-Gunnar Larsson and Alina Castell. Characterization of a high affinity MYC-binding compound. Manuscript.

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LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
APAF-1	Apoptotic protease activating factor 1
ATM	Ataxia Telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia Telangiectasia and Rad3-related
AURKA	Aurora kinase A
BAD	Bcl-2 associated agonist of cell death
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2 associated protein X
BCL-2	B-cell lymphoma gene 2
BCL-XL	B-cell lymphoma extra large
BH3	Bcl-2 homology domain 3
bHLHZip	Basic helix-loop-helix leucine zipper
BiFC	Bimolecular fluorescence complementation
BID	BH3-interacting domain death agonist
BIM	Bcl-2 interacting mediator of cell death
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CDT	Cytotoxic distending toxin
ChIP	Chromatin immunoprecipitation
CHK	Checkpoint kinase
CIP	CDK interacting protein
CTD	C-terminal domain
DED	Death effector domain
DNA	Deoxyribonucleic acid
DDR	DNA damage response
DISC	Death-inducing signaling complex
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
FADD	FAS-associated protein with death domain
FRET	Fluorescence resonance energy transfer
FBW7	F-box WD-40 domain protein 7
GAP	GTPase activating proteins
GDP	Guanosine diphosphate
GLUT	glucose transporter
GPCR	protein coupled receptors
GTP	Guanosine triphosphate
GSK3	Glycogen synthase kinase 3
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HIF	Hypoxia inducible factor
IL-2	Interleukin-2
IR	Ionizing irradiation
IsPLA	In situ proximity ligation assay
KIP	Kinase inhibitory protein
Luc	Luciferase
MAD	Max dimerizer
MAPK	Mitogen activated protein kinase
MAX	MYC-associated protein X
MB	MYC box
MDM2	Mouse double minute 2
MEK	Mitogen-activated protein kinase kinase
MIZ-1	MYC-interacting zinc finger protein 1
MNT	Max's next tango
MST	Microscale thermophoresis
mTOR	Mammalian target of rapamycin
MXI	Max-interacting protein

MYC	Myelocytomatosis
MYCMI	MYC:MAX inhibitor
NF- κ B	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
ODC	Ornithine decarboxylase
OIS	Oncogene-induced senescence
PCA	Protein fragment complementation assay
PKD1	Phosphoinositide dependent kinase 1
PI3K	Phosphatidylinositol-3 kinase
PPI	Protein-protein interaction
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PTEN	Phosphatase and tensin homologue
pRb	Retinoblastoma protein
REF	Rat embryonic fibroblast
PUMA	p53 upregulated modulator of apoptosis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Tyrosine kinase receptor
SASP	Senescence-associated secretory phenotype
SCF	Skp, Cullin, F-box containing complex
SKP2	S phase kinase-associated protein 2
SOS	Son of sevenless
SPR	Surface plasmon resonance
TAD	Transcription activation domain
TNF	Tumor necrosis factor
TSC1	Tuberous sclerosis complex protein 1
TSC2	Tuberous sclerosis complex protein 2
VEGF	Vascular endothelial growth factor

YFP

Yellow fluorescent protein

1 INTRODUCTION

1.1 CANCER

Cancer, by definition, quoted from the WHO, is the rapid creation of abnormal cells with the potential to grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs, the latter process is referred to as metastasis. So, in short, cancer is a subset of neoplasms with the potential to invade or spread to other parts of the body.

How does cancer arise? Research during the last decades has demonstrate that cancer is caused by genetic or epigenetic aberrations, leading to loss of control over cell proliferation and cell division. Cancer is a heterogeneous malignant disease, that the mechanisms of cancer development are still not fully elucidated, so as the optimal treatment strategies for different cancers.

However, there are several barriers against transformation of normal cells to malignant cells, as was summarized in 2000 by Hanahan and Weinberg. They defined six hallmarks of cancer, capabilities that pre-malignant cells have to acquire step by step to evolve progressively to a neoplastic state. These include sustaining proliferative signaling; evading growth suppressors; evading apoptosis; limitless replicative potential; sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

In addition to these six, two new hallmarks, genome instability and tumor promoting inflammation, were included in the updated in the latest version of the article in 2011, as well as two emerging hallmarks, reprogramming energy metabolism and evading the immune system (Hanahan and Weinberg, 2000, 2011).

1.1.1 Sustaining Proliferative Signaling

The growth and proliferation of normal cells are under control of growth factors, mitogens and hormones produced by other cells in the microenvironment or from distant locations in the body. These ligands bind receptors, such as transmembrane proteins on the cell surface or intracellular receptors such as the inositol trisphosphate receptor (InsP3R) and nuclear hormone receptors. Once bound by ligand, the receptors become activated and initiate downstream signaling cascades, for instance by phosphorylation. Cancer cells bypass the external growth stimuli as a result of genetic or epigenetic alterations the receptors or affecting further downstream component in the signaling pathway, such as mutant RAS, which becomes constitutively active. In this way, tumor cells have self-sufficiency in growth signaling.

1.1.2 Evading Growth Suppressors

Nevertheless, despite uncontrolled growth stimulation, not all cells will develop into tumor cells because of the existence of “gatekeepers” tumor suppressor genes. The two most important and well characterized tumor suppressors are pRB and p53, which are often found to be altered or mutated in cancers, leading to loss of function (Dick and Rubin, 2013; Hemann et al., 2005; Indovina et al., 2013; Vousden and Lane, 2007). Without these critical “gatekeepers”, cells become insensitive to negative regulators of proliferation, and anti-growth signals will not be able to pass on to command the cell to stop proliferation or to undergo programmed cell death by apoptosis.

1.1.3 Evading apoptosis

Apoptosis is defined as programmed cell death, which together with cell senescence, is considered as the most important barriers towards cancer development (Collado et al., 2007). Normal cells usually respond to transformation-associated stress, including irreparable DNA damage, uncontrolled proliferation, or matrix detachment, by apoptosis, while cancer cells are often able to surpass apoptotic response by various mechanisms (Koff et al., 2015; Lopez and Tait, 2015; Polyak et al., 1997), thereby allowing genetic aberrations to accumulate and be passed on to the daughter cells.

1.1.4 Limitless replicative potential

Unlike normal cells, which have a limited lifespan and restricted number of growth-and-division cycles, also known as Hayflick Limit beyond which cells enter replicative senescence, cancer cells can overcome this limitation, keep on dividing and eventually become immortal. Replicative senescence, which is part of the cellular aging process, is due to telomere erosion. Telomeres are regions at the ends of each chromosome containing repetitive nucleotide sequences that protect the end of the chromosome from deterioration or from fusion with neighboring chromosomes (Dick and Rubin, 2013; Indovina et al., 2013). Normal cells progressively get shorter in telomeres the more cycles they have divided, until the critical shortening causes chromosomal fusion and breakage (Shay, 2016). This due to end replication problem that the synthesis of Okazaki fragments during DNA replication requires RNA primers attaching ahead on the lagging strand. After the last RNA primer is attached and degraded, part of the telomere is lost during each cycle of replication at the 5' end of the lagging daughter strands where the RNA primers attached. Telomerase is a ribonucleoprotein that adds a species-dependent telomere repeat sequence to the 3' end of telomeres. It is a reverse transcriptase (TERT, or hTERT in humans), which is normally

active in normal stem cells but absent from, or at very low levels in most somatic cells. Telomerase maintains telomere ends by catalyzing addition of nucleotides to the ends of the telomere repeats (Gunes and Rudolph, 2013). In order to escape from the irreversible shortening of telomeres, cancer cells can stabilize telomeres length by the reactivation of telomerase. Telomerase allows each offspring to replace the lost bit of DNA, facilitating the cell to divide without ever reaching the Hayflick Limit.

1.1.5 Sustained angiogenesis

The uncontrolled growth of cancer cells requires blood vessels to transport oxygen and nutrients to the tumor, as well as removal of waste products (Carmeliet and Jain, 2000; Sakurai and Kudo, 2011). Angiogenesis refers to the formation of new blood vessels from pre-existing ones by proliferation of endothelial and other cells supporting the vessels. Pathological angiogenesis triggered for instance by excess vascular endothelial growth factor (VEGF), facilitates the aggressiveness and metastasis of cancerous tissue (Ferrara et al., 2003).

1.1.6 Tissue invasion and metastasis

Cancerous invasion and metastasis are responsible for over 90% of cancer-related deaths (Steeg, 2006). A successful metastasizing cancer cell, much like a seed, need to survive extreme stress to be able to colonize into foreign soil. It first has to migrate through the extracellular matrix (ECM), passing through basement membrane, thereafter penetrate vasculature and survive in the circulation, finally invade and colonize distant organs (Massague and Obenauf, 2016; Valastyan and Weinberg, 2011). The invasion and metastasis process may involve multiple steps of cell-biological and molecular changes, which also offer therapeutic targets for cancer therapy.

1.1.7 Reprogramming energy metabolism

Another important characteristic of cancer cells is their reprogramming of metabolism, for instance their utilization of glucose and glutamine for energy production and biosynthesis as compared to normal cells (Dang, 2010). Due to uncontrolled growth, cancer cells apparently require more energy, but they metabolize glucose less efficiently, which is one of the paradoxes with cancer cells (Altman et al., 2016; Pavlova and Thompson, 2016). One plausible reason behind this phenomenon might be that cancer cells not only use glucose purely for energy production, but the intermediate products of glucose are used for biosynthesis to support cell division (Cairns et al., 2011).

There are multiple mechanisms and signaling pathways could promote glucose uptake in cancer cells. Increased expression of hypoxia-inducible factor (HIF) in cancer cells, for instance, increases the expression of glucose transporter (GLUT) and hexokinase (trapping the inflowing glucose), and aberrant PI3K/AKT signaling increases expression of GLUT1 and protein translocation to the cellular membrane, and so do oncogenic KRAS and BRAF signaling (Hay, 2016; Szablewski, 2013). Glutamine is the most abundant nutrition in human plasma, and it acts as a source of carbon and nitrogen in cancer cells as well. In glutaminolysis, the enzyme glutaminase converts glutamine to glutamate, which will be further catabolized in the tricarboxylic acid cycle (TCA cycle). The glutamine transporter SLC1A5 is often upregulated in cancer cells, thus affecting the metabolic pathways (Hsieh et al., 2015).

1.1.8 Evading the immune system

Long before the capability of avoiding immune detection became a cancer hallmark, the theory of ‘cancer immune surveillance’ was developed (Burnet, 1957; Dunn et al., 2002). The immune system protects the organism against foreign pathogens and diseases, but it also plays a very important role in clearing the body’s own unhealthy or abnormal cells. The immune system is also able to recognize and eliminate cancer cells, an important process which is utilized and enforced for immune therapy.

Human immune system is classified into two categories: innate and adaptive system, both of which play a role in eliminating tumor cells. During the process of immunoediting initial immunosurveillance is followed by tumor progression during different phases that can be divided into elimination, equilibrium and escapes phases. Interestingly immunoediting by cancer cells and immune cells was proposed to exhibit a dual role in both promoting host protection against cancer and facilitating tumor escape from immune destruction (Malmberg, 2004; Schreiber et al., 2011).

Normal cells evolve progressively to become cancerous, and the eight hallmarks of cancer describe above are acquired step by step and not necessarily in a particular order. Can those hallmarks be served as cancer targets? Some aspects of the hallmarks will be explored the further in the following chapters.

1.2 ONCOGENES, TUMOR SUPPRESSOR GENES AND CELL SIGNALING

Activation of oncogenes and inactivation of tumor suppressor genes are the main critical cause of cancers. Therefore, in the following chapters, oncogene and tumor suppressor genes, as well as cell cycle, cell signaling pathways involved in the tumorigenesis will be examined further.

1.2.1 Proto-oncogene and oncogenes

Oncogenes, by definition, refer to those genes that have the potential to cause cancer, and they are often found mutated or overexpressed in tumor cells (Adamson, 1987; Chial, 2008; Weinstein and Joe, 2008). A proto-oncogene is a normal gene that could become an oncogene due to point mutations, translocation, amplification or increased expression. In fact, many proto-oncogenes are necessary for survival, they only cause cancer when they acquire a “gain-of-function” due to, for instance, point mutations, chromosomal translocation or deregulated of amplification. Examples of well characterized proto-oncogenes are *RAS*, *PI3K* (phosphatidylinositol 3-kinase) and *MDM2* (mouse double minute 2). There are several systems for classifying oncogenes, and one of the widely accepted methods is to categorize oncogenes by functions, for instance, growth factors/mitogens (*PDGF*, *VEGF*); receptor tyrosine kinases (*EGFR*, *PDGFR*, *VEGFR*); cytoplasmic tyrosine kinases (*SRC*, *BTk*); serine/threonine kinases (*RAF*, *AKT*); regulatory GTPases (*RAS*), transcription factors (*MYC*, *JUN*) and anti-apoptotic genes (*BCL-2*, *BCL-X_L*).

RAS family proto-oncogenes (*HRAS*, *KRAS* and *NRAS*), named after Rat sarcoma, were discovered originally as part of oncogenic retroviruses in rats during the 1960s (Harvey, 1964). They encode *RAS* proteins, which are cellular signal transducers. *RAS* belongs to the GTPases superfamily and is associated with the plasma membrane to transduce extracellular signals from the plasma membrane to the cytoplasm and into the cell nucleus (Malumbres and Barbacid, 2003).

Mutations in certain oncogenes is often linked to specific cancers, for example *HER2* mutation in breast cancers (Krishnamurti and Silverman, 2014; Moasser, 2007; Petrelli et al., 2017). Identification of point mutations or other aberrations in oncogenes plays an important role cancer diagnosis, as well as in targeted treatment and prediction of prognosis of benefit for many cancer patients today and even more in the future.

1.2.2 Tumor suppressor genes

Tumor suppressor genes are, on the contrary, genes that protect cells from transforming into tumor cells. They function through, for instance, slowing down cell division by regulating the

cell cycle, by repairing DNA damage, or by initiating apoptosis in abnormal cells, such as *p53*. Basically, tumor suppressor genes serve as antagonists to oncogenes. In many tumors, these genes are lost or inactivated, leading to lack of negative regulators of cell proliferation and survival, thereby contributing to abnormal proliferation and tumor development, much like the scenario of a speeding car without having a brake.

The retinoblastoma protein (pRb) was the first tumor suppressor protein discovered in humans. pRb, which is a transcription factor, can prevent excessive cell growth by inhibiting cell cycle progression from G1 to S phase, resulting in G1 arrest (Lee et al., 1984; Murphree and Benedict, 1984).

Another tumor suppressor protein, phosphatase and tensin homolog (PTEN) is a phosphatase that dephosphorylates PIP3 to PIP2, thereby antagonizing the PI3K/AKT-pathway, which regulates proliferation and survival (Manning and Dyson, 2011; Milella et al., 2015).

p53, known as the guardian of the genome, is another classic tumor suppressor (Lane, 1992). p53 is a transcription factor and plays an important role in regulation of the cell cycle, apoptosis, and genomic stability. p53 is activated in response to diverse stress, including DNA damage (for instance caused by UV irradiation, ionizing irradiation or chemical agents), osmotic shock, oxidative stress, ribonucleotide depletion and deregulated oncogene expression. Mechanisms leading to p53 activation can be stimulus-dependent: DNA damage induces p53 phosphorylation mediated by ATM/CHK1, blocking MDM2-mediated degradation by preventing p53:MDM2 interaction (Kastenhuber and Lowe, 2017; Shieh et al., 1997; Zhang et al., 1998), whereas oncogenic signaling induces the ARF tumor suppressor to sequester MDM2 into the nucleolus or by inhibiting the E3 ligase activity of MDM2, thus protecting p53 from degradation (Quelle et al., 1995; Pomerantz et al., 1998; Moll and Petrenko, 2003).

p53 arrests or eliminate cells following DNA damage, it is crucial for a reversible DNA damage-induced G1 phase checkpoint (Kastan et al., 1991) via activation of p21, which is a cyclin-dependent kinase inhibitor (Harper et al., 1993; Harper et al., 1995). Cell cycle arrest facilitates DNA repair, after which the cell cycle can restart. p53, in association with pRb, can alternatively induce cellular senescence following oncogenic stress and DNA damage in certain cell contexts. Upon severe DNA damage that cannot be repaired, p53 induction can lead to apoptosis by promoting of pro-apoptotic members of the BCL-2 family (BAD, BAX and BAK etc.), or alternatively increasing other p53 regulated modulators of apoptosis (PUMA, NOXA etc.).

Inherited abnormalities sometimes contribute to the tumorigenesis, like inherited mutation in the *BRCA1* or *BRCA2* genes; acquired mutations are more common in tumor suppressor genes mutation like *p53*, which has been found in more than half of human cancers (Mei and Wu, 2016).

1.2.3 Cell signaling

As it is described in the previous chapter, cells respond to external signals such as growth stimuli through plasma membrane surface receptors, which can be classified as tyrosine kinase receptors (RTK); G-protein couple receptors (GPCR); NOTCH receptors and etc. Upon binding, receptors transmit signals into cell interior via activation of different signaling pathways. There are several signaling pathways which are highly related to tumorigenesis, therefore will be discussed in more detail.

As an example, the epidermal growth factor (EGF) signaling pathway plays an important role in cell proliferation and the prevention of apoptosis (Chang et al., 2003). Upon binding of EGF ligand to the EGF receptor (EGFR), which is a transmembrane receptor, EGFR will homodimerize and become activated leading to auto-phosphorylation of the receptor at multiple sites. The phosphorylated tyrosine residues at the Y992, Y1045, Y1068, Y1148 and Y1173 EGFR becomes a docking sites for adaptor proteins such as GRB2, which recognizes phosphorylated tyrosine residues and conformational changes via its SH2 domain, and transduces the signal further downstream into the RAS signaling pathway. GRB2 and the guanine nucleotide exchange factor (GEF) or “son of sevenless” (SOS), will interact with the GTPase RAS by exchanging binding of GDP for GTP, thereby turning RAS into the active state. This in turn will trigger downstream signaling through for instance the RAF/MEK/MAP kinase and PI3K/AKT pathways. The active state of RAS can be reversed by GTPase activating proteins (GAPs). In many cancers RAS is constitutively activated by mutations that prevent GTP hydrolysis, thus locking RAS into a permanently active state. In addition to mutations, overexpression and amplification of growth factors signaling through RAS, inactivation of GTPase activating protein (GAPs), as well as upregulation of GRB2 adaptor proteins also lead to hyperactive RAS thereby promoting tumorigenesis (Malumbres and Barbacid, 2003).

The RAF family of proteins (RAF1, ARAF, and BRAF) are serine/threonine kinases that bind to the effector region of RAS-GTP, thus inducing translocation of the protein to the plasma membrane. Following activation of GTP-bound RAS, RAF is in turn activated by interaction with RAS and by homodimerization and phosphorylation (Chong et al., 2001; Luo et al., 1996; Weber et al., 2001). The RAF kinase initiates a phosphorylation cascade by phosphorylating

and activating the serine/tyrosine/threonine kinase MEK, which in turn phosphorylates and activates a mitogen-activated protein kinases (MAPK) including extracellular signal regulated kinase (ERK), which are serine/threonine-selective protein kinases.

Finally, the ERK/MAPK component in this kinase cascade phosphorylates target proteins such as the transcription factor MYC and FOS and thereby regulate gene transcription (Leone et al., 1997; Lo et al., 2006; Sears et al., 1999). In addition, SRC family kinases can promote the above signaling pathway by phosphorylating RAF in the presence of RAS (Weber et al., 2001; Williams et al., 1992), or binding to and phosphorylating RAS directly on a conserved tyrosine at position 32 (Bunda et al., 2014).

Apart from the MAPK pathway, phosphatidylinositol 3-kinase (PI3K) is another main effector pathway of RAS, regulating cell growth, cell cycle entry, cell survival, cytoskeleton reorganization, and metabolism (Castellano and Downward, 2011; Tsai et al., 2012). PI3K is also a key component in insulin and growth factors pathways, growth factor receptors such as IGF1 receptor, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and others can activate PI3K (Vivanco and Sawyers, 2002). Similar as activation of GRB2 discussed above, PI3K activation triggered by binding with tyrosine kinase receptors (RTK), dimerization and autophosphorylation at tyrosine residues of the RTKs, which allows them to interact with SRC homology 2 (SH2) domain-containing molecules (Pawson and Nash, 2003; Schlessinger, 2002). It was reported that PI3K activation pathways depend on the adaptor protein GRB2 as well through a large complex including RAS, SOS and GRB2-associated binder (GAB).

The second messenger PIP3 is generated by phosphorylation of PIP2, where tumor suppressor PTEN can stop the process by dephosphorylating PIP3 to PIP2. PIP3 is a membrane-docking site for AKT and it binds to phosphoinositide dependent kinase 1 (PDK1), which further activates AKT by phosphorylating it at threonine 308 at the plasma membrane. Activated AKT subsequently inhibit pro-apoptotic BCL-2 family members BAD and BAX thereby promoting cell survival (Cantley, 2002; Engelman et al., 2006; Manning et al., 2002). AKT also downregulates transcription factor NF- κ B, and phosphorylates MDM2, resulting in increased p53 degradation, in turn leading to decreased p53 mediated apoptosis.

In addition, PI3K/AKT activates mTOR-signaling. mTOR (mammalian target of rapamycin) serves as a core component of two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which regulate different cellular processes. mTORC1 functions as a sensor of nutrition and redox, responding to growth factors, energy

status, amino acid levels, and cellular stress, its activation stimulates increased translation. mTORC1 is regulated by tuberous sclerosis complex proteins 1 and 2 (TSC1 and 2), which form a protein complex. TSC2 is a heavily phosphorylated protein that senses a variety of growth factors and stress signals such as EGF, phorbol esters, and constitutively active mutant RAS. TSC2 contains a GTPase Activating Protein (GAP) domain, via which, TSC2 subsequently releases TSC inhibition of the GTPase Ras homolog enriched in brain (RHEB), resulting in the activation of mTORC1 (Mendoza et al., 2011). In turn, mTORC1 phosphorylates S6K and promotes mRNA translation and cell growth. TSC1 does not have a GAP domain but it acts as a stabilizer of TSC2 by protecting it from degradation (Sengupta et al., 2010).

Besides the AGC (protein kinase A, G, and C) family kinase p70 ribosomal S6 kinase (S6K) as mentioned above, mTORC1 also phosphorylates eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). S6K1 and 4E-BP1 are two main regulators of mRNA translation and ribosome biogenesis, their inactivation stimulates protein synthesis and cellular growth (Mamane et al., 2006; Proud, 2009). 4E-BP1 blocks translation initiation factor eIF4E which is considered as oncogenic and a target of MYC. 4E-BP1 undergoes caspase-dependent cleavage in apoptotic cells. The cleaved 4E-BP1 binds strongly to eIF4E, fails to become sufficiently phosphorylated and thus inhibits cap-dependent translation (Tee and Proud, 2002). In addition, consecutively active 4E-BP1 mutant with all phosphorylation sites mutated to Ala delays G1 progression in cell cycle and blocks MYC-induced transformation by increasing apoptosis (Lynch et al., 2004), and suppresses tumor growth in breast carcinoma (Avdulov et al., 2004).

mTORC2 phosphorylates AKT (also known as protein kinase B) and SGK (serum glucocorticoid-induced kinase), and regulates cytoskeleton organization, cell survival, as well as lipid metabolism (Julien et al., 2010).

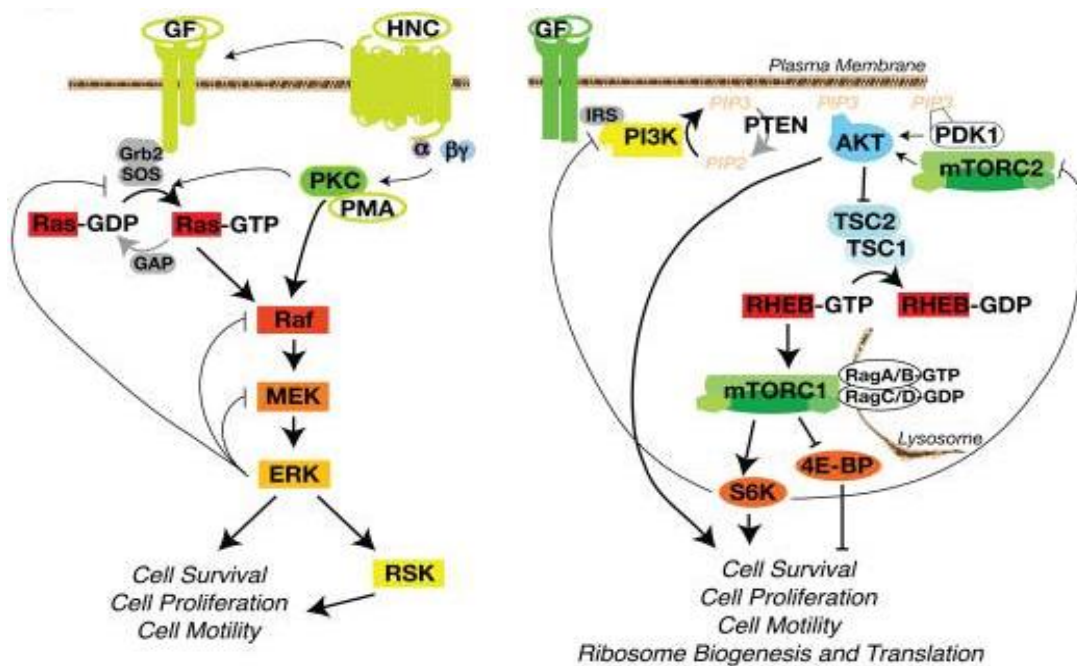


Figure 1. Cell signaling via the RAS/MAPK and PI3K/AKT pathways. The RAS/RAF/MEK/ERK and RAS/PI3K/mTOR pathways respond to extracellular and intracellular cues to control cell survival, proliferation, motility, and metabolism. Adopted from Mendoza & Blenis, *The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and Compensation*, Trends Biochem Sci. 2011 Jun; 36(6): 320–328.

1.3 CELL CYCLE

In eukaryotic cells, the cell cycle is divided into several different phases and involves two major events; DNA replication to duplicate the genome, which occur in the synthesis (S) phase and mitosis followed by cell division, which occurs in the M phase, in order to create. The S and M phase are preceded by the G1 and G2 phases, respectively. The G1, S and G2 phases are collectively called the interphase.

In G1 phase, cells synthesize mRNA and proteins in preparation for the S phase. The G1phase contains a restriction point (R) before S phase. G2 phase follows the successful completion of S phase, with rapid cell growth and protein synthesis which prepare cells for the M phase, which consists of mitosis and cytokinesis. Mitosis can be divided into prophase, prometaphase, metaphase, anaphase and telophase. During the telophase, replicated chromosomes are separated, as well as separation of other component such as membrane and cytoplasm. Cytokinesis is the part of the cell division process during which the cytoplasm of a single eukaryotic cell divides into two daughter cells. Cells can also residue in a quiescent/resting state called G0, from which the cell cycle can be activated. This G0 phase

may be a temporary resting period or more permanent if a cell has reached an end stage of development and will no longer divide.

The cell cycle is tightly monitored and controlled by positive and negative regulators called cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. CDKs are a family of serine/threonine kinases whose activity depends on association with cyclins, which are non-catalytic positively regulatory subunits interacting with specific CDK substrates.

In general cyclin/CDKs complexes are the positive regulators to promote cells enter the next phase, such as cyclin D/CDK4 (upregulated in G0/G1), cyclin E/CDK2 (upregulated in G1/S transition) and cyclin B/CDK1 (upregulated in M and G2). The activities of the cyclin/CDK complexes are counteracted by CDK inhibitors (CKIs), which are negative regulators of the cell cycle. These consist of the CIP/KIP (CDK interacting protein/Kinase inhibitory protein) family including the p21, p27 and p57, and INK4/ARF family (Inhibitor of Kinase 4/Alternative Reading Frame) which includes p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, p19^{INK4d}. p21, p27 and p57 primarily bind and inhibit cyclin E/CDK2, cyclin A/CDK2 and cyclin B/CDK1 complexes (Bouchard et al., 1999; Harper et al., 1995; Seviour et al., 2016), limiting G1/S phase and cell proliferation progression. As a major target of p53, p21 is induced following p53 activation during oncogenic stress and DNA damage to rapidly arrest the cell cycle in order to prevent tumorigenesis (Abbas and Dutta, 2009; Claassen and Hann, 2000; Passos et al., 2010). The INK4 family proteins bind to CDK4 and CDK6 and induce an allosteric change that leads to the formation of CDK/INK4 complex rather than CDK/cyclin complex thereby preventing G1-S phase transition (Sherr and Roberts, 2004).

Checkpoints are surveillance mechanisms that monitor the order, integrity, and fidelity of the major events of the cell cycle, such as cell size control, DNA damage control, DNA replication control etc.(Barnum and O'Connell, 2014).The well characterized checkpoints are G1 checkpoint, G2/M (DNA damage) checkpoint and metaphase or spindle checkpoint.

In the presence of mitogenic stimuli, the MAPK kinase cascade, which is activated by RAS signaling pathway, will in turn activate cyclin D/CDK4 and cyclin D/CDK6 complex, therefore promoting G1 progression. On the contrary, tumor suppressor pRb prevents G1 to S phase transition by binding the transcription factor E2F/DP1, which controls S phase genes (Datta et al., 2003). The pRB/E2F/DP complex acts as transcriptional repressor by recruiting histone deacetylase (HDAC) complexes to the chromatin, which successively results in the transcription inhibition and DNA synthesis repression, thereby executing the late G1 phase restriction point.

Cyclin E/CDK2 complex “hyperphosphorylates” pRb, which ensures complete inactivation of pRb. In the absence of pRb, E2F1 along with its binding partner DP1, mediates the transactivation of E2F1 target genes that facilitate the G1/S transition and S phase. The hyperphosphorylation of pRB by cyclin D/CDK4/6 and cyclin E/CDK2 thereby overrides the late G1 restriction point, after which the cell is committed to progress into the S phase of the cell cycle. On the other hand, signals activating CKIs that inhibit Cyclin D/CDK4 or cyclin E/CDK2, such as p15, p16, p12 and p27, will keep pRb in active state and prevent passing of the R point.

The G2/M checkpoint serves to prevent the cell from entering mitosis (M phase) with genomic DNA damage. The cyclin B/CDK1 complex promotes the G2-phase transition wherein CDK1 is maintained in an inactive state by the tyrosine kinases WEE1 and MYT1, while phosphatase CDC25 family protein activates CDK1 by dephosphorylation (Boutros et al., 2007).

It is reported that once approaching M phase, Aurora A and the cofactor BORA activate PLK1, which in turn activates CDC25 and downstream CDK1 activity, hence establishing a feedback amplification loop that efficiently drives the cell into mitosis (Ciccia and Elledge, 2010). Importantly, DNA damage triggers ATM/ATR kinases, which involves phosphorylation of p53 and allows for its dissociation from MDM2 and MDM4, which prevent p53 degradation thus upregulating p53 target genes p21, 14-3-3 σ , and GADD4, and downregulating CDC25. p21 inhibits the cyclin B/CDK1 complex, as well as its activating kinase CAK; GADD45 promotes dissociation of the Cyclin B/CDK1 complex; 14-3-3 σ sequesters CDC25 in the cytoplasm (Varmeh and Manfredi, 2009), thereby preventing G2-M phase transition.

1.4 APOPTOSIS AND SENESCENCE

Apoptosis is referred to a programmed cell death, which means that the cell death process follows a controlled, predictable and energy-dependent route. The initiation or activation of apoptosis could be categorized into intrinsic (mitochondrial) pathway and extrinsic (death receptor) pathway (Koff et al., 2015). Both pathways involve caspases, which are protease enzymes which cleave target proteins via their cysteine protease activity. They can be classified into three groups: initiator (caspase 2,8,9,10), executioner (caspase 3,6,7) and inflammatory (caspase 1,4,5,11,12,13). The first two groups play very important roles in apoptosis.

The complex apoptosis process involves many modulators that coordinate the event. Multiple stress-inducible molecules, for example, c-Jun N-terminal kinase (JNK), mitogen-activated

protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK), nuclear factor kappa B (NF- κ B) or ceramide, have been implied in transmitting the apoptotic signal (Davis, 2000; Fulda and Debatin, 2016; Karin et al., 2002).

Apoptosis normally occurs during development and aging and as a homeostatic mechanism to maintain cell populations in tissues (Elmore, 2007). It also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Therefore, apoptosis is considered as an important barrier against cancer and involved crucially in the regulation of tumor formation, it protects cells from uncontrolled proliferation and transformation. Furthermore, apoptosis critically determines the treatment response, to example, the failure to undergo apoptosis may result in treatment resistance.

The intrinsic pathway is usually activated by cellular stresses, such as radiation exposure or growth factor deprivation. The regulation of the intrinsic pathway is balanced by the pro-apoptotic (BAX, BAK and others) and the anti-apoptotic (BCL-2 and BCL-X_L and others) members of the BCL-2 family (Eischen et al., 2001; Vogler et al., 2011). There are three functionally important BCL-2 homology domains (BH1 BH2 and BH3) which are in close spatial proximity. They form an elongated cleft that may provide the binding site for other BCL-2 family members. In more detail, intracellular stress activates the pro-apoptotic BH3-only members, which have only one BCL-2 homology domain, including BIM, PUMA, NOXA, DP5, BID and others (Strasser, 2005). These proteins initiate apoptosis signaling by binding to the BCL-2 pro-survival proteins associated with the mitochondrial membrane including BCL-2, BCL-X_L and others, resulting in the recruitment of BAX and/or BAK from cytosol to mitochondrial membrane (Jeong et al., 2004; Schellenberg et al., 2013).

This leads to the permeabilization of mitochondrial membrane, followed by the release of cytochrome c from mitochondria. Cytochrome c thereby activates apoptosome (APAF-1) which in turn cleaves the pro-enzyme of caspase-9 into the active dimer form, by dephosphorylation inducing conformational changes. They induce the activation of caspase-9, and downstream caspase-3, 6, and 7, eventually leading to apoptosis (Hotchkiss et al., 2009).

It was reported that the activation of BCL-2 pro-apoptotic members is cell type specific and dependent on the different sources of stresses. Moreover, the pro-apoptotic members can downregulate pro-survival members (Youle and Strasser, 2008).

The other major apoptosis pathway, extrinsic pathway, is initiated by activation of tumor necrosis factor (TNF) super-family such as FasL, followed by binding to the cell-surface death receptors such as FAS or TNFR that have an intracellular death domain. FAS-associated death

domain (FADD) binds to above death receptors on death domain (DD) at the plasma membrane so to recruit caspase-8 via death effector domain (DED) binding. Caspase-8 is then activated by FADD by formation of the death inducing signaling complex (DISC), and subsequent effectors downstream caspase-3, 6, and 7 for eventual proteases activation. The BH3-only protein BID is essential for death receptor-mediated apoptosis in beta cells, thereby providing cross-talk between the two apoptotic pathways (Hotchkiss et al., 2009; Thomas et al., 2009).

Oncogenes like *BCL2* are potent negative regulator of apoptosis whereas poor inducers of cell proliferation. Therefore, it is not sufficient to drive tumorigenesis as a single oncogenic event. The cooperation of oncogenes such as *MYC* and *BCL2* lead to suppress apoptosis and drive proliferation at the same time, thereby resulting in cellular transformation. Oncogene *BCR-ABL*, which is generated by translocation of *c-ABL1* on chromosome 9 with the *BCR* gene on chromosome 22, can activate signaling pathways that simultaneously induce cell proliferation (PI3K, JAK/STAT, and RAS/RAF/MEK/ERK pathways) and suppress apoptosis (by downregulating tumor suppressors), thereby leading to transformation (Fernald and Kurokawa, 2013).

Cellular senescence is defined as an irreversible growth arrest, irrespective of the presence of growth factors. Senescence is characterized with a high metabolically activity, enlarged morphology and a secretory phenotype. Apart from its normal functions which facilitates wound healing, tissue repair, and development, senescence is considered as a potent anticancer mechanism.

Senescence can be triggered telomere erosion, so called “replicative senescence”, and is associated with cellular aging process. Therefore, senescence is recognized as an important contributor to aging and age-related diseases. In addition, premature senescence can be induced by different types of stress, inducing oxidative stress, DNA damage, and oncogene deregulation. Therefore, it is also suggested as a potential anti-tumor therapy (Debacq-Chainiaux et al., 2009). Senescence is considered as a barrier against tumorigenesis as it suppresses tumor formation by arresting cell cycle and eliminaing the tumor cells via the immune system (Kuilman et al., 2010). SASP (senescence-associated secretory phenotype) is a major trait of senescence cells, which include secreted proteins such as proinflammatory cytokines, lymphokines, growth factors, angiogenic factors, and matrix metalloproteases, comprising the senescent cell secretome. The SASP factors therefore promote angiogenesis, recruit immune cells for clearance of senescent cells and remodel the extracellular matrix (Coppe et al., 2010; Kim et al., 2018; Malaquin et al., 2016). Senescence could potentially be utilized for anti-tumor therapy (pro-senescence therapy). However, the role of the SASP is

controversial and may favor tumorigenesis depending on the context, such as driving chronic inflammation that promote cancer and degenerative diseases (de Visser et al., 2006; Grivennikov et al., 2010; Grivennikov and Karin, 2010; Saijo et al., 2010; Terzic et al., 2010). Learning how to modulate the SASP (Kuilman and Peeper, 2009) could reduce the negative sides of inflammation, promote tissue repair and the immune-mediated clearance of senescence tumor cells that otherwise may escape treatment and drive cancer progression.

1.5 DNA DAMAGE

To maintain the homeostasis of cells, multiple mechanisms are developed including DNA damage detection and repair, cell cycle regulation, apoptosis and senescence. One of the hallmarks characterizing cancer cells is genomic instability that result in the accumulation of chromosomal rearrangements such as deletions, amplifications, inversions, and translocations, resulting in extensive aneuploidy. The stability of the genome is guarded by DNA damage stress response (DDR) pathway. The DDR pathway is independent of ARF-MDM2-p53 tumor suppressive pathway, and is a mechanism that detects DNA lesions, pass through signals in the cell that promote DNA repair or alternatively cell cycle arrest, and - if the DNA damage cannot be repaired - apoptosis or senescence (Harper and Elledge, 2007; Maclean et al., 2007; Rouse and Jackson, 2002).

DDR is a multi-steps pathway that initiates with recognition of damage DNA by specialized “sensor proteins”, followed by DNA repair after activation of DNA damage check point, which halts the progression of cell cycle to prevent passing genomic abnormalities to the daughter cells during cell division.

The DDR pathway is tightly controlled by post-translational modifications (PTM), including phosphorylation, ubiquitination, sumoylation, methylation, acetylation and other modifications (Gorgoulis et al., 2005; Jackson and Bartek, 2009; Norbury and Hickson, 2001; Rouse and Jackson, 2002; te Poele et al., 2002). ATM (ataxia telangiectasia mutated) and ATR (adenosine triphosphate), as well as protein kinase catalytic subunit (DNA-PKcs), which all belong to the PIKK (phosphatidylinositol 3-kinase-related kinase) protein family, are central in DDR signaling. ATM and ATR are activated in response to double strand breaks (DSBs) and replication stress, respectively, with overlapping but nonredundant activities (Blackford and Jackson, 2017; Jackson, 2009; Jackson and Bartek, 2009). ATM, ATR and DNA-PKcs all require a specific co-factor for stable recruitment to DNA damage sites, which is the MRN

complex for ATM (Falck et al., 2005), ATRIP for ATR (Zou and Elledge, 2003), and Ku80 for DNA-PKcs (Gell and Jackson, 1999; Hanakahi and West, 2002; Singleton et al., 1999).

The MRN complex consists of MRE11, RAD50 and NBS1. It plays an important role of initiating DDR signaling and DSB repair. MRE11 is involved in the detection of DSB and the repair pathway selection (Lavin, 2007; Lee and Paull, 2007; Williams et al., 2007), as well as coordinating the alignment of broken chromosomes (Williams et al., 2008). RAD50 collaborates with MRE11, holding different DNA molecules together thereby facilitating ligation of DNA ends (de Jager et al., 2001). NBS1 protein was identified from Nijmegen breakage syndrome (NBS) which is characterized by increased sensitivity to ionizing radiation (IR) and a high frequency of malignancies (Rodier et al., 2009; Weemaes et al., 1981). NBS1 forms complex with MRE11 and RAD50 (MRN complex), and it is considered as the one of the first proteins recruited to DSB, to form radiation-induced nuclear foci. NBS1 acts as a damage sensor/mediator that recruits the key transducer ATM kinase to DSB sites (Chapman and Jackson, 2008; Kang et al., 2002; Nakanishi et al., 2002). There have been evidence indicating that NBS1 could be used to measure the DNA damage initiation and it has multifunctional roles in response to DNA damage from a variety of genotoxic agents.

A variety of sophisticated DNA repair machineries can be activated in response to endogenous and exogenous genotoxic attacks. When a single strand damage occurs, nucleotide excision repair (NER) removes a variety of helix-distorting lesions such as typically induced by UV irradiation, whereas base excision repair (BER) targets oxidative base modifications. Mismatch repair (MMR) scans for nucleotides that have been erroneously inserted during replication.

DNA double strands breaks (DSBs) can be repaired either by homologous recombination (HR) or by non-homologous end joining (NHEJ). HR is a genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. In contrast to HR, NHEJ does not require homology direction, it utilizes short homologous DNA sequences called microhomologies to guide repair. Inappropriate NHEJ can cause translocations and telomere fusion which lead to the tumorigenesis. Whether HR or NHEJ is used to repair DSB is mainly determined by the phase of cell cycle: HR is usually utilized after DNA replication, in the S and G2 phases when sister chromatids are available. NHEJ is predominant in the G1 phase of the cell cycle (Li and Heyer, 2008; Weterings and Chen, 2008). Upon induction of double strands breaks (DSBs) by agents such as ionizing radiation (IR), chemotherapeutic agents, as well as by-products of normal cell metabolism, notably reactive oxygen species (ROS), chromatin relaxation occurs rapidly. The stress-activated protein kinase, c-Jun N-terminal kinase (JNK), phosphorylates SIRT6 on serine 10 in response to oxidative

stress and it facilitates the mobilization of SIRT6 to DNA damage sites and the activation of poly (ADP-ribose) polymerase 1 (PARP1) by mono-ADP ribosylation of PARP1 on lysine 521. PARP1 in turn mediates the initial recruitment and activation of the MRN/ATM complex at DSBs. ATM kinase is thereby activated by MRN and histone acetyltransferase TIP60, resulting in the phosphorylation of checkpoint kinase 2 (CHK2) on Thr68. CHK2 in turn phosphorylates downstream targets including CDC25, E2F1 and PML (Promyelocytic leukemia protein). It also phosphorylates p53 on serine 20, which interferes with MDM2 binding thereby leading to stabilization of p53 (Hirao et al., 2000; Van Meter et al., 2016).

ATM also phosphorylates the histone variant H2AX on serine 139 (γ H2AX). The γ H2AX-dependent signaling cascade is thereafter induced, which involved the recruitment of DNA damage response proteins such as MDC1, RNF8, RNF168, BRCA1 and 53BP1 to DSBs for initiating DNA repair (Panier and Boulton, 2013; Scully and Xie, 2013; Starita and Parvin, 2003).

Unlike typical IR-induced DSBs, DNA lesions induced by UV light or replication stress, rather than ATM activation, lead to replication fork stalling and accumulation of replication protein A (RPA)-coated ssDNA regions, which recruit the ATR/ATRIP (ATR-interacting protein) complex and phosphorylate the RAD17/RFC2-5 complexes which are bound to the DNA, thus activating the ATR signaling cascade and checkpoint kinase 1 (CHK1) phosphorylation (Zou and Elledge, 2003). ATR phosphorylates substrates such as p53, BRCA1, CHK1, and RAD17. The phosphorylation of ATR substrates collectively inhibits DNA replication and mitosis and promotes DNA repair, recombination, or apoptosis. Many substrates of CHK1 have been identified such as p53, CDC25, p21 etc. (Zhang and Hunter, 2014), and CHK1 play an important role in transmitting the signal from ATR leading to cell cycle arrest and repair upon DNA damage.

As a downstream target of ATM and ATR, p53 plays an important role in DDR. Treatment of cells with DNA damaging agents such as ultraviolet light (UV), ionizing radiation (IR), and numerous cancer therapeutic and/or DNA damage-inducing compounds, increases P53 protein levels, either by phosphorylation and degradation of MDM2, or by phosphorylation of p53 which affects the association of MDM2. Moreover, by inducing p21, p53 causes cell cycle arrest, either in the G1 phase before DNA replication or in the G2 phase before mitosis (Fritsche et al., 1993; Hartwell and Kastan, 1994; Kastan et al., 1991; Maltzman and Czyzyk, 1984; Zhan et al., 1993). This provides a window for DNA repair or elimination of cells with severely damaged DNA through apoptosis by transcriptional activation of pro-apoptotic genes by p53 or through transcription-independent mechanisms (Lakin and Jackson, 1999).

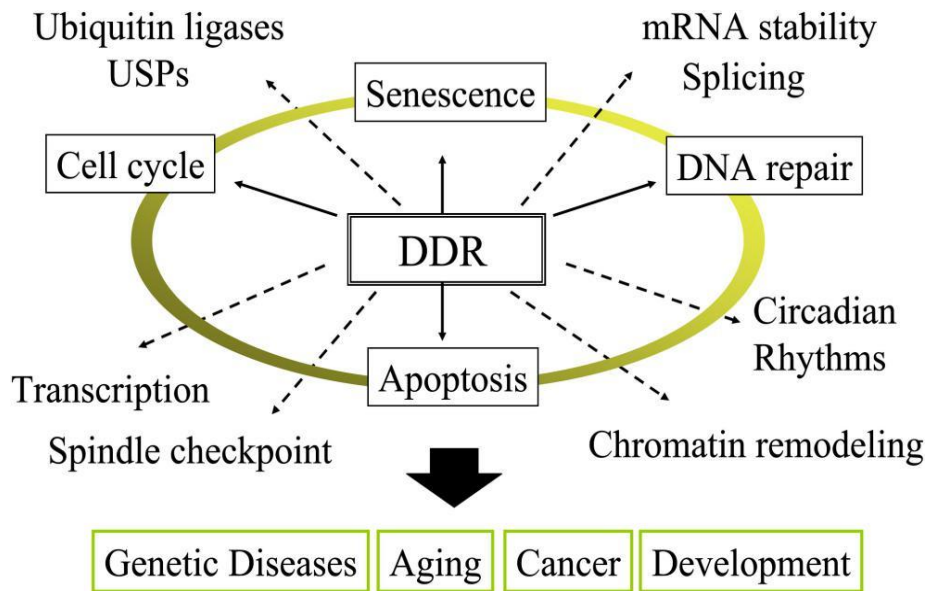


Figure 2. Biochemical and Physiological Consequences of the DNA Damage Response. Adopted from *The DNA Damage Response: Ten Years After*, J.W. Harper and S.J. Elledge, *Molecular Cell*, 2007

1.6 THE MYC FAMILY

Over 40 years ago, *MYC* was discovered as a viral oncogene (v-*MYC*) carried by some avian retroviruses that cause **myelocytomatosis**, a type of leukemia, from which the name originates. After identifying the cellular homologue (*MYC* or *c-MYC*) in chicken, the homologous gene in humans was identified in Burkitt lymphoma and was found altered and over-expressed in various cancers. Later, *N-MYC* (*MYCN*) and *L-MYC* (*MYCL*) were discovered and became part of the *MYC* family of proto-oncogenes (Brodeur et al., 1984; Kohl et al., 1983; Nau et al., 1985; Schwab et al., 1983).

Most of the functional studies of *MYC* family members have focused on *MYC* and *MYCN*, but all *MYC* family members share highly conserved functional domains. Moreover, the expressions of the three genes are differently regulated.

MYC is commonly expressed in most rapidly proliferating cells/tissues throughout development and continues to be expressed in dividing cells of adult tissues (Alt et al., 1986; DePinho et al., 1987; Depinho et al., 1986; Zimmerman et al., 1986).

Whereas *MYCN* is mostly expressed during embryogenesis in pre-B cells, kidney, forebrain, hindbrain, intestine, and particularly highly expressed in the developing brain. After embryonic development, *MYCN* is downregulated and not expressed in most adult tissues but only

expressed in immature cells of many lineages in regenerating tissues (Okubo et al., 2005). The *MYCN* oncogene was first identified as an amplified gene in neuroblastoma, which are the most common solid tumor in early childhood, and from where *MYCN* got its name. It is also found implicated in retinoblastoma, glioblastoma, medulloblastoma, astrocytoma and small cell lung cancer cells (Beltran, 2014; Breit and Schwab, 1989; Fletcher et al., 1991; Gasperi-Campani et al., 1998; Ingvarsson, 1990; Leanna Cheung, 2013; Lee et al., 2016; Siegfried and Delisle, 2018; Yokota et al., 1989).

The *MYCL* gene is less well characterized, but it is homologous to *MYC* and *MYCN*, and is found amplified in lung cancers therefore named as *L-MYC* (Barrett et al., 1992; Nau et al., 1985; Oster et al., 2002; Zimmerman et al., 1986).

1.6.1 MYC AS A TRANSCRIPTION FACTOR

MYC functions mainly as a transcription factor, it is known to both activate and repress transcription from target genes by binding to DNA and to different cofactors. MYC regulates expression of a large number of genes that involved in cell cycle progression, cell growth, apoptosis, senescence and metabolism, hence modulates the wide range of cellular events that when deregulated promote tumorigenesis. (Conacci-Sorrell et al., 2014; Fletcher and Prochownik, 2015).

MYC belongs to the basic/helix-loop-helix/leucine zipper (bHLHZip) family of transcription factors. In order to bind DNA, bHLHZip proteins need to dimerize. MYC homodimerization does not occur under physiological conditions, and therefore MYC to dimerize with another bHLHZip protein, MAX, which results in stable heterodimer formation with specific DNA-binding activity. The MYC:MAX complex binds to the DNA sequence 5'-CACGTG-3' or similar sequences, known as an E boxes, and thereby MYC can activate transcription (Berg, 2011; Blackwell et al., 1990; Tu et al., 2015).

MAX was initially identified as a ubiquitous bHLHZip protein essential for MYC target gene expression. In addition, MAX forms homodimers, or forms heterodimers with MXD family of bHLHZip proteins such as MXD1 (MAD1), MXI1, as well as MNT, which act as antagonists to MYC function (Conacci-Sorrell et al., 2014; Luscher and Vervoorts, 2012; Conacci-Sorrell et al., 2014; Grandori and Eisenman, 1997; Hann, 2014). The bHLHZip family members show distinct preferences of dimer formation. All these above heterodimers also bind E-boxes, as do Max homodimers (Amati and Land, 1994; Grandori et al., 2000; Hu et al., 2005).

Functionally distinct classes of target genes differ in the enhancer box sequences (E-box) bound by MYC, suggesting that different cellular responses to physiological and oncogenic MYC levels are controlled by promoter affinity (Walz et al., 2014).

MYC has been reported to regulate the expression up to about 15% of all human genes involved in many physiological functions (Patel et al.). This view, however, has been challenged by recent work that suggested that MYC works as a global amplifier of transcription, which would mean that MYC does not simply target specific genes but universally amplifies transcription of all expressed genes (Lin et al., 2012; Nie et al., 2012). There have been considerable debate regarding these two views on MYC's transcriptional functions (Levens, 2013; Rahl and Young, 2014; Sabo et al., 2014; Kress et al., 2015). One of the critical disagreements is how to explain transcriptional repression by MYC. MYC has been shown to interact with the zinc finger protein MIZ1 to represses distinct target genes (Walz et al., 2014; Wiese et al., 2013). The global amplification theory seems to argue that “repressed” genes, rather than being directly repressed by recruitment of repressor proteins by MYC, may actually also be upregulated by MYC, only less than the average compared with other genes.

In contrast, the new data provided by Sabo et al and Walz et al suggest that the promoter/enhancer invasion and RNA amplification by MYC are two independent events. By either up or down regulating distinct sets of target genes, MYC overexpression modifies cellular status, which indirectly affects global RNA amplification according to this view (Walz et al., 2014; Sabo et al., 2014; Kress et al., 2015).

1.6.2 MYC STRUCTURE AND INTERACTION WITH COFACTORS

MYC harbor several conserved so called MYC homology boxes: MBI, MBII, MBIII and MBIV in addition to the C-terminal bHLHZip domain, which binds DNA in a heterodimeric complex with MAX, as described in the previous section.

The N-terminally located transactivation domain of MYC (TAD) is essential for MYC-mediated transformation, differentiation and apoptosis. This region serves as an interaction platform for proteins involved in chromatin and histone modification as well as ubiquitination and subsequent degradation (Barrett et al., 1992).

MBI serves as a phosphodegron and is involved in the ubiquitylation and proteasomal degradation of MYC involving threonine 58 (Thr58). Point mutations at this phosphorylation

site block ubiquitin E3 ligase FBXW7 binding and augment MYC stability. MB1 also contains signals for activation linked to phosphorylation of serine 62 (Ser62). These phosphorylations will be discussed further in section Regulation of MYC.

MBII is the most well studied region within the TAD, it harbors sites for MYC cofactor binding. The molecular and biological functions of MYC are suggested to be regulated by binding to different cofactors, which potentially could yield a diverse range of outcomes in a cell-type- and/or context-dependent way. These different complexes of cofactors are thought function differently in the regulation of MYC's transcriptional activity (Agrawal et al., 2010; Koch et al., 2007).

One of the factors binding MBII is transformation/transcription domain-associated protein (TRRAP), which belongs to the phosphatidylinositol 3-kinase-related kinase (PI3K) protein family. TRRAP is found in various large chromatin complexes with histone H3 and H4 acetyltransferase activity (HAT) involved in epigenetic regulation of transcription. By recruitment of TRRAP to chromatin, MYC's stimulation of RNA polymerase II, I and III (pol II and pol III) transcription is enhanced (Liu et al., 2003; McMahon et al., 2000), as well as cell transformation (McMahon et al., 1998). It is also required for P53, E2F1, and E2F4 mediated transcription activation. TRRAP forms complexes with the HATs GCN5, p300/CBP-associated factor (PCAF), and TIP60, which by relaxing the chromatin structure at gene promoters allow MYC to promote transcription driven by pol II, I and III (Ikura et al., 2000).

Reptin proteins TIP48 and TIP49 also interact with MYC through MBII independent of TRRAP binding (Cheng et al., 1999; Wood et al., 2000), they enhance MYC transformation capability through their ATPase and helicase activities.

Moreover, MBII is also involved in MYC protein turnover. S phase kinase-associated protein 2 (SKP2), an F-box protein, is the substrate-binding subunit of SCF ubiquitin ligase complexes that targets MYC, E2F-1 and p27^{KIP1} (p27) for degradation, and suppresses p53-mediated apoptosis (Hydbring et al., 2017). Since SKP2 targets MYC for destruction, one could assume that SKP2 downregulates the transcriptional activity of MYC. However, SKP2-mediated ubiquitylation of MYC was shown to have a dual function, stimulating MYC transcriptional activity but at the same time targeting MYC for destruction by the proteasome. Further, SKP2 stabilizes the MYC protein level indirectly by promoting ubiquitylation and degradation of the E3 ligase TRUSS (Jamal et al., 2015), which targets MYC for degradation (Farrell and Sears, 2014). SKP2 binds to two regions within MYC: residues 129–147 in the amino terminus, containing MBII, and 379–418 in the carboxy terminus, which contains the bHLHZip region.

The absence of phosphorylation sites within the Skp2 binding sites of MYC suggests the interactions might differ from other SCF interactions. MYC can in turn upregulate SKP2 expression by binding to E box in the promoter.

MBIII is mostly related to transcription repression and mediating apoptosis, transformation, and tumorigenesis (Herbst et al., 2005; Kurland and Tansey, 2008). MBIII also involves in binding with WDR5, a WD40-repeat protein that is present in multiple chromatin regulatory complexes, including H3K4 methyltransferases (Ang et al., 2011; Chen et al., 2008; Thomas et al., 2015).

MBIV overlaps with nuclear localization signal (NLS), and it is necessary for DNA binding, required for apoptosis and transformation via binding with p300, FBXO28 (residues 294-439) and p27 (residues 294-366) (Adhikary and Eilers, 2005; Bahram et al., 2016; Cepeda et al., 2013; Cowling et al., 2006).

The C-terminal MYC:MAX dimeric HLHZip region presents a large solvent-accessible surface area ($\sim 1000 \text{ \AA}^2$) forming a platform for binding by other factors, such as MIZ-1 and SKP2 (Cheng et al., 1999; Nair and Burley, 2003; Peukert et al., 1997; Staller et al., 2001; von der Lehr et al., 2003). MYC:MAX complex formation with MIZ-1 mediates repression of multiple target genes by MYC and the ratio of MYC and MIZ-1 bound to each promoter correlates with the direction of response (Staller et al., 2001).

INI1, which is the core factor of the SWI/SNF chromatin remodeling complex, binds with MYC via bHLHZip region, and without interfering with MYC:MAX interaction. INI1 functions as a tumor suppressor, and share many common target genes with MYC (Cheng et al., 1999; Sammak et al., 2018; Stojanova et al., 2016).

The positive transcription elongation factor b (P-TEFb) which consists of the cyclin dependent kinase 9 (CKD9) and its regulatory subunit cyclin T1, is another crucial cofactor for MYC transactivation. MYC:MAX heterodimer recruit P-TEFb complex to its targets, allowing P-TEFb phosphorylates the carboxyl-terminal-domain (CTD) of the larger subunit of RNA polymerase II (pol II) and negative elongation factors to facilitate transcription elongation (Gargano et al., 2007; Peterlin and Price, 2006).

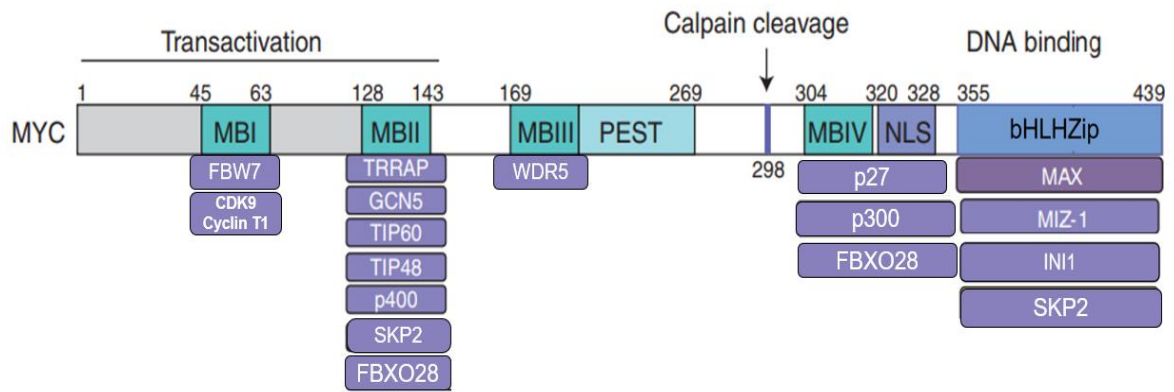


Figure 3. The structure of MYC and its cofactors. Proteins shown in purple boxes are MYC-interacting factor regulating different aspects of MYC function.

1.6.3 REGULATION OF MYC

MYC is regulated tightly at every level from RNA synthesis to protein degradation, its transcription is particularly responsive to multiple diverse physiological and pathological signals. Aberrant MYC expression is usually due to induction caused by upstream signals, but can also be caused by amplification or translocation of the *MYC* gene.

MYC transcription can be activated by several mitogenic signaling pathways, including tyrosine kinases receptors such as EGFR, PDGFR and their downstream effectors RAS/RAF/MAPK/ERK, or WNT, Janus kinase/signal transducers and activators of transcription (JAK/STAT), NOTCH as well as NF- κ B. Transcription factors harnessed by these pathways bind to the *MYC* promoter to regulate transcription initiation and elongation, dependent on cellular context and chromatin conformation (Liu and Levens, 2006; Wierstra and Alves, 2008).

MYC is rapidly turning over with a half-life of 30 minutes (Andresen et al., 2012). There are many modulators that regulate MYC's stability and turnover. In MYC's transactivation domain, two phosphorylation sites are controlling its degradation (Farrell and Sears, 2014): serine 62 (Ser62) and threonine 58 (Thr58), both of which are located in the highly conserved region of MYC designated MYC Box I (MBI) within the second exon of MYC. The phosphorylation of Thr58 needs to be primed by the phosphorylation of Ser62. Phosphorylation of Thr58 is mediated by glycogen synthase kinase 3 β (GSK3 β). The F-box protein FBXW7, acting as the substrate receptor of an SCF (SKP1/cullin1/F-box) complex, recognizes the phosphorylated Thr58 residue, thereby promoting ubiquitination and subsequent proteasomal degradation of

MYC(Welcker et al., 2004b; Yada et al., 2004;Becker, 2012).The phosphorylation of Ser62 by MAPK/ERK can stabilize MYC and thus promote proliferation (Dworakowska et al., 2009; Tsai et al., 2012). Also, CDK1 and CDK2 are involved in Ser62 phosphorylation, and the latter is highly correlated with MYC's function of repressing Ras-induced senescence (Hydbring et al., 2010).

Posttranslational modification players (Aurora A, SIRT1), acetyltransferases that block ubiquitination (CBP/p300, TIP60) are also involved in MYC's stability regulation (Koepp et al., 2001; Popov et al., 2010; Welcker et al., 2004a; Yada et al., 2004).

The regulation of MYC and its therapeutic implication will be further discussed in later chapters.

1.6.4 MYC AND CANCERS

The first implication of MYC in human cancer came from observations in Burkitt's lymphoma, which is highly aggressive B cell neoplasm. It is characterized by the translocation of MYC gene on chromosome 8 to immunoglobulin heavy locus (IGH) on chromosome 14, juxtaposing the IGH enhancer within the vicinity of the MYC, resulting in deregulation of MYC expression.

Amplification of the MYC gene is another reason for MYC overexpression, and was first identified in the human leukemia cell line HL60 in 1982 (Collins and Groudine, 1982). In fact, the *MYC* family genes are the most frequently amplified oncogenes in human cancer (Beroukhim et al., 2010). According to data from The Cancer Genome Atlas (TCGA), MYC amplification occurs in 40% of basal-like breast cancer, 34% in ovarian cancer, 30% in lung adenocarcinoma and 29% in colon cancer. Likewise, amplifications of MYCN and MYCL were discovered in tumors of about 50% of neuroblastoma and 20% of small cell lung cancer, respectively (Brodeur et al., 1984; Gasperi-Campani et al., 1998; George et al., 2015; Jung et al., 2017b; Nau et al., 1985; Nesbit et al., 1999). MYC is rarely point mutated, with the exception of lymphomas. Apart from amplification and translocation, deregulation of MYC can be caused by deregulation of signaling upstream of MYC (Poole and van Riggelen, 2017), such as the constitutive activation of growth factor signaling, loss of checkpoint components such as p53, loss of E3 ubiquitin ligases targeting MYC etc. It is reported that over 50% of human cancers have increased or deregulated MYC expression. Moreover, MYC over expression is often highly correlating with aggressive, poorly differentiated tumors, so as poor prognosis of patients and low survival rate.

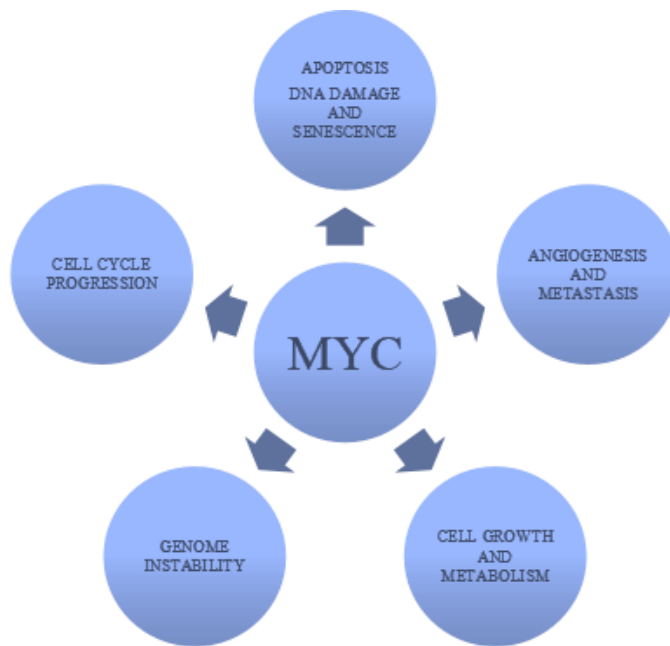


Figure 4. Biological functions of MYC and impacts of MYC dysregulation.

1.6.5 CELLULAR FUCTIONS OF MYC

1.6.5.1 MYC and apoptosis

MYC is well known to participate in the apoptotic response. It is tipping the equilibrium balance of pro- and anti-apoptotic proteins, by suppressing the expression of anti-apoptotic proteins such as BCL-2 and BCL-X_L, (Eischen et al., 2001; Fulda and Debatin, 2016).

MYC also induces apoptosis through the ARF-MDM2-p53 pathway, which stabilizes p53 production thereby triggers the tumor suppressive response. The high ARF levels as consequence of MYC activation or deregulation, inhibit MDM2 which is a ubiquitin ligase that in turns ubiquitylates p53, finally result in allowing a robust p53 transcriptional response that induce apoptosis (Grandori et al., 2000; Nesbit et al., 1999; Ponzielli et al., 2005; Zindy et al., 1998).

It might sound controversy that MYC involves in both proliferation and apoptosis pathways. One of the explanations for how MYC determine the cell's fate is that different levels of MYC expression might engage different sets of target genes, which might preferentially head toward adverse directions (McMahon; Prendergast, 1999).

For instance, upon growth factors withdraw, cells with high MYC levels experience activation of surveillance mechanisms, such as p53 induction. However, in the situation of constitutively elevated MYC in tumor cells, and additionally loss of surveillance mechanisms

such as p14ARF or p53 mutation, MDM2 overexpression, and/or by gain of prosurvival signals such as BCL-2 and NF- κ B pathway alterations, tumor cells are able to tolerate high level of MYC and avoid apoptosis.

Alternatively, MYC might engage the same set of target genes regardless of expression levels but only the extent of target gene transcription is altered. In this scenario, MYC functions more as a universal modulator of preexisting transcriptional programs (Lin et al., 2012; Nie et al., 2012; Wali et al., 2013).

1.6.5.2 MYC and senescence

MYC is a potent apoptosis inducer as a safeguard mechanism for cells, while it is not a potent inducer of senescence. On the contrary, MYC has been found to overcome RAS-induced senescence in rat embryonic fibroblasts (REFs), thereby leading to successful transformation via the direct upregulation of *E2F* genes and repression of p16 and p21. Further, MYC is reported to override BRAFV600E-induced senescence in vivo, leading to accelerated mouse lung tumor development (Hydbring and Larsson, 2010; Tabor et al., 2014).

1.6.5.3 MYC and DNA damage

MYC is highly involved in DDR pathways. For instance, reactive metabolic intermediates such as reactive oxygen species (ROS), was shown to increase upon MYC deregulation, and induce oxidative damage (Vafa et al., 2002). Upon oxidative damage, in response to hypoxia, hypoxia inducible factor 1 (HIF-1) is stabilized by ROS, and significantly contributes to the induction of VEGF for angiogenesis and the conversion of glucose to lactate for tumor glucose metabolism (Gao et al., 2007; Kaelin and Ratcliffe, 2008; Podar et al., 2008).

ATM/CHK2 and ATR/CHK1 are two different branches of DDR signaling pathways. ATM activity constitutes a barrier to malignant transformation, while, on the other hand, full activation of ATR and CHK1 is also essential for tumor maintenance (Campaner and Amati, 2012). Thus MYC-induced DDR acts as a double-edged sword in tumor progression. On one hand, it restrains proliferation and promotes apoptosis against cancer cells. On the other hand, after cancer cells have evolved to overcome the anti-proliferative effects of DNA damage, continuing to replicate in the presence of DNA damage, the overload of DDR leads to increased frequency of double strand breaks (DSBs), inappropriate cell cycle progression and genomic instability (Gorgoulis and Kotsinas, 2019; Gorgoulis et al.; Hartwell and Kastan, 1994; Jackson, 2009; Jackson and Bartek, 2009).

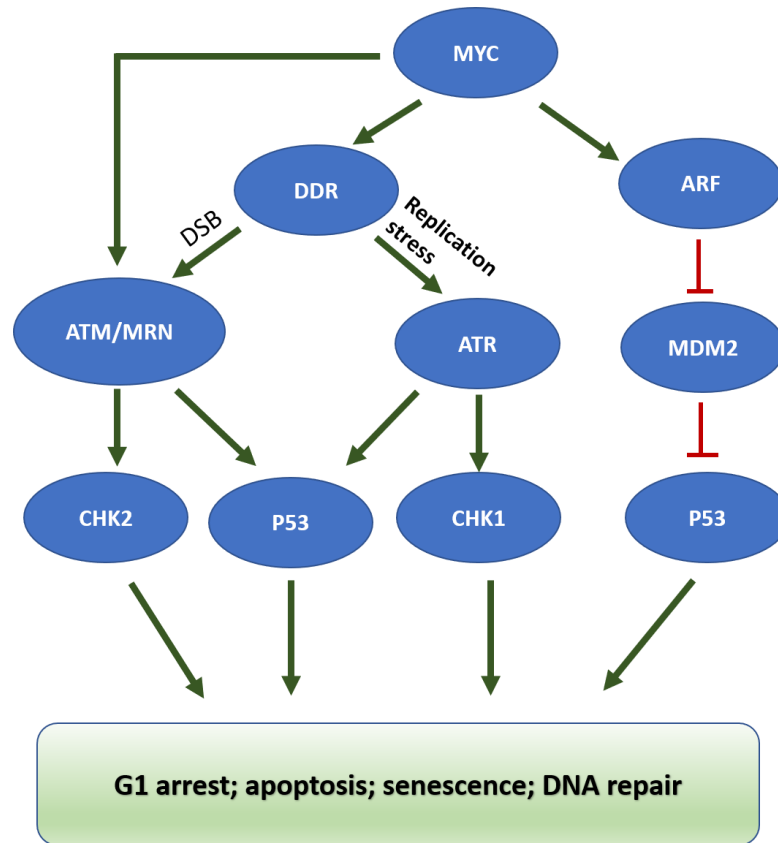


Figure 5. Effects of MYC on DDR signaling, G1 arrest, apoptosis, senescence and DNA repair. DSB (double strand breaks).

1.6.5.4 MYC and cell cycle, cell proliferation

MYC plays an important role in the regulation of the cell cycle. It pushes cell cycle progression and response to mitogenic signals. Forced MYC expression alone is sufficient to drive quiescent G0 phase cells to re-enter the cell cycle, and increase cell size. The mechanism is, at least in part, the direct activation of cyclin/CDKs expression and overriding cell cycle checkpoints. For instance, at G0/G1 checkpoint, MYC can promote cell-cycle entry and progression by increasing cyclin D/CDK4 and cyclin D/CDK6 complex. Further at G1/M checkpoint, cyclin E/CDK2 complex replace cyclin D/CDK4/6 complex to be the driven force for DNA replication. MYC involves in the cyclin E/CDK2 complex modulation by either increasing its activity or alternatively inhibiting p27. On the contrary, P53 antagonize above cell cycle progression by inducing p21. Similarly, pRB is present in G0/G1 phase of cell cycle and inhibits E2F transactivation thereby blocks downstream effectors, while cyclin/CDK complex can phosphorylate and inactivate pRB in late G1 to release E2F.

In addition, MYC induces loss of contact inhibition. As the role of MYC in sensitivity of growth stimulation also varies with cell types, the impact on proliferation is cell type dependent (Perna et al., 2012). It is speculated that cell type or other environment context determined the regulation of alternative subsets of target genes thereby affecting proliferation differently in different contexts (Ellwood-Yen et al., 2003).

1.6.5.5 MYC and metabolism

Tumor cells have enhanced conversion of glucose to lactate even in the presence of normal levels of oxygen, known as Warburg effect, was discovered by Otto Heinrich Warburg (Warburg et al., 1927). It demonstrated that cancer cells show an increased dependence on glycolysis to meet their energy needs, regardless of oxygen condition. Converting glucose to lactate, rather than metabolizing it through oxidative phosphorylation in the mitochondria, is far less efficient because less ATP is generated per unit of glucose metabolized. Therefore, a high rate of glucose uptake is required to meet increased energy needs to support rapid tumor progression. The controversies have been discussed and considered as a potential cancer target (Altman et al., 2016; Cairns et al., 2011; Dang, 2010; Dang and Kim, 2018).

To sustain the high rate of proliferation of cancer cells, there is a high demand for nutrition and energy resource like glucose, glutamine. MYC is a key regulator of cellular metabolism and drive the Warburg effect in cancer cells. For example, it cooperates with HIF-1 α to activate several genes encoding glycolytic proteins such as LDH-A and stimulates mitochondrial biogenesis. MYC upregulates glucose transporters as well as hexokinase to increase glucose import (Osthus et al., 2000). Moreover, MYC promotes glutamine import by directly inducing the expression of glutamine transporter ASCT2 (Wise et al., 2008). In addition, Myc increases the conversion of glutamine to glutamate for subsequent oxidation in the tricarboxylic acid cycle (TCA cycle) by upregulating glutaminase (GLS) both transcriptionally and post-transcriptionally (Dang et al., 2009).

1.6.6 MYC AS A CANCER TARGET

Although the MYC has been widely studied during several decades years, no MYC targeting drug is available today in clinic. The main reason for this is that MYC, like other transcription factors, lacks enzymatic activity and is an intrinsically disordered protein. Nevertheless, a lot of effort has been done to seek a promising therapeutic way of combating MYC's tumorigenic function.

1.6.6.1 MYC is a challenging cancer target

The concept of “oncogene addiction” has been proposed in the literature during recent years, meaning that certain tumor cells are dependent on a single activated oncogenic protein or pathway to maintain their malignant properties, despite the likely accumulation of multiple gain- and loss-of-function mutations that contribute to tumorigenicity (Weinstein and Joe, 2008). MYC in particular has been suggested to cause oncogene addiction in tumor cells. This is based on observations that even a short inactivation of MYC could lead tumor regression or survival improvement (Felsher, 2008; von Eyss and Eilers, 2011). This phenomenon indicates that transient or prolonged MYC inactivation may be sufficient for sustained reversal of the tumorigenic process (Boxer et al., 2004).

This offers a rationale for targeting MYC as a cancer therapy, for instance by interfering with MYC synthesis, stability or transcriptional activity. Another important reason is that MYC lies downstream of multiple key signaling pathways, for example, RAS/PI3K/AKT AND Ras/RAF/MAPK/ERK pathways that show a high degree of redundancy. Targeted therapies against mutant oncogenic proteins within these pathways therefore rapidly results in resistance development due to activation of various drug escape pathways, while the MYC pathway is considered non-redundant and therefore cannot be bypassed (Sodir and Evan, 2011). However, although MYC was one of the first oncogenes identified several decades ago, MYC targeted therapy is not clinically available today. There are multiple reasons why MYC is such a challenging therapeutic target (Horiuchi et al., 2014).

First, the most common concern is that MYC is an essential pleiotropic transcription factor that controls the expression of thousands of genes, therefore a complete inhibition of MYC could lead to severe toxicity in normal cells since MYC is generally expressed in proliferating cells. However, recent publications showed that systemic inactivation of endogenous MYC in a K-Ras driven tumor lead to tumor regression, the side effects on normal cells are reversible and tolerated (Castell et al., 2018; Sodir and Soucek, 2013; Soucek et al., 2008; Soucek et al., 2013).

Secondly, MYC is an intrinsically disordered protein that lacks enzymatic activity or recognizable pockets or clefts, making it a difficult target for structure-based drug design.

Despite all these challenges, great effort is put into MYC targeting in the scientific community and in the pharmacological industry using different strategies as discussed in the next section.

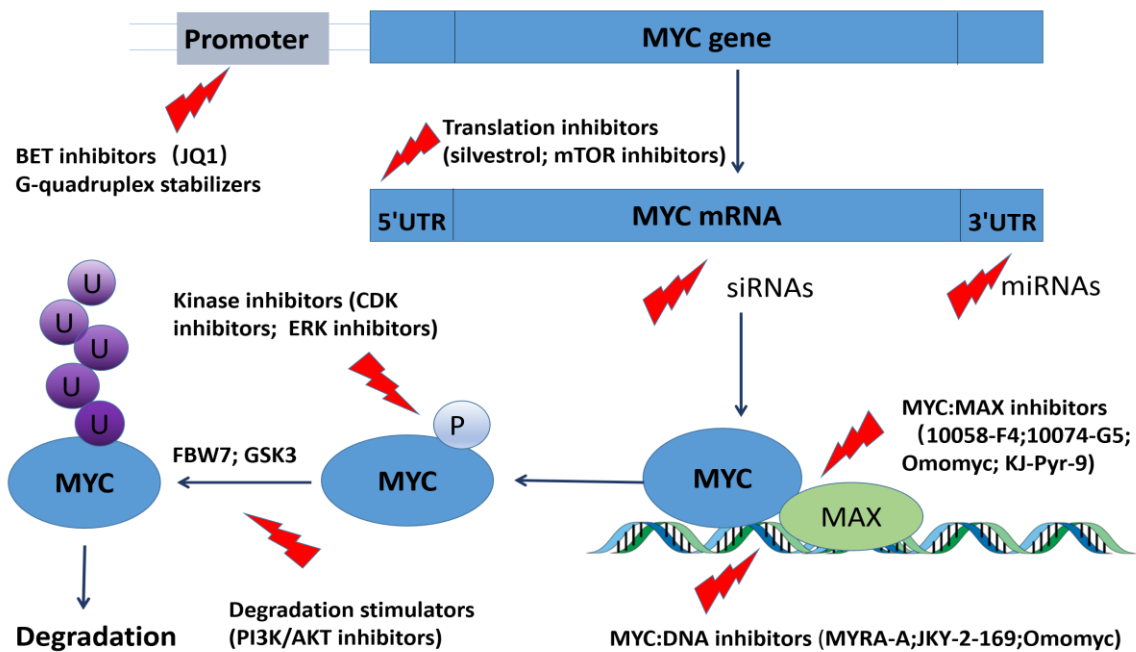


Figure 6. Different classes of inhibitors targeting MYC and signaling pathways regulating MYC.

1.6.6.2 Targeting MYC at the Transcriptional Level

JQ1 was recently discovered as a small molecule bromodomain protein inhibitor. Bromodomain and extraterminal motif (BET) proteins, are well known to be overexpressed in multiple tumor types, such as melanoma (Segura et al., 2013). BET family of proteins consist of BRD2, BRD3, BRD4 and BRDT, they are chromatin adaptor modules which recognize acetylated lysine residues on histone tails and other nuclear proteins (Shi and Vakoc, 2014), thereby contributing to the activation of RNA polymerase II-mediated initiation and elongation of transcription (Denis et al., 2006; Devaiah and Singer, 2013; Jiang et al., 1998; LeRoy et al., 2008). Research on BET inhibitors has identified them as a potential means of targeting MYC (Fu et al., 2015; Mertz et al., 2011; Shu and Polyak, 2016).

It has been shown that JQ1 downregulates transcription of the *MYC* gene through the inhibition of the BET protein BRD4 (Alderton, 2011; Delmore et al., 2011; Filippakopoulos et al., 2010). Downregulation of the MYC-dependent transcriptional network thereby results in tumor cell growth inhibition and apoptosis. It also reduced tumor growth in patient derived PDX mouse models in vivo. The underlying mechanisms include an effect on cell cycle arrest in the G1 phase and a decrease in the percentage of cells in the S phase as well as induction of apoptosis. This suggests that JQ1 has potential for cancer therapy. As the first well described and

characterized BET inhibitor, despite its inferior pharmacology property, JQ1 served as a useful tool for pre-clinical study as well as basic cancer research (Moyer, 2011).

Currently there are a dozen of BET inhibitors in the early phase of clinical trials (Andrieu et al.), and preliminary results of BET inhibitors in NUT midline carcinoma (NMC) and hematological malignancies have been modest. Further, MYC dysregulation does not appear to predict response, and *vice versa*, good response does not seem to correlate with MYC down regulation. This suggests that the BET inhibitors also have other important targets than MYC depending on cell type and context (Dombret et al., 2014; Amorim et al., 2016; Doroshow et al., 2017; Lewin et al., 2018; O' Dwyer et al.; Odore et al., 2016; Postel-Vinay et al., 2019; Stathis et al., 2016).

Reports have shown that a purine-rich strand in the nuclease hypersensitive element III1 (NHE III1) of the MYC promoter can form a G-quadruplex (G4) from duplex DNA, which was shown to be positively and negatively regulated by nucleolin and NM23-H2, respectively. The stabilization of G4s can halt transcription of downstream gene products, therefore offering a potential targets for MYC targeting therapeutic development (Brooks and Hurley, 2010; Flusberg et al., 2019; Whitfield et al., 2017).

1.6.6.3 Targeting MYC at the Post-Transcriptional Level

MYC is reported to regulate many post-transcriptional mechanisms, such as promoting mRNA capping (Cowling and Cole, 2006), regulating the abundance of splicing factors (Nadiminty et al., 2015). MYC also indirectly regulates several pathways of RNA degradation, as well as modulating miRNAs (Psathas and Thomas-Tikhonenko, 2014) and non-coding RNAs (Deng et al., 2014) transcriptionally, in which way MYC is therefore able to greatly expand the number of its indirect targets. Based on the theory above, antisense oligonucleotides were designed to target MYC mRNA and they have shown efficacy in certain tumor type (Balaji et al., 1997) .

Inhibition of MYC translation by targeting the translation initiation factors: cap-binding protein eIF4E, the RNA helicase eIF4A, and the scaffolding protein eIF4G was reported recently. It results in inhibition of MYC-dependent proliferation of tumor cells *in vitro* and *in vivo* (Castell and Larsson, 2015; Wiegering et al., 2015). mTOR kinase inhibitors could also be used to inhibit MYC mRNA translation by blocking phosphorylation/inactivation of 4E-BP1, which in turn blocks eIF4E, which promotes cap-dependent translation of MYC mRNA (Bhat et al., 2015; Castell and Larsson, 2015). However, due to induction of the negative feedback loop,

and an increased expression of several growth factor receptors and activation of the MAPK pathway, this does not work in all tumor cells, such as in colorectal cancer cells. Here, silvestrol, which is a direct inhibitor of eIF4A is an alternative (Sansom et al., 2007; Wiegering et al., 2015). By increasing the affinity between eIF4A and RNA, silvestrol sequesters and depletes eIF4A from translation initiation complexes (Bhat et al., 2015; Bordeleau et al., 2008).

1.6.6.4 Targeting MYC on protein stability and turnover

As described above, MYC is rapidly turning over through ubiquitin/proteasome-mediated degradation through the phospho-Thr58/GSK3 β /FBXW7 pathway. Therefore, the mutations of Thr58 or loss of FBXW7 will result in constitutive MYC stabilization in tumors. Active Ras induces ERK that stabilizes Myc by phosphorylation at Ser62, moreover PI3K activation blocks Myc degradation by inhibiting phosphorylation at Thr58 by GSK3 β . Small molecule inhibitors could potentially affect the MEK-ERK, CDK or PI3K-AKT pathways so to modulate MYC's stability (McKeown and Bradner, 2014; Hydbring, Castell, et al., 2016).

Phosphorylated Ser62 is also targeted by protein phosphatase 2 (PP2A), which is a family of heterotrimeric enzyme complexes that acts as tumor suppressors and are often lost in cancer, and thus modulates MYC protein stability (O'Connor et al., 2018). Cancerous inhibitor of protein phosphatase 2A (CIP2A) is one of the endogenous inhibitors of PP2A and prevents PP2A mediated dephosphorylation of MYC at Ser62, thereby resulting in stabilization of MYC in cancer cells (Soofiyan et al., 2017). Small molecule compounds targeting CIP2A (Wu et al., 2017) or PP2A activation (Gutierrez et al., 2014) were reported, but are still far from pharmaceutical application (O'Connor et al., 2018; Soofiyan et al., 2017).

It reported that GSK3- β phosphorylates and destabilizes both the MYC and MYCN protein (Chesler et al., 2006; Kenney et al., 2003) (Gustafson and Weiss, 2010; Kenney et al., 2004). Since GSK3 β is inhibited by the PI3K/AKT pathway, Dual PI3K/mTOR (Chanthery et al., 2012) show promise as means of decreasing MYC/MYCN protein levels. Upon low level of PI3K activity in neuronal cells, MYCN, which also contain equivalents to Ser62 and Thr58, undergo sequential phosphorylation by cyclin B/CDK1 and GSK3 β in G2/M phase of the cell cycle, thereby gets degraded by the FBXW7 ubiquitin ligase complex (Otto et al., 2009; Bonelli et al., 2017; Tsai et al., 2012). Aurora A kinases (AURKA) can interact with both MYCN and FBXW7 and thereby counteract degradation of MYCN (Otto et al., 2009; Gustafson et al., 2014). AURKA is therefore another feasible target to destabilize MYCN. Two ATP-competitive azepine inhibitors of AURKA) were shown to disrupt AURKA binding to MYCN

and to promote MYCN degradation via FBXW7 and showed anti-proliferative effect in MYCN-driven neuroblastoma cells (Brockmann et al., 2013).

Apart from PP2A and AURKA, the MYC-associated histone acetyltransferases CBP/P300, GCN5, and TIP60 were reported to regulate MYC stability. These bind MYC at the N-terminal TAD and induce acetylation of MYC at lysines used for ubiquitylation by E3 ligases, leading to MYC stabilization (Cowling et al., 2006). This offers another opportunity to destabilize MYC via targeting these HATs.

The natural compound oridonin was reported to promote the FBXW7-mediated proteasomal degradation of MYC, and to induce cell growth inhibition and apoptosis, and should be studied further (Huang et al., 2014; Owona and Schluesener, 2015);Liang et al., 2018; Sechet et al., 2018).

1.6.6.5 Targeting interactions of MYC and its cofactors

Activation of transcription by MYC through specific binding to E-boxes is dependent on the interaction with its partner MAX. Therefore, targeting the heterodimerization between MYC and MAX is an alternative approach to target MYC apart from trying to reduce the MYC level in cancer cells.

Omomyc is a mutant version of MYC where residues of the leucine zipper that are critical for its dimerization specificity have been mutated (Soucek et al., 2002; Soucek et al., 2008). Though Omomyc preferentially forms homodimers, it can form heterodimers with both MYC and MAX, and was shown to retain the interaction with MIZ-1 but did not bind to MXD1 or other selected bHLH proteins (Jung et al., 2017a). As a result, Omomyc functions as a dominant negative mutant and prevent MYC from triggering DNA transcriptional activation. In addition, Omomyc does not seem to affect MIZ-1-mediated MYC binding to promoters and transrepression (Savino et al., 2011; Soucek et al., 2002).

Multiple studies in mouse models of cancer demonstrated Omomyc's therapeutic impact in different types of cancer, independently of their driving mutation or tissue of origin, pointing to the key role of MYC in tumorigenesis downstream of the diverse oncogenic lesions (Annibali et al., 2014; Galardi et al., 2016; Sodir et al., 2011; Soucek et al., 2013). Notably, the systemic inhibition of MYC in vivo with Omomyc was well tolerated by normal regenerating tissues, and the effects of MYC inhibition could be reversed completely and quickly (Sodir and Evan, 2011; Soucek et al., 2008; Soucek et al., 2013).

Most recently, purified Omomyc mini-protein, was shown to possess intrinsic cell penetrating properties, possibly due to the amphipathic helical basic region of the mini-protein, which share features with cell-penetrating peptides (CPPs) and protein transduction domains (PTDs). It was demonstrated that Omomyc was taken up by tumor cells in culture, displaced MYC from target genes, reversed MYC-driven transcription and caused apoptosis or growth arrest (Beaulieu et al., 2019). Further, by intranasal administration Omomyc was shown to inhibit growth of lung tumors in a transgenic KRAS-driven lung tumor mouse model, and acted synergistically with paclitaxel in a lung cancer xenograft model after intravenous administration.

During recent years, a number of small molecule inhibitors of MYC/MAX dimerization have also been developed. These compounds work by interfering with the association between MYC and MAX, thereby reducing MYC function in cells (Follis et al., 2008; Xu et al., 2006; Yin et al., 2003; Castell et al., 2018), by binding to and stabilizing the monomeric form of MAX (Jiang et al.; Struntz et al.) or by interfering with MYC:MAX association with DNA such as JKY-2-169, KSI-3716 and MYRAs (Jeong et al., 2010; Jung et al., 2015; Mo and Henriksson, 2006; Mo et al., 2006; Kiessling et al., 2006).

The peptide mimetic compounds IIA6B17 was first reported as a small-molecule inhibitor of MYC:MAX dimerization, identified through a fluorescence resonance energy transfer (FRET) screen (Berg et al., 2002). Later, the compounds 10058-F4 and 10074-G5 were identified (Wang et al., 2007; Yin et al., 2003) to inhibit MYC:MAX association using a yeast two-hybrid screen. Moreover, chemical modifications were carried out upon 10058-F4 and several analogues such as 10074-G5 was identified with improved efficacy *in vitro* (Clausen et al., 2010; Wang et al., 2013). However, when studied *in vivo*, although showing some effect in a xenograft neuroblastoma mouse model (Zirath et al., 2013), due to rapid metabolism none of them were potent enough to proceed into preclinical studies (Clausen et al., 2010; Fletcher and Prochownik, 2015; Guo et al., 2009; Raffeiner et al., 2014; Yap et al., 2013).

Mycro1 and Mycro2 were identified by a fluorescence polarization screening, both Mycros have shown MYC:MAX inhibition *in vitro*. Mycro3 was built upon the two predecessor compounds and interferes with MYC:MAX interaction over MAX:MAX and other bZip proteins dimers like FOS/JUN. Furthermore, Mycro3 has good pharmacokinetic properties, as well as improved efficacy in mouse models with pancreatic, mammary and prostatic adenocarcinoma by oral gavage (Kiessling et al., 2006; Kiessling et al., 2007; Stellas et al., 2014).

KJ-Pyr-9, a trisubstituted pyridine compound, was identified using a fluorescence polarization screen, and was reported to bind MYC with a K_D of 6.5 nM and to inhibit MYC:MAX heterodimerization at 13.4 nM (Hart et al., 2014). Further, KJ-Pyr-9 inhibited proliferation breast cancer and other cancer cell lines in culture with an IC_{50} of 5-10 μ M, and reduced tumor volumes of MDA-MB-231 breast cancer xenografts in nude mice *in vivo*.

Most recently a novel small molecule sAJM589 was identified from a high-throughput *Gaussia* luciferase fragment complementation screen using cell lysates. sAJM589 has shown MYC:MAX inhibition in a dose dependent manner with an IC_{50} of 1.8 ± 0.03 μ M, and it downregulates MYC protein levels as well. sAJM589 suppressed cellular proliferation in diverse MYC dependent cancer cell lines and anchorage independent growth of Raji cells (Choi et al., 2017).

Most of the published MYC:MAX inhibitor screens were conducted *in vitro* or in Y2H screens, and though many of them showed promising results *in vitro* they often display moderate or poor efficacy and selectivity in cells, and have therefore not entered clinical studies. In most cases the selectivity of published compounds has not been reported extensively and are therefore difficult to judge. As presented below in papers II-IV, our group also developed a cell based BiFC screening platform for small molecule inhibitor targeting MYC:MAX interaction, and several interesting candidates were identified and undergone validation (Castell et al., 2018).

2 HYPOTHESIS AND AIM OF THE STUDY

An overall aim of this study is to target MYC via identification of small molecules suppressing tumorigenic function of MYC or alternatively enhancing its anti-tumorigenic function by targeting MYC:MAX interactions. Another overall aim is to characterize MYC's role in response to DNA damage and function in apoptosis.

The specific aims of studies presented in this thesis are as follows:

Paper I

MYC is required for activation of the ATM-dependent checkpoints in response to DNA damage.

The aim of paper I was to address MYC's function in regulating effectors acting upstream of the mitochondrial apoptotic pathway, which is known to be triggered by MYC activation. Although it is known from previous work that MYC sensitizes cells to apoptosis induced by DNA damaging drugs, the mechanism behind this has not been elucidated. The specific aim in this paper was to determine whether MYC affects ATM and/or ATR signaling in response to DNA damage induced by ionizing irradiation, UV irradiation and the bacterial toxin CDT.

Paper II

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

By using a Bimolecular Fragment complementation (BiFC) screening strategy, our aim was to set up a reliable cell-based platform to identify low weight small molecules targeting the MYC:MAX interaction in living cells. The top hits from the BiFC screening were further characterized and validated in paper II-IV. Paper II focus on MYCMI-6, -11 and -14, and the aim was to clarify if these MYCMIs could selectively inhibit MYC:MAX interaction in cells and *in vitro*. Another aim was to determine if these MYCMIs bind directly to MYC or to MAX. A fourth aim was to verify if MYCMIs could efficiently suppress tumor cell growth in culture and in tumors *in vivo* in a MYC dependent manner and to evaluate their effect on normal cells.

Paper III

MYCMI-7 - a small MYC-binding compound that inhibits MYC:MAX interaction and tumor cell growth in culture and in vivo in a MYC-dependent manner.

Paper III focuses on MYCMI-7, which was identified in paper II. Also here, the aim was to clarify if MYCMI-7 could selectively inhibit MYC:MAX interaction in cells and *in vitro*, and to find out if MYCMI-7 binds directly to MYC or to MAX. Since MYCMI-7, in contrast to MYCMI-6, -11 and -14, downregulates MYC expression, another aim was to elucidate the mechanism by which MYCMI-7 accomplishes this. Like for the other MYCMIs, an aim was also to determine the efficacy and selectivity of MYCMI-7 in cells as well as *in vivo* and to evaluate its effect on normal cells.

Paper IV

Identification of a high affinity MYC-binding compound targeting the MYC:MAX protein interaction.

Paper IV focuses on MYCMI-2, which was also identified in paper II. Like for the other MYCMIs, the aim was to validate MYCMI-2 and to determine its efficacy with respect to disrupting MYC:MAX interaction in cells and *in vitro*, and to clarify if MYCMI-2 binds directly to MYC or to MAX. Another task was to do a limited structure-activity relationship (SAR) analysis by using MYCMI-2 analogues with the aim of improving efficacy in cells and *in vitro*. Like for the other MYCMIs, another aim was to verify if MYCMI-2 could efficiently suppress tumor cell growth in culture in a MYC dependent manner and to evaluate its effect on normal cells.

3 METHODS TO STUDY PROTEIN-PROTEIN INTERACTIONS

3.1 PROTEIN FRAGMENT COMPLEMENTATION ASSAY (PCA)

Since protein-protein interactions are of great importance for MYC functions, they may offer a way of targeting MYC in cancer cells. To quantify protein-protein interactions we are utilizing different types of protein fragment complementation assays (PCAs). In this type of assay a reporter protein is split into two inactive fragments. One of the two interacting proteins of interest is fused to one of the reporter fragments and the other to the other fragment, respectively. As these proteins of interest bind to each other, the two inactive fragments of the reporter protein get in close proximity and can refold to its native structure. The signal, in our case fluorescence or luminescence, can be monitored in a fluorescence or luminometer reader, respectively.

3.1.1 Biomolecular Fluorescence Complementation (BiFC)

The bimolecular fluorescence complementation assay (BiFC) is a PCA based on split yellow fluorescent protein (YFP) as a reporter protein, where two YFP fragments were fused to MYC and MAX respectively in our case. It allows investigation in living cells and direct visualization of MYC:MAX interaction by fluorescence microscopy. The two fragments with protein of interest, MYC and MAX respectively, together with Cyan Fluorescent Protein (CFP) as an internal control of the assay, were co-transfected into HEK293T cells. Quantification of the positive YFP BiFC signals by a CCD camera allows effective cell-based high-throughput screenings for protein-binding partners and drugs that modulate PPIs (Kerppola, 2006; Miller et al., 2015). We used this assay to screen the chemical library (The NCI/DTP Open Chemical Repository, <http://dtp.nci.nih.gov>) of 1900 compounds for inhibitors of the MYC:MAX interaction.

3.1.2 Gaussia luciferase (GLuc) protein fragment complementation assay

Gaussia luciferase (GLuc) was originally identified from the marine copepod *Gaussia princeps*. GLuc as the smallest known luciferase (molecular mass of 19.9 kDa), besides its strong luminescence activity, it is attracting more and more attention as a reporter protein (Luker and Luker, 2014; Wille et al., 2012). GLuc exhibits an activity up to 1,000-fold higher than to *Renilla reniformis* luciferase (RLuc), firefly luciferase (FLuc) (Tannous et al., 2005), or bacterial luciferases (LuxAB) (Wiles et al., 2005).

The high sensitivity, good signal-to-noise ratio, and simplicity of assays made split GLuc assay to the main alternative PCA for validation of MYC:MAX interactions in our study, both as transient and stable transfections. Stable cell lines HEK293 expressing GLuc reporter fused to MYC or MYCN and MAX have been established, and a well-developed GLuc based PCA platform showing a good z-factor has been created, allowing the assay to be used in high throughput screening.

3.2 *IN SITU* PROXIMITY LIGATION ASSAY (ISPLA)

in situ Proximity Ligation Assay (*isPLA*) is another method to measure protein-protein interactions in cells which that developed by Ola Söderberg and Ulf Landegren's research groups in Uppsala together with our research group. The principle of *isPLA* is based on the immunodetection of two antigens with a pair of primary antibodies raised in different species. Then the two primary antibodies will be recognized by two species-specific secondary antibodies, which were specially designed antibody-oligonucleotide conjugates, also known as PLA probes. The PLA probes link to a unique short DNA strand. When come into close proximity (maximum 40 nm), the DNA strands will in turn ligate by recruiting two additional oligonucleotides as connector, and jointly give rise to DNA circles, which is a template followed by a single-stranded rolling circle PCR (rolling circle amplification). Thereafter the interaction is visualized by a fluorescence labelled complementary oligonucleotide probe concatenated complements of the DNA circle.

One of the benefits with this assay is that interactions between endogenous proteins can be measured at single molecule level in fixed cells and tissues (Bagchi et al., 2015; Soderberg et al., 2006; Soderberg et al., 2008). The requirement for dual recognition of the target proteins improves selectivity by avoiding any cross-reactivity not shared by the antibodies, and it allows detection of both protein-protein interactions, post-translational modifications and expression of proteins *in situ* (Klaesson et al., 2018). This facilitates the study of multiple interactions and we have adopted this system to be able to study interactions which are important for MYC function.

3.3 FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) ASSAYS

Fluorescence resonance energy transfer (FRET) is another powerful method for studies of molecular interactions. Many previously published MYC:MAX inhibitors were identified using FRET *in vitro* (Berg et al., 2002; Shi et al., 2009; Xu et al., 2006). For study protein-protein interaction studies, two fluorophores known as donor and acceptor which have spectral overlap between the pair, are attached to the interacting proteins either by fusion or through

fluorophore-tagged antibodies. The fluorescence emission spectrum of the donor molecule must overlap the absorption or excitation spectrum of the acceptor. In the FRET assay, the donor fluorophore becomes excited by a light source within certain spectral range for excitation. The acceptor fluorophore absorbs the energy from the donor fluorophore and then produces a detectable light emission.

Using FRET, the interaction between MYC and MAX become measurable *in vitro* when the two recombinant proteins are in close proximity as they bind each other.

3.4 SURFACE PLASMON RESONANCE (SPR)

Surface plasmon resonance (SPR) is an optical technique that is utilized for detecting molecular interactions, including protein-protein interactions, and interactions between proteins and other classes of molecules. The advantages of SPR are it is real-time, label-free, and noninvasive nature in medical or biological research.

The principal of SPR assay is by binding of a mobile molecule (analyte) to a molecule immobilized on a thin metal film (ligand), which changes the refractive index of the film. The angle of extinction of light that is completely reflected after polarized light impinges upon the film, is altered and monitored as a change in detector position for a dip in reflected intensity (the surface plasmon resonance phenomenon). Because the method strictly detects mass, there is no need to label the interacting components, thus eliminating possible changes of their molecular properties (Douzi, 2017; Drescher et al.; Leonard et al., 2017; Tang et al., 2010).

3.5 MICROSCALE THERMOPHORESIS (MST)

Microscale thermophoresis (MST) is a technology based on the detection of a temperature induced change in fluorescence of a target, which is altered in temperature gradients due to a variety of molecular properties such as different sizes, charges and conformation.

In the MST experiment, an infrared laser is usually used to generate the temperature gradient. The directed movement of molecules through the temperature gradient in the cell lysate is then detected and quantified using either covalently attached or intrinsic fluorophores. Especially in the case of study of low molecular weight compounds such as potential drug candidates and their target binding, which does not often significantly change the charge or size of a molecule, but only weak conformational alternation upon binding (Jerabek-Willemsen et al., 2014; Wienken et al., 2010).

By recording the precision of fluorescence, plus the sensitivity of thermophoresis and very small amount of sample consuming, MTS has been employed to study the protein-protein interaction/dissociation in a fast and robust way. Moreover, MST facilitates the sophisticated analysis of binding processes of small molecules with proteins in biological liquids.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I: MYC IS REQUIRED FOR ACTIVATION OF THE ATM-DEPENDENT CHECKPOINTS IN RESPONSE TO DNA DAMAGE.

In paper I, we demonstrated that MYC contributes to the regulation of the ATM-dependent checkpoint responses to DNA damage, while cell death induced by UV irradiation, known to activate the ATR-dependent checkpoint, worked independently of the MYC status.

Dysregulation of MYC can induce DNA damage both in a ROS-dependent and ROS-independent manner, thereby inducing apoptosis. However, upon loss of surveillance modulators such as p14, p53 deficiency and MDM2 overexpression, and/or gain of prosurvival signals such as BCL-2 and NF- κ B pathway alterations, MYC deregulation can also contribute to genome instability, chromosomal abnormalities and transformation (McMahon, 2014) (Gorgoulis et al., 2005; Karlsson et al., 2003; Vafa et al., 2002).

Further, MYC activation has previously been shown to enhance the apoptotic effect of DNA damage induced by agents such as etoposide and camptothecin (Afanasyeva et al., 2007; Albiñ et al., 2007), but the mechanism for this phenomenon has not been clarified.

In paper I, engineered Rat1 cell lines were employed to measure the apoptotic effect upon DNA damage in cells with different MYC status. TGR-1 is the wild type parental line, expressing physiological level of MYC, while both MYC alleles has been deleted by homologous recombination in HO15.19 cells (Mateyak et al., 1997). HOmyc3 cells has been generated by reconstitution of the murine *MYC* gene into the HO15.19 cells, and these express higher level of MYC compared with TGR-1 cells. In paper I, we have shown that cell deaths upon ionizing irradiation (IR) or bacterial cytolethal distending toxin (CDT) intoxication were delayed in HO15.19 cells with MYC deletion compared with the MYC expressing cells, as shown by the increased sub-G1 population in the former cells as assessed by PI staining and flow cytometry. Both IR and CDT are known to cause DNA double strand breaks (DSBs) and activate ATM-dependent checkpoint responses.

UV irradiation, which causes dipyrimidine photoproducts and activates mainly the ATR-dependent DNA damage response, on the contrary, induced cell death with similar amplitude and kinetics in all the cell lines tested independently of the MYC status.

We next assessed whether the delayed cell death induced by IR in the MYC null cells was associated with an altered ATM response. Indeed, phosphorylation of ATM and induction of

γ H2AX were strongly reduced 2 hrs after IR exposure in MYC null cells compared to wt and MYC-reconstituted cells, suggesting that activation of ATM and downstream responses are MYC dependent. Moreover, p53 accumulated after 24 hrs of IR exposure in TGR-1 and HOMyc3 cells as expected, presumably as a consequence of MDM2 phosphorylation by ATM (Cheng and Chen, 2010; Gannon et al., 2012; Maya et al., 2001). However, this was not the case in MYC null cells, where p53 induction was delayed.

We next looked at the NBS1 dependency of ATM activation. NBS1 is a sensor of DNA damage, as well as a MYC target gene (Chiang et al., 2003). The induction of NBS1 foci upon IR showed different kinetics in MYC expressing and MYC nulls cells, namely an enhanced expression of the NBS1 protein in HOMyc3 cells, and attenuated level of NBS1 in HO15.19 null cells compared to the parental TGR-1. Further, after knocking down the expression of MYC in HCT116 human colon cancer cells by siRNA, we observed similar kinetics of ATM phosphorylation in wild type and MYC depleted cells, but the total level of phosphorylated ATM was significantly lower.

Our results demonstrated that MYC regulates the ATM DDR/DNA repair pathway. MYC was shown to be required for activation of ATM, NBS1 accumulation and phosphorylation of downstream effectors (H2AX, CHK2 and p53), in response to DNA damage inducers such as irradiation and CDT intoxication, thereby leading to apoptosis. Other studies have shown that inactivation of ATM reduces MYC-induced apoptosis thereby increases MYC-driven tumor development (Maclean et al., 2007; Pusapati et al., 2006; Reimann et al., 2007). This would be coherent with our result that MYC is necessary for ATM-mediated apoptosis.

Also, other publications addressing the role of MYC in DDR and DNA repair are consistent with our findings; Chiang et al. have shown that NBS1 is a direct target gene of MYC. MYC-mediated induction of NBS1 gene transcription was shown to occur in different tissues independent of cell proliferation (Chiang et al., 2003). Louto et al. showed that MYC occupies most DSB repair gene promoters and regulates their expression, and that MYC knockdown alone resulted in loss of long-term clonogenic survival, independent of apoptosis induction (Luoto et al., 2010). MYC therefore seems to play a dual role in apoptosis regulation upon DNA damage. It could promote both apoptosis and DNA repair, and the outcome of this may depend on the context, such as the extent of DNA damage, the MYC level, as well as the status other pro-apoptotic/ pro-survival cofactors. This clearly needs further investigation. Our new findings could contribute to a better understanding of the mechanisms of drug-induced cytotoxicity as well as for resistance to drug treatment in cancer therapy.

4.2 PAPER II: A SELECTIVE HIGH AFFINITY MYC-BINDING COMPOUND INHIBITS MYC:MAX INTERACTION AND MYC DEPENDENT TUMOR CELL PROLIFERATION.

In paper I, a BiFC screening platform was established to screen for small molecule inhibitors which interfere with MYC:MAX protein interactions in cells. This was based on split YFP fusions with MYC and MAX proteins, respectively, and CFP as internal control. A few candidates, named MYCMI, were identified from the NIC/NIH small molecule library of 1900 compounds, according to the ratio of BiFC/CFP fluorescence intensities. Compared with other methods such as FRET, fluorescence polarization *in vitro* and yeast-two-hybrid (Y2H) assay, the cell-based BiFC assay has several advantages. For instance, already at the screening step, molecules with cell compatible features that target MYC:MAX interactions in their natural cellular environment will be selected for.

Candidates from the screening, MYCMI-2, MYCMI-6, MYCMI-7 MYCMI-9, MYCMI-11 and MYCMI-14, were thereafter evaluated and validated in other cell-based interaction assays. Paper II mainly focused on compounds specifically targeting MYC:MAX interaction without affecting MYC protein levels, including MYCMI-6, -11 and -14 in Paper II. MYCMI-7 and MYCMI-2 are characterized in Paper III and Paper IV, respectively.

MYCMI-6, MYCMI-11 and MYCMI-14, and in particular MYCMI-6, were shown to be potent and selective inhibitors of the MYC:MAX bHLHZip interaction in cells by the split *Gaussia* luciferase (Gluc) assay, whereas no signs of inhibition of other bZip transcription factors interactions such as those between FOS and JUN, and GCN4 homodimerization were detected at the active concentrations of disrupting MYC:MAX bHLHZip. Furthermore, MYCMI-6, MYCMI-11 and MYCMI-14 were found to inhibit endogenous MYC:MAX interaction in cells as shown in *in situ* proximity ligation (*isPLA*) assay, with IC₅₀ values of 1.5, 6 and 6 μ M, respectively, while they did not interfere with the interaction between bZip transcription factors FRA1:JUN or the interaction between MAX and the bHLHZip protein MXD1(MAD1), which is an intracellular competitor of MYC for MAX. MYCMI-6 exhibited its inhibitory effect already after 3 hours of treatment in the co-IP assay.

MYCMI-6 in particular exhibited strong selective inhibition of MYC:MAX interaction in cells in the Gluc and *isPLA* assays at single-digit micromolar concentrations. Next, we addressed if MYCMI-6 inhibits MYC:MAX interaction also *in vitro* by microscale thermophoresis (MST) and surface plasmon resonance (SPR) assays using recombinant bHLHZip domains of MYC and MAX. In the MST assay, interaction between molecules is measured by detection of a

change in the movement of a fluorescence labeled target through a temperature gradient (thermophoresis) upon binding with a non-fluorescent ligand. MST were used in our study to measure interaction between recombinant bHLHZip domains of MYC and MAX. We used this MST system to measure the disturbance by MYCMI of MYC:MAX interaction during thermophoresis of fluorescently labeled MAXbHLHZip interacting with MYCbHLHZip, which was pre-incubated with MYCMIs. All three MYCMIs, in particular MYCMI-6, shifted MAX thermophoresis during interaction with MYCbHLHZip pre-mixed with MYCMI-6 relative to DMSO.

We next characterized the efficiency of MYCMI-6 and investigated if it could discriminate between MYC:MAX and MAX:MAX interactions in MST assay. MYCMI-6 was titrated together with fluorescent labeled MAXbHLHZip together with MYCbHLHZip, or together with MAXbHLHZip. The results showed that MYCMI-6 caused a thermophoresis shift with a K_d of $4.3 \pm 2.9 \mu\text{M}$ with respect to MYC:MAX interaction while only having minor effect on MAX:MAX interaction.

To further investigate the potency of MYCMI-6 in inhibiting MYC:MAX interaction, we also conducted an SPR assay. In SPR, the interaction between protein and ligand is measured kinetically in real time. The ligand is immobilized on a gold sensor surface, followed by the injection of the receptor over the ligand fixed surface, and subsequent changes in the refractive index of the medium close to the sensor surface is then monitored. In our case, MAXbHLHZip was covalently attached onto the sensor chip, and MYCbHLHZip was injected over the surface, thereby allowing the measurement of MYC:MAX association and dissociation rates. Various concentrations of MYCMI-6 up to $10 \mu\text{M}$ were pre-incubated with MYCbHLHZip before flowing over MAXbHLHZip. The SPR results showed that MYCMI-6 inhibited the MYC:MAX heterodimer formation with an IC_{50} of $3.8 \pm 1.2 \mu\text{M}$. 10058-F4, used as a reference, was not as efficient as MYCMI-6 and KJ-Pyr-9, which is another MYC:MAX inhibitor reported previously, did not show any effect on MYC:MAX heterodimer formation up to a concentration of $10 \mu\text{M}$.

We next investigated whether MYCMI-6 selectively binds to MYC or MAX using MST and SPR assays. Recombinant MYC bHLHZip or MAX bHLHZip proteins were titrated, respectively, in a fixed concentration of MYCMI-6 in the MST assay. Changes in fluorescence was detected for MYC but not MAX, indicating binding of MYCMI-6 to MYC but not to MAX at these concentrations. In addition, the binding affinity of MYCMI-6 to MYC bHLHZip domain was analyzed by SPR, using a 1:1 Langmuir model to calculate the association and

dissociation rates of the compound. In this case, MYC bHLHZip was immobilized on the chip and injected MYCMI-6 was titrated. The association (on rate) and dissociation (off rate) was measured kinetically and showed a K_D value of $1.6 \pm 0.5 \mu\text{M}$ for the affinity of MYCMI-6 to MYC bHLHZip. This is clearly a higher affinity than reference molecules 10074-G5 and #474 (an analogue of 10058-F4), which showed K_D s of 28 and $15 \mu\text{M}$, respectively. Binding of KJ-Pyr-9 to MYC was not detected up to $8 \mu\text{M}$ in this assay. MYCMI-6 did not bind to the MAX bHLHZip in neither the MST or SPR assays, nor to the bHLHZip domain of MAD1, the p53 core domain, BSA and YFP as shown by SPR.

We continued to investigate whether MYCMI-6 would interfere with the biological activities of MYC. A panel of neuroblastoma cell lines with different status of MYCN was utilized to determine the effect of MYCMI-6 on MYC dependent tumor cell growth. The growth inhibition of 50% (GI_{50}) values were about $2.5\text{-}6 \mu\text{M}$ for MYCN-amplified cell lines and around $20 \mu\text{M}$ or higher for MYCN-non-amplified cell lines, which indicate that MYCMI-6 inhibit tumor cell growth in a MYC dependent manner. Moreover, MYCMI-6, MYCMI-11 and MYCMI-14 also inhibited anchorage-independent growth of MYCN-amplified neuroblastoma cells efficiently with GI_{50} values of <0.4 , 5 and $0.75 \mu\text{M}$, respectively.

To investigate if MYCMI-6 inhibited MYCN:MAX interaction and the transcriptional output of MYCN in MYCN-amplified neuroblastoma cells, we performed MYCN:MAX *is*PLA and measured the expression of a panel of verified MYC family target genes in neuroblastoma. It showed that MYCMI-6 significantly blocked MYCN:MAX interaction and the expression of MYC/MYCN target genes at a concentration of $2.5 \mu\text{M}$, while maintaining the expression of both MYCN and MAX.

MYCMI-6 also suppressed growth of Burkitt's lymphoma (BL) cells, which carry MYC translocation, with high efficacy (GI_{50} about $0.5 \mu\text{M}$). To have a better understanding of the correlation between the levels of MYC expression in tumor cells and the growth inhibitory response, we extracted GI_{50} data from the NCI-60 diverse human tumor cell line panel available for MYCMI-6 by the Developmental Therapeutics Program of the U.S. National Cancer Institute (DTP-NCI). Statistical analysis of the data showed a strong correlation between MYC protein expression and growth inhibitory in response to MYCMI-6. In other words, cell lines with higher MYC protein level had significantly higher probability to respond to MYCMI-6 treatment than cell lines with lower MYC protein levels.

We next studied the effect of MYCMI-6 on normal human cells. Importantly, while MYCMI-6 caused apoptosis in MYCN-amplified neuroblastoma cells, it was not cytotoxic to normal human cells at active concentrations.

To analyze the effects of MYCMI-6 *in vivo*, we next utilized a mouse xenograft tumor model based on human MYCN-amplified SK-N-DZ neuroblastoma cells. The SK-N-DZ cells were injected into the flank of athymic nude mice for tumor formation, a daily intraperitoneal injection of a dose of 20 mg/kg body weight of MYCMI-6 or vehicle were administered for 1–2 weeks. MYCMI-6 reduced proliferation and induced massive apoptosis in tumor tissue measured by Ki67 and TUNEL staining. CD31 staining of endothelial cells in xenograft model indicated a significantly reduced microvascular density (MVD) upon MYCMI-6 treatment. Moreover, MYCMI-6 treatment did not cause severe side effects but only temporal body weight loss of the mice. Importantly, *isPLA* in the tumor tissue showed a significant reduction in MYCN:MAX interaction from MYCMI-6 treatment compared to vehicle-treated mice, showing that MYCMI-6 had active effect on the target, MYCN:MAX inhibition, *in vivo*.

In conclusion, using a cell-based BiFC screen several new MYC:MAX heterodimer inhibitors were identified. Three of these, MYCMI-6, -11 and -14, which did not affect MYC expression, were studied in paper II. MYCMI-6 in particular showed strong potency and selectivity with respect to inhibition of MYC:MAX interaction both in cells and *in vitro*. Further, MYCMI-6 selectively bound directly to MYC with low micromolar range affinity. Moreover, MYCMI-6 it inhibited tumor cell growth in a MYC-dependent manner at low micromolar concentrations, while discriminating well between cancer cells and normal cells, thus showing a good therapeutic window. Importantly, MYCMI-6 exhibited MYC:MAX inhibitory bioactivity in tumor tissue *in vivo*, and thus could be of interest for further pharmacological development.

4.3 PAPER III: MYCMI-7 - A SMALL MYC-BINDING COMPOUND THAT INHIBITS MYC:MAX INTERACTION AND TUMOR CELL GROWTH IN CULTURE AND IN VIVO IN A MYC-DEPENDENT MANNER.

In paper III we characterized small molecule MYCMI-7, which was one of the top hits identified in paper II using a cell-based BiFC screen for MYC:MAX interaction inhibitors. In this study, we further validated the efficiency of MYCMI-7 with respect to inhibition of MYC:MAX interaction, binding to MYC and MYC-driven tumor cell growth and normal cell growth, as well as in mouse models of MYC-driven cancer models.

The inhibitory effect of MYCMI-7 was validated using a number of other cell-based protein interactions assays in addition to BiFC, including the GLuc, isPLA, and coimmunoprecipitation (co-IP).

Firstly, we verified that MYCMI-7 had a MYC:MAX inhibitory effect in the GLuc assay; it strongly reduced both MYC:MAX and NMYC:MAX interaction in cells but had only minor effect on homodimerization of the bZip protein GCN4, whereas previous published experimental inhibitor 10058-F4 and the bromodomain inhibitor JQ1 had much weaker effects on MYC:MAX interaction. A structural analogue of MYCMI-7 was inactive in the GLuc assay, therefore served as a reference compound in this and other assays. Further, MYCMI-7 inhibited endogenous MYC:MAX interaction in breast cancer cells as measured by isPLA, but did not inhibit interaction between the bZip proteins FRA1 and JUN. Kinetic experiments showed that MYCMI-7 started to reduce the MYC:MAX interaction already after 1 hour of treatment, and after 4 hours it had almost reached the minimum level, as shown by co-IP.

Chromatin immunoprecipitation (ChIP) was also carried out in breast cancer cells to examine the effect of MYCMI-7 on the association of MYC with target gene promoters. In agreement with the rapid reduction in MYC:MAX dimerization observed in the co-IP, MYCMI-7 led to disassociation of MYC from the gene promoters starting already within 2 hours, and a maximum level of inhibition was reached after 4 hours of treatment and thereafter. In U2OS MYCER cells containing a 4-hydroxytamoxifen (HOT) regulatable MYC-estrogen receptor fusion protein, the HOT-induced expression of the MYC target gene CR2 was dramatically repressed by MYCMI-7 treatment. Therefore, we conclude that MYCMI-7 not only inhibited both exogenous and endogenous MYC:MAX protein interactions in cells, it also rapidly reduced MYC association with chromatin and blocked MYC target gene expression.

Next, we looked at MYCMI-7 inhibitory effect on MYC:MAX *in vitro*. In the SPR assay, the recombinant MAXbHLHZip was immobilized on a gold chip, and titration of recombinant

MYCbHLHZip pre-mixed with MYCMI-7 was injected, flowing over MAXbHLHZip. The affinity of MYC to MAX on the chip was then measured. SPR assay showed that MYCMI-7 decreased the MYC:MAX interaction, which however stabilized at a level of approximately 45% of control. Using SPR to study potential binding of MYCMI-7 to MYC, we could also show that MYCMI-7 bound to recombinant MYC bHLHZip domain with an affinity approximately of 4 μ M. Taken together, the results indicated that MYCMI-7 inhibited the interaction between recombinant bHLHZip domains of MYC and MAX by binding directly to MYC at low micromolar concentration.

Looking at longer treatments (24-48 hours) with MYCMI-7 in cells, we noticed that not only the interaction between MYC or MYCN with MAX decreased, but also the steady state levels of both MYC and MYCN proteins were downregulated in Hela and Kelly cells, respectively. This suggested that MYCMI-7 might inhibit MYC and MYCN in both direct and indirect ways.

We next addressed if the decrease in MYC protein levels was due to reduced MYC mRNA expression, therefore RT-qPCR was carried out after treatment with MYCMI-7 at different time points. The results demonstrated that MYCMI-7 did not decrease MYC mRNA levels in RT-qPCR significantly up to 24 hours. To investigate how MYCMI-7 regulates the steady level of the MYC protein, a cycloheximide (CHX) chase experiment, in which protein synthesis is blocked by CHX, was performed in HCT116 cells. A slightly increase of MYC turnover was detected.

To further address how MYCMI-7 stimulates MYC degradation, we focused on FBXW7, which is the main E3 ligase targeting MYC for ubiquitylation and degradation (Yada et al., 2004). Interestingly, MYCMI-7 did not affect MYC protein levels in the HCT116 FBXW7 deficient cells, whereas both wild type and p53 null HCT116 cells showed a significant decrease of MYC protein level after MYCMI-7 exposure. CHX chase in FBXW7 deficient HCT116 cells confirmed that MYCMI-7 did not cause any change in MYC turnover rate in these cells. However, protein level of cyclin E, another target of FBXW7 (Cole et al., 2011; Koepp et al., 2001; Weber and Ryan, 2015) was not affected after MYCMI-7 treatment, excluding a general effect of FBXW7 on target proteins upon MYCMI-7 treatment.

It has been reported that FBXW7 recognizes the Thr58- phosphorylated form of MYC and thereby target MYC for degradation. We therefore utilized Thr58A and Ser62A MYC mutants to validate the contribution of FBXW7 to MYCMI-7-induced downregulation of MYC protein level. Wild type MYC, T58A and S62A-mutant MYC were transiently transfected into U2OS cells, and also transduced stably in HO15.19 MYC null cells. Surprisingly, the levels of both

wild type and mutated MYC proteins were reduced in response to treatment in U2OS cells as well as in the HO15.19 cells. This argued against the hypothesis that the downregulation of MYC protein level upon MYCMI-7 treatment always go through the “classical” phospho-Thr58/FBXW7 pathway.

Intriguingly, when CHX chase experiments were performed in HeLa cells, no change MYC turnover rate was observed after MYCMI-7 treatment even though the MYC level was reduced, suggesting that the mechanism of MYC protein reduction is cell type or context dependent. To examine this further, we next measured MYC proteins synthesis in human U-937-MYC-6 cells, which contains a viral *v-myc* gene expressed from a retroviral promoter and lacking 5' and 3' mRNA regulatory elements, also expresses the endogenous MYC protein. MYCMI-7 only reduced synthesis of the endogenous MYC protein, but not the exogenous v-MYC protein. This suggests that regulatory sequences present in the human MYC mRNA species but absent in the mRNA expressed from the v-MYC construct may affect MYC mRNA translation. Therefore, it suggested the effect of MYCMI-7 on MYC turnover was cell type or context-dependent.

We next investigated anti-tumor growth/viability effect of MYCMI-7, and its correlation with MYC level. We first utilized Rat1 fibroblasts with different MYC status to address if whether MYCMI-7 would affect cell growth in a MYC-dependent manner. H015.19 is a MYC null cell line derived from TGR-1 (parental cell line), while H0MYC3 was generated from the MYC null cells by reconstitution of the MYC gene (Mateyak et al., 1997). After 48 hours of MYCMI-7 treatment, the metabolic activity of cells measured by MST-1 assay differed dramatically between MYC expressing cells (TGR-1 and H0MYC3 with IC50 around 2 μ M), and the MYC null cells HO15.19 which were unaffected even at MYCMI-7 concentrations of 12 μ M, with IC50 close to 20 μ M after 96 hours of MYCMI-7 exposure.

An analogue of MYCMI-7, which did not affect MYC:MAX interaction, declined the viability of both HO15.19, TGR-1 and H0MYC3 cells and therefore did not discriminate between cells with different MYC status. The MYC selectivity of MYCMI-7 was further investigated in MCF7 breast cancer cells upon MYC knockdown, showing that cells depleted of MYC by siRNA-mediated knockdown were less sensitive to MYCMI-7 compared with the MYCMI-7 analogue, while cells exposed to control-siRNA were equally sensitive to the two compounds.

We next utilized a panel of neuroblastoma cell lines with or without MYCN-amplification. Our results showed that MYCMI-7 reduced tumor growth and viability in all the cell lines, but the effect was clearly stronger in the *MYCN*-amplified cell lines. MYCMI-7 also reduced growth of three Burkitt's lymphoma cell lines with MYC translocation. In addition, we utilized

mRNA and protein expression data and GI₅₀ data for the NCI-60 diverse human tumor cell line panel as described previously in Paper II. The data showed a strong correlation between MYC expression and growth inhibitory response to MYCMI-7 in human cancer cell lines. The results collectively indicated that MYCMI-7 reduced cell viability or inhibit cell growth in a MYC dependent manner.

We next investigated the MYCMI-7's anti tumorigenic capacity in a RAS + MYC cotransformation assay in primary rat embryonic fibroblasts (REFs). At a concentration of 0.5 μ M, MYCMI-7 blocked transformation of the REFs by MYC together with activated HRAS in focus assays. MYCMI-7 also inhibited agarose colony formation of both MYC/HRAS transformed REFs and *MYCN*-amplified neuroblastoma cell line SK-N-DZ.

To find out whether MYCMI-7 induced cytotoxic or cytostatic effects in tumor cells and normal cells, we utilized the Rat1 cells with different MYC status described above and human P493-6 B-cells with Tet-regulatable MYC. MYCMI-7 did not induce apoptosis in MYC null HO15.19 cells, but triggered apoptosis the wild type TGR-1 and MYC reconstituted HOMYC3 cells. The P493-6 cells were synchronized in the G0 phase by shutting off MYC with the addition of doxycycline, then released into the G1 phase of cell cycle by doxycycline withdrawal in the presence or absence of MYCMI-7, after which cell cycle distribution was analyzed by FACS. MYCMI-7 treatment induced significant apoptosis compared with DMSO treatment as evident from the increased subG1 population as well as G1 arrest. However, MYCMI-7 did not reduce viability of either normal REFs, which arrested in the G1 phase of the cell cycle, nor of human normal diploid fibroblasts (HNDF) cells, but reduced viability in A375 melanoma cells in a dose dependent manner by induction of apoptosis. Taken together, our results showed that MYCMI-7 strongly inhibited tumor cell growth and induced apoptosis in a MYC dependent manner without affecting viability of normal cells, thus indicating a good therapeutic window for MYCMI-7.

We also conducted in an ex vivo screen of MYCMI-7 efficacy on cells derived from primary glioblastoma tumor biopsies of 42 patients. The results showed that MYCMI-7 had potent anti-tumor growth effect with EC₅₀ in the submicromolar range in most of the glioblastoma cultures. In addition, MYCMI-7 inhibited growth of three patient-derived acute myeloblastic leukemia (AML) cell cultures with EC₅₀ in a range of 0.15-1.3 μ M.

Finally, MYCMI-7 was tested in several mouse tumor models, representing a MYC driven acute myeloid leukemia (AML) in which hematopoietic stem and myeloid progenitor cells that have gone through malignant transformation. In the AML model, hematopoietic stem cells

(HSCs) were isolated and purified from mouse bone marrow, then transduced with MYC and BCL-X_L retroviral vectors, followed by expansion *in vitro* and injection into sub-lethally irradiated recipient syngeneic mice (Hogstrand et al., 2012). This leads to reconstitution of the bone marrow stem cell compartment to some extent but also leads to rapid development of very aggressive AML-like leukemia. After the first signs of AML in the blood, treatment with MYCMI-7 or vehicle was initiated, and mice were sacrificed at different days post treatment and bone marrow and spleen samples were collected. At day 11 post treatment, very few leukemic cells were seen in the bone marrow under any conditions, but at day 15, there was a dramatic increase in leukemic cells in vehicle treated mice, which was essentially blocked in MYCMI-7 treated mice. At the end point, MYCMI-7 treated mice had still less leukemic cells in both bone marrow and spleen, and the latter retained a more normal spleen structure compared with the collapsed spleen structure in vehicle-treated mice. In summary, MYCMI-7 inhibited MYC/BCL-X_L-driven tumor growth *in vivo* with tolerable side effects.

We also studied the anti-tumor effect of MYCMI-7 in mouse xenograft tumor models of solid tumors representing breast cancer and neuroblastoma. Here we utilized the human basal-like breast cancer cell line MDA-MB-231 and MYCN-amplified SK-N-DZ neuroblastoma cells. The tumor cells were injected into the flank of mice. After tumors had become palpable, MYCMI-7 was administered intratumorally every fourth day until sacrifice when tumor volume reached the endpoint. MYCMI-7 treatment in both the breast cancer and the neuroblastoma xenograft model resulted in reduced tumor growth as well as prolonged survival. Immunohistochemical Ki67 staining of the tumor tissues showed that MYCMI-7 treatment in triple negative breast cancer xenograft model considerably slowed down the tumor cell proliferation.

In conclusion, MYCMI-7 inhibited both exogenous and endogenous MYC:MAX protein interaction in cells at low micromolar concentrations, as well as *in vitro*. It bound to recombinant MYC with an affinity of approximately 4 μ M, as well as decreased the steady state levels of MYC protein in cells. It also induced apoptosis in a MYC-dependent manner in tumor cells and immortalized cells while causing only G1 arrest with maintained viability in normal cells. In comparison with other reference MYC inhibitors such as 10058-F4 and JQ1, MYCMI-7 was more potent with respect to inhibition of cell growth and induction of cell death. Importantly, MYCMI-7 inhibited tumor growth in AML, breast cancer and neuroblastoma mouse models. The mechanism of how MYCMI-7 affects the steady state level of MYC protein still remains elusive, and there are indications that these effects may be cell type-specific, which

requires further investigation. MYCMI-7 is a potent and selective MYC inhibitor that has potential for further development towards anti-MYC drugs for clinical relevance in the future.

4.4 PAPER IV: IDENTIFICATION OF A HIGH AFFINITY MYC-BINDING COMPOUND TARGETING THE MYC:MAX PROTEIN INTERACTION.

We utilized the cell-based GLuc and *is*PLA protein interaction assays to validate MYCMI-2's MYC:MAX inhibitory efficacy in cells. MYCMI-2 inhibited both MYC:MAX and MYCN:MAX dimerization to about 60% and 70% of the DMSO control at 10 μ M after 24 hours treatment, without interfering with homodimerization of the bZip protein GCN4. The *is*PLA assay demonstrated an IC₅₀ of about 5-6 μ M in MCF7 cells, where cells started to respond already after 6 hours of MYCMI-2 treatment. Taken together, MYCMI-2 inhibited both exogenous and endogenous MYC:MAX interaction in cells.

We next examined whether MYCMI-2 targets the MYC:MAX interaction directly or indirectly and therefore performed experiments *in vitro*. For this we utilized an *in vitro* Gluc assay, based on split *Gaussia* luciferase fused to recombinant MYC and MAX, respectively. The recombinant proteins were translated separately *in vitro* using the TNT T7 coupled reticulocyte lysate system, mixed together in a ratio 1:1 together with compound after which *Gaussia* luciferase was measured by adding the substrate coelenterazine. The results showed that MYCMI-2 dramatically inhibited the MYC:MAX and MYCN:MAX interactions *in vitro* to 10-15% of DMSO control, with little interference with GCN4:GCN4 homodimerization. The MAX:MAX interaction was only reduced slightly, suggesting that MYCMI-2 preferentially inhibits the MYC:MAX over the MAX:MAX interaction. Fluorescence resonance energy transfer (FRET) was utilized to further validate MYCMI-2's ability to interrupt the MYC:MAX interaction *in vitro*, using recombinant proteins MYCbHLHZip fused to mTorq and MAXbHLHZip fused to eYFP as donor and acceptor, respectively. The compounds were incubated with the already heterodimerized MYC-mTorq:MAX-eYFP proteins which had a ratio of 1:1.1. IC₅₀ for MYCMI-2 was estimated to approximately 240 +/-80 nM after a titration up to 3200 nM. The reference compounds 10058-F4 and 10074-G5, however, did not obtain any FRET signal in the same set up. Next, we modified the experiment setting which was to incubate the compounds with MYC-mTorq first before MAX-eYFP was added. In this case, only 10074-G5, in addition to MYCMI-2, showed an inhibitory effect, indicating that only MYCMI-2 had the capacity to disrupt pre-formed MYC:MAX heterodimers. Titration of 10074-G5 indicated an IC₅₀ of approximately 25 μ M, corresponding to previous published data. The other reference compound 10058-F4 was not active even up to 100 μ M in the FRET assay, which is consistent with other study that 200 μ M is needed to split the MYC:MAX heterodimer (Choi et al., 2017).

The surface plasmon resonance (SPR) assay, described above, was also utilized to study the inhibitory effect of MYCMI-2 on MYC:MAX interaction inhibition *in vitro*. In the SPR assay, MYCbHLHZip was immobilized onto the sensor chip, while MAXbHLHZip was injected together with MYCMI-2, with a titration of MYCMI-2 in concentrations ranging from 1 nM up to 40 nM. The results showed that MYCMI-2 inhibited the MYC:MAX interaction with an IC_{50} below 1 nM.

To address whether MYCMI-2 binds to MYC or MAX, we next conducted an MST experiment. In the MST assay, fluorescence labelled MYCbHLHZip was used for the thermophoresis after incubation with various concentrations of MYCMI-2 up to 500 nM. The MST assay determined a high affinity of MYCMI-2 binding to with MYC with an approximate K_d of 7 nM. SPR assay was performed to further validate the results. For the binding study of MYCMI-2, MYCbHLHZip was immobilized on the sensor chip, and MYCMI-2 was injected at different concentrations. An affinity with the extraordinary K_D of approximately 1.3 +/- 0.2 nM was determined in this assay.

We next investigated the biological effect of MYCMI-2 in cells. MYCMI-2 inhibited growth of Burkitt's lymphoma (BL) cells Mutu and Daudi, which have MYC translocation, and inhibited cell growth/viability of the MYCN-amplified neuroblastoma cell line SK-N-DZ with an EC_{50} between 1.5-6 μ M, while there was little effect on the MYCN-non-amplified cell line SK-N-F1 up to 50 μ M. MYCMI-2 reduced viability in the cervical cancer cell line HeLa and the breast cancer cell line MCF7, with EC_{50} s about 5 μ M for both cell lines, while it did not affect growth and viability of normal melanocytes at concentrations up to 25 μ M. In addition, in Rat1 cells with different MYC status, MYCMI-2 barely affected growth HO15.19 MYC null cells while inhibiting growth of MYC-reconstituted HOMYC3 cells. Further, MYCMI-2 potently inhibited anchorage independent growth of SK-N-DZ neuroblastoma cells, with EC_{50} values below 1 μ M. Taken together, these results suggest MYCMI-2 inhibited tumor cell growth in a MYC dependent manner, while it was not cytotoxic to normal cells.

To optimize the efficacy of MYCMI-2 in cells, we attempted to identify analogues with improved efficacy in cells while maintaining activity *in vitro*. A number of MYCMI-2 analogues were obtained from NIH/NCI, and their efficacy were screened both in the cell-based and the *in vitro* Gluc assay.

One of the analogues, compound MYCMI-2:47 had a similar activity as MYCMI-2 *in vitro*, but showed a poor GLuc activity in cells. Several other candidates were not as good as MYCMI-2 in the *in vitro* screen, such as MYCMI-2:7, MYCMI-2:16, MYCMI-2:17 and

MYCMI-2:18, but showed improved GLuc activity in cells compared with MYCMI-2. The selected analogues were tested further for selectivity in the GLuc screen but unfortunately many analogues did not pass this test. One analogue, MYCMI-2:7 showed lower but acceptable potency in the *in vitro* GLuc screen, demonstrated slightly better MYC:MAX inhibitory effect in the cell based GLuc screen compared with MYCMI-2 ($p < 0.05$). Further, MYCMI-2:7 maintained selectivity towards MYC:MAX heterodimerization over MAX:MAX or GCN4:GCN4 in Gluc assay both in cells and *in vitro*. Moreover, in the *is*PLA assay, MYCMI-2:7 inhibited MYC:MAX interaction down to about 40% of DMSO treatment, although here the difference towards MYCMI-2 was not significant.

To further investigate MYCMI-2:7 inhibitory capability *in vitro*, a FRET assay was performed, as described above. MYCMI-2:7 exposure at a concentration of 25 μ M showed an inhibitory effect below 50% on the MYC-mTorq:MAX-eYFP protein interaction. Using SPR, the affinity of MYCMI-2:7 to MYC and MYCN was estimated to be approximately 17-20 μ M in K_D .

Unlike MYCMI-2, MYCMI-2:7 downregulated the endogenous MYC protein level in MCF7 cells, indicating that MYCMI-2 and MYCMI-2:7 work differently mechanistically. Similar to MYCMI-2, MYCMI-2:7 treatment selectively reduced cell growth of MYCN-amplified SK-N-DZ neuroblastoma cells with an IC_{50} of approximately 10 μ M while not inhibiting growth of the MYCN-non-amplified SK-N-F1 neuroblastoma cells. MYCMI-2:7 inhibited cell proliferation in a MYC dependent manner when evaluated in Rat1 cells with different MYC status. The growth of HOMYC3 cells with reconstituted MYC declined down to 40% at 6.25 μ M of MYCMI-2:7, while the HO15.19 MYC null cells were unaffected at the same concentrations.

In summary, we have demonstrated that MYCMI-2 has an extraordinary potency *in vitro* binding to MYC with a K_D of 1.3 \pm 0.2 nM as determined by SPR, and an activity in cells in the lower μ M range, while the analogue MYCMI-2:7 was less active *in vitro* and only marginally better in cells. With help of structure-activity relationship (SAR) studies, potentially a more biologically active lead molecule can be designed based on the mapping of binding site of MYCMI-2 to MYC using MYC mutants, NMR and X-ray crystallography.

5 CONCLUSIONS AND PERSPECTIVES

The MYC transcription factor family is a classic oncogene involved in cancer formation and progression. As a “master regulator” MYC plays a critical role in cell cycle progression, biomass increase, apoptosis and tumorigenesis. MYC is reported to be dysregulated in roughly 70% of human cancers, often as a consequence of gene amplification.

In addition, deregulation of MYC proteins is often associated with aggressive forms of tumors and poor prognosis in the clinic (Meyer and Penn, 2008). Evidence is emerging that MYC is both a prioritized and suitable target for anti-cancer therapy. Many studies demonstrated that MYC is required not only for formation and maintenance of typical MYC-driven tumors, but also in a number of different tumors as well, including KRas- or BRAF-induced lung and pancreatic cancer (Soucek et al., 2008) (add some more ref, including the latest from Laura, Tabor, Juan). 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.

Considering the two opposing functions of MYC-induced DDR, it would be of interest to investigate the deeper mechanism so to open up new therapeutic opportunities for targeting MYC. As a tumor suppressor mechanism, DDR is induced upon oncogene activation, such as activation of RAS or MYC, and involves ATM/CHK2, ATR/CHK1, DNA-PK, TIP60, WIP1 and p53 to restrain tumor development, often resulting in apoptosis or senescence. However, the MYC-induced DDR also engages the ATR/CHK1 and DNA repair pathways, allowing cell proliferation and avoiding cytotoxic DNA damage accumulation (Murga et al., 2011; Smith et al., 2010; Weber and Ryan, 2015).

Since MYC is tightly regulated at multiple levels such as transcription level, mRNA translation and protein turnover, etc., inhibition of MYC through any of these mechanisms may result in activation of alternative routes of MYC activation and therefore resistance development (Prochownik and Vogt, 2010). Therefore, targeting MYC directly, or through its obligatory partner Max, although challenging, seems to be a more fruitful way to attack MYC in cancer.

If specific protein-protein interactions within the MYC network can be inhibited, the inhibitors may not only be used as drug candidates but also in the research community for studying the fundamental mechanism of the many MYC actions within the cell.

However, as one of the earliest identified cellular oncogenes, MYC was considered as “undruggable” for such a long time due to the fact that the intrinsic disordered structure of MYC and MAX, as well as the extensive interfaces via large surface lacking of virtually natural

“pocket” to hold firmly the small molecule drug. With science and research methods development, many breakthroughs have been made in MYC biology which indicate that “undruggable” might need to be re-define as “difficult to drug” or “yet to be drugged” (Dang et al., 2017).

In this work, we identified and characterized several small molecules targeting the interaction between MYC and MAX. We found that MYCMI-6 selectively targets MYC:MAX interaction both *in vitro* and in cells without affecting MYC expression and binds directly to MYC with single digit micromolar affinity, thus was qualified as a unique molecular tool to specifically target MYC: MAX pharmacologically and it has good potential for drug development. MYCMI-7 also binds to MYC, and besides its MYC:MAX inhibitory effect also reduces MYC protein expression, it inhibits MYC-dependent tumor cell growth at single digit micromolar concentrations, thereby inducing apoptosis in tumors while sparing normal cells, and most importantly shows good potential *in vivo*. Though the mechanism of how MYCMI-7 targets MYC protein level remains unclear, it could be used as a tool to increase our understanding of pathways for MYC inhibition. MYCMI-2 possesses a very high activity with respect to inhibition of MYC:MAX interactions in all *in vitro* assays including GLuc, FRET (IC₅₀ of 240 +/-80 nM in a 1:1.5 ratio of MYC:MYCMI-2) and SPR (IC₅₀ < 1 nM). Importantly, MYCMI-2 bound to the bHLHZip region of MYC with very high affinity with a K_D of 1.3 +/- 0.2 nM as determined by SPR, compared with MYCMI-6 has a K_D of 1.6 μM for MYC. The affinity of MYCMI-2 to MYC makes it outstanding compared to previously described MYC:MAX PPI inhibitors, such as 10058-F4 and 10074-G5, with an IC₅₀ of approximately 25 μM and no effect up to 100 μM in FRET, respectively. Another well studied MYC:MAX inhibitor, KJ-Pyr-9 was previously reported to bind directly to MYC with a K_D of 6.5 nM, and with an IC₅₀ for MYC:MAX dissociation of 13.4 nM. However, we were not able to confirm this in our protein interaction and binding assays. MYCMI-2 shows mediocre effect on MYC:MAX interaction in cells probably due to inefficient cellular uptake or high intracellular turnover, but it is worthy to identify analogues with improved cellular or *in vivo* bioactivity.

Take all the evidence as described above, our study indicated that despite of MYC's intrinsically disordered nature and other challenges, inhibitors of MYC/MAX interaction is a promising therapeutic approach to combat MYC's tumorigenic function.

Identification of small molecules that specifically inhibits the interaction between MYC:MAX will be of importance not only to increase the basic knowledge on mechanisms of tumor

development but will also contribute to the exploring of new therapeutic strategies to combat cancer in the future.

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