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Targeting MYC and Exploring the Role of Mitochondrial Metabolism in Childhood Neuroblastoma

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Till Olle & Judit

"It always seems impossible until it's done"
Nelson Mandela

ABSTRACT

MYCN is a member of the *MYC* family of proto-oncogenes, encoding transcription factors (c-MYC, MYCN and L-MYC) that play crucial roles for normal cellular functions and during development. However, the expression of MYC (here referring to c-MYC and MYCN) is found elevated in a large number of human cancers where it is implicated in most aspects of tumorigenesis and correlates to poor clinical outcome.

Neuroblastoma is a heterogenous childhood cancer of the sympathetic nervous system. Tumors harboring amplification of the *MYCN* gene are highly aggressive and these patients have a poor prognosis. Consequently, new treatments directed against high MYC expressing tumors could help to improve the survival rates of these children.

In **Paper I**, we screened 80 chemotherapeutic drugs and small chemical compounds to assess their selectivity against MYC-overexpression, using cancer cells with conditional c-MYC or MYCN expression. Positive hits belonged to distinct classes of chemical agents acting on selective cellular processes, including RNA, DNA and protein synthesis and turnover, and those inhibiting microtubules and topoisomerases. These results may provide indications for future drug development and treatment optimization towards MYC.

One important goal in cancer research is to identify small molecules, which can interfere with MYC's function, since today, no therapeutically relevant therapy acting directly against MYC exists. In **Paper II** we demonstrated that a previously identified c-MYC binding molecule, 10058-F4, showed selectivity towards high MYCN expressing neuroblastoma cells and resulted in prolonged survival in a MYCN-driven transgenic mouse model of neuroblastoma. In **Paper IV**, we further demonstrated that 10058-F4 and a few additional c-MYC-binding small molecules bind directly to the corresponding region of MYCN, and that their binding affinities correlated with the level of growth suppression in cells.

Metabolic rewiring is an important feature in aggressive tumors. In **Paper II** we showed that downregulation of MYCN in neuroblastoma cells leads to accumulation of cytoplasmic lipid droplets caused by mitochondrial dysfunction. In this regard, MYCN was found to be linked with an overall elevated mitochondrial metabolism important for mediating tumor aggressiveness in neuroblastoma.

In **Paper III**, we carried out a systematic investigation of metabolic alterations associated with MYCN in neuroblastoma, using patient gene expression data, quantitative proteomics and functional studies of metabolic pathway fluxes. MYCN was found to positively regulate glycolysis, respiration as well as oxidation of exogenous fatty acids in neuroblastoma cells, suggesting that MYCN mediates metabolic plasticity, which could account for an important survival mechanism during neuroblastoma tumor progression.

Together the work comprised in this thesis support the development of targeted therapy against MYCN and identified MYCN-induced metabolic signals as a potential approach to target high risk neuroblastoma.

LIST OF SCIENTIFIC PAPERS

I. Anna Frenzel, **Hanna Zirath**, Marina Vita, Ami Albihn, Marie Arsenian Henriksson. Identification of Cytotoxic Drugs That Selectively Target Tumor Cells with MYC Overexpression. PLoS One. 2011;6(11):e27988.

II. **Hanna Zirath**, Anna Frenzel, Ganna Oliynyk, Lova Segerström, Ulrica K. Westermark, Karin Larsson, Matilda Munksgaard Persson, Kjell Hultenby, Janne Lehtiö, Christer Einvik, Sven Påhlman, Per Kogner, Per-Johan Jakobsson, and Marie Arsenian Henriksson. MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells. Proc Natl Acad Sci U S A. 2013 Jun 18;110(25):10258-63.

III. Ganna Oliynyk*, **Hanna Zirath***, Marcus Klarquist, Janne Lehtiö, Christer Einvik, and Marie Arsenian Henriksson. MYCN mediates metabolic plasticity in human neuroblastoma. Manuscript, 2014.

IV. Inga Müller, Karin Larsson, Anna Frenzel, Ganna Oliynyk, **Hanna Zirath**, Edward V. Prochownik, Nicholas Westwood and Marie Arsenian Henriksson. Targeting of the MYCN with small molecule c-MYC inhibitors. Plos One, in press, 2014

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CONTENTS

LIST OF ABBREVIATIONS

3BrPA	Bromopyruvic acid
ACAA2	Acetyl-CoA acetyltransferase 2
AKT/PKB	Protein kinase B
ALK	Anaplastic Lymphoma Receptor Tyrosine Kinase
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
BCL2	B-cell CLL/lymphoma 2
BET	Bromodomain and extraterminal (BET)
BL	Burkitt´s lymphoma
BRD4	Bromodomain containing 4
CAF	Cancer associated fibroblast
CDK	Cycline dependent kinase
ChiP	Chromatin immunoprecipitation
Chip-seq	Chromatin immunoprecipitation sequencing
CIS	Cisplatin
CPT1	Carnitine palmitoyltransferase 1
CPT	Champtothecin
CSC	Cancer stem cell
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DXR	Doxorubicin
E-box	Enhancer box
ECS	Embryonic stem cell
EGF	Epidermal growth factor
EMSA	Electrophoeretic mobility shift assay
EMT	Epithelial to mesenchymal transition
ETO	Etoposide
FAO	Fatty acid oxidation

FADH2	Flavin adenine dinucleotide
FASN	Fatty acid synthase
GLS	Glutamine
GLUT1	Glucose transporter 1
GTPase	Guanosine triphosphate (hydrolyze enzyme)
HADH	Hydroxyacyl-CoA dehydrogenase
HK2	Hexokinase 2
HIF1	Hypoxia inducible factor 1
HLHLZip	Basic helix-loop-helic leucine zipper
Ig	Immunoglobulin
LDHA	Lactate dehydrogenase A
LC-MS	Serum liquid chromatography mass spectrometry
MAPK/ERK	Mitogen-activated protein kinase kinase
MAX	MYC Associated Factor X
MNA	*MYCN* amplified
MYC	*v-myc* avian myelocytomatosis viral oncogene homolog
NADP	Nicotinamide adenine dinucleotide phosphate
NB	Neuroblastoma
NGF	Nerve growth factor
NMNA	Non- *MYCN* amplified
Notch	Notch homolog 1, translocation-associated (Drosophila)
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1
OH-CPT	10-OH-camptothecin
Oxphos	Oxidative phosphorylation
PEITC	B-phenylethyl isothiocyanate
PHOX2B	Paired-like homeobox 2b
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
POD	Podophyllotoxin
RAC	A subfamily of the Rho family of GTPases
RAS	A protein superfamily of small GTPases
RHO	A subfamily of the Ras superfamily

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SNP	Single nucleotide polymorphism
shRNA	Short hairpin RNA
TAX	Paclitaxel
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor β
TH	Tyrosine hydroxylase
TOP	Topoisomerase
TRKA	Receptor tyrosine kinase A
TTFA	2-thenoyltrifluoroacetone
TWIST	Twist basic helix-loop-helix transcription factor 1
VBL	Vinblastine
VEGF	Vascular endothelial growth factor
WNT	Wingless-type MMTV integration site family

1 INTRODUCTION

1.1 CANCER

1.1.1 Definition

Cancer is a collective name for over 100 different types of diseases affecting different tissues, in which cells become abnormal and grow out of control, beyond their natural boundaries and can spread (metastasize) to other organs, which is a major cause of death from the disease (1, 2). Throughout the world, especially in Western countries, cancer is a major health problem and cause of mortality, accounting, for instance, for 20 % of all deaths in Europe (3, 4).

1.1.2 Cancer is a disease of deregulated growth signals and dysfunctional control systems in cells

More than 35 years of empirical observation and testing have supported cancer as a gradual "Darwinian" evolutionary process (see *Figure 1*), driven by clonal selection of somatic (or germ-line, in case of familial cancers) gene alterations (DNA mutations, copy number alterations, or epigenetic changes) affecting oncogenes and/or tumor suppressor genes, ultimately conferring selective growth and survival advantages (1, 5).

In multicellular organism there are highly efficient cancer suppressive control mechanisms operating to both prevent and ensuing elimination of pre-malignant cells or lesions (1). Hence, cancer is only "allowed" to develop when the various control systems of tissue homeostasis which are normally under tight regulation have gone awry and the cancer cells hence can escape from being "held in check" (1). In normal tissues, cell growth is strictly regulated by both growth-suppressive and promoting (mitogenic) signals. As a simplified example, cell growth can be mediated through growth factor molecules, which bind to cell surface receptors, in turn harboring intracellular tyrosine kinase receptors. These transfer the signal further, leading to a cascade of downstream signaling events in the cells which together impact on cell cycle progression and cell growth (2). There are also negative feedback-loops of regulatory signals as well as tumor suppressor molecules whose function is to inhibit tumor growth by sensing genetic and functional imbalances and thereafter dampening or halting proliferation, or inducing cell senescence or apoptosis to eliminate defective cells (2). In tumors, various mechanisms operate to evade these control systems, consequently leading to constitutive activation of growth-inducing signals and avoidance of cell death (2), which is a major cause of treatment failure (6).

1.1.3 Cancer involve the activation of oncogenes and inactivation of tumor suppressors

Proto-oncogenes are genes that during normal physiological conditions are necessary for processes such as proliferation, differentiation and survival, while their activation leads to tumorigenesis. Genetic alterations that cause activation of proto-oncogenes and consequently leads to elevated expression or increased activity of the encoded onco-proteins, promote cancerous events by providing growth and survival advantages (7). Examples of onco-

proteins include transcription factors (e.g. MYC and TWIST) (8, 9), receptor tyrosine kinases (RTK) (e.g. vascular endothelial growth factor receptor (VEGF)) (10), growth factors (e.g. epidermal growth factor (EGF)) (11), serine/threonine kinases (e.g. cycline dependent kinases, CDKs) (12) regulatory GTPases (such as RAS) (13), cell cycle regulators (including cyclins) (12) and regulators of cell death (e.g. members of the BCL2 family of proteins) (14). Likewise, as already mentioned, tumor formation and progression also involve the inactivation of so called tumor suppressor genes, encoding for tumor suppressor proteins. The normal function of tumor suppressors in cells is to protect cells from tumor formation, mediated by suppression of cell growth, detecting and repairing damage to DNA, regulating the ubiquitination and degradation of proteins, and inducing apoptosis (7, 15, 16). The transcription factor p53 is a classic example of a tumor suppressor since it is deleted or mutated in ~50 % of all human tumors, and is often referred to as "the guardian of the genome" due to its important anti-tumorigenic functions (17).
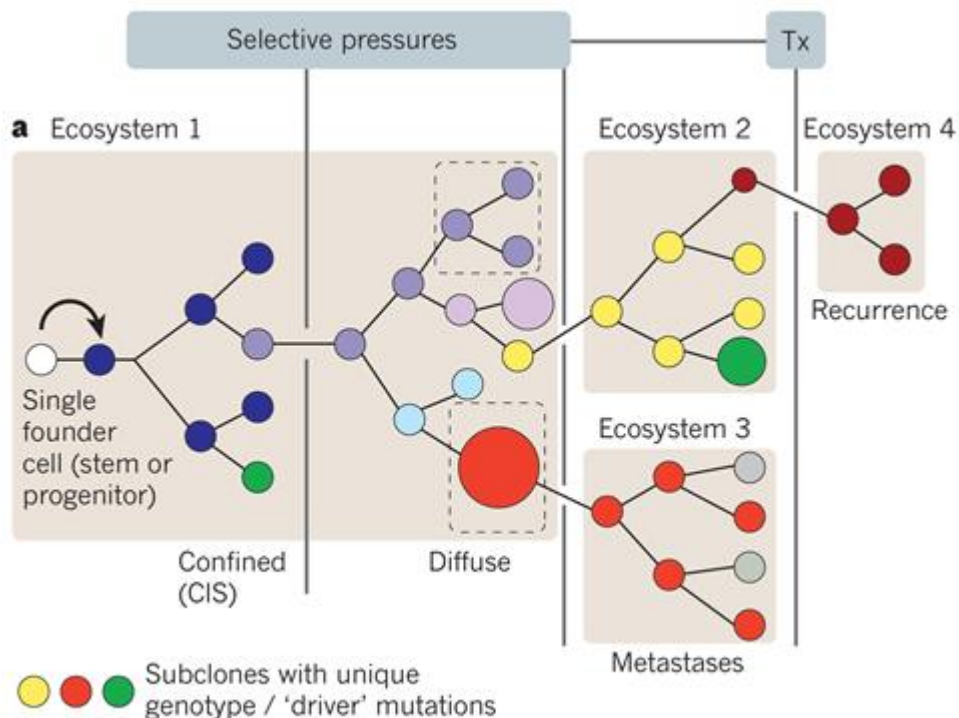
Figure 1. Representative pattern of clonal evolution in solid cancers. The development of cancer is a complex process involving the expansion of cancer clones acquiring selectively advantageous lesions to survive selective pressures or restraints (vertical lines), genetic diversification of co-existing cell clones (represented by differentially colored circles) driven e.g. by additional mutations and other genetic or epigenetic changes and the tumor milieu, as well as clonal selection. These processes occur in close connection with the tumor microenvironment of "tissue ecosystems". CIC: carcinoma in situ, Tx: treatment. From Greaves M & Maley CC, 2012, with permission from the publisher (1).

Accumulating gene mutations and/or gene copy number alterations during tumor progression can furthermore trigger genomic instability, leading to events such as chromosomal gains and losses. Consequently the genetic diversity is further increased and accelerates the malignant

development (2, 5). Importantly, the tumor formation process is furthermore believed to progress by natural selection, similar to in organisms, driven by the competition for space and resources of the cancer cells (see *Figure 1*) (1, 18).

1.1.4 Recurring themes in cancer despite an enormous complexity

Cancer is an extremely heterogeneous disease; in fact each tumor is unique, originating from different types of tissues and cells of origin, and harbors diverse types of mutations and genetic alterations (1, 19-21). Moreover, due to the clonal evolvement of cancer, each tumor can be viewed as multiple different cancers or subclones, occupying distinct or intersecting tumor territories (1, 22).

The rapid development of second generation high throughput sequencing techniques has allowed for deep analyses of cancer genetics and epigenetics and has consequently further revealed the complexity of human tumors (1, 19, 20). However, despite the enormous genetic and histological heterogeneity seen both within specific tumor types as well as between different forms of cancers, these techniques have also allowed for the discovery that a relatively small number of recurring alterations or "driver events" exists across cancers. These identified events or "oncogenic signatures" are coupled to distinct, well recognized oncogenic pathways (including MYC driven proliferation) and can be used to characterize tumors independently of tissue of origin (19). Similarly, a limited number of phenotypic traits or "hallmarks of cancer" which are the functional outcome of accumulated genetic alterations, can be delineated across cancers, and have been recognized since decades. These traits include the acquired ability of: unlimited cell growth by sustaining proliferative signaling and evading growth suppressors, avoidance of apoptosis, allowing for replicative immortality by taking control over the machinery that regulates chromosomal telomere shortening, and inducing angiogenesis (new intra-tumor blood vessels) and metastasis (2). Also, cancers share some common attributes of metabolic aberrations, as will be discussed in further detail later (page 10).

1.1.5 Clues from the biology of embryonic development

Importantly, the phenotypic traits of cancer just described (2) are not restricted to tumors but also occur naturally during physiological processes (e.g. cell migration during wound healing) and during embryonic development (e.g. cell migration and invasion of neural crest cells). In fact, there is a large amount of evidence suggesting that similar signaling programs regulate cell fates during both normal and embryonic development and tumorigenesis. For instance, factors involved in mediating rapid cell growth during morphogenesis (including MYC), differentiation, as well as the process of epithelial to mesenchymal transition (EMT) needed for cells to detach from their original position and migrate in the developing embryo, are readily implicated in cancer (23, 24).

1.1.6 Tumors consist of many different cell types

The tumor microenvironment is an important component of tumors and is crucial for cancer progression. In fact, tumors are not simply made up by malignant cancer cells. Instead, diverse cell types are recruited from the tumor surroundings such as fibroblasts, vascular and inflammatory cells that together constitute the cancer associated fibroblasts (CAFs), or stroma (see *Figure 2*) (25, 26). Together, these cells are potent modulators of the tumorigenic process. In addition, both the tumor cells and the tumor microenvironment are modulated by factors such as nutrients, inflammation and hormones and by lifestyle factors such as diet and the degree of genotoxic exposure (1).

Although there is still some controversy regarding the origins of CAFs, these cells are in general believed to originate in the bone marrow and are known to promote cancerogenous events such as inducing EMT important for the metastatic process, inducing a stem cell phenotype, angiogenesis, therapy resistance and tumor-relapse (1, 26-29).

In this way, cancer can be viewed as complex tissues involving cross-talk between different cell types (2, 26).
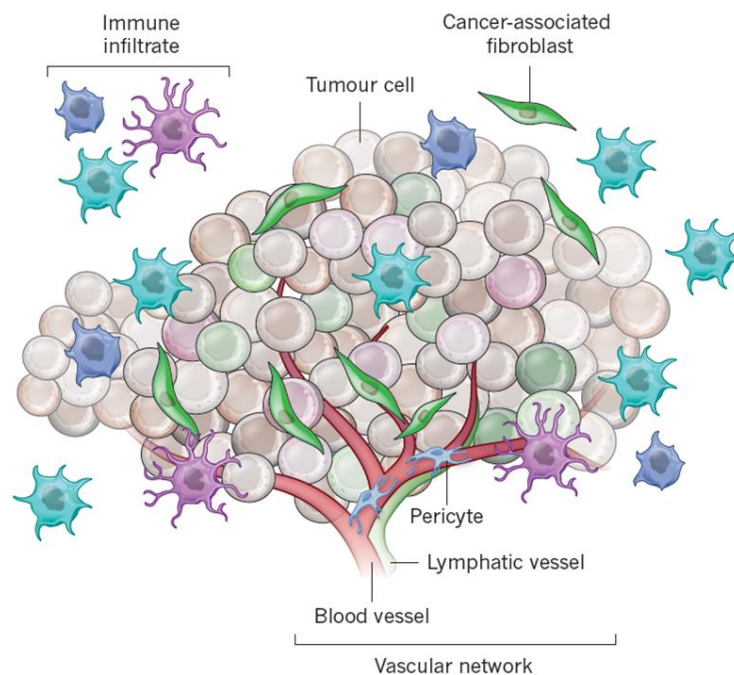
Figure 2. The tumor microenvironment. Tumors are heterogeneous and are composed not only of neoplastic tumor cells, but also of extracellular matrix, endothelial cells, fibroblasts and immune cells. These different cell types can confer both pro- and anti-tumorigenic functions, and together form the tumor microenvironment, which can vary radically even within the same tumor. From Junttila MR & de Sauvage FJ (2013), with permission from the publisher (26).

1.1.7 Cancer stem cells

The view of cancer is further complicated by the increasing evidence for the existence of cancer stem cells (CSC) in the majority of human tumors (30). Such tumor initiating cells are defined by their efficient ability to form new tumors when implanted into recipient immune-deficient mice (2). Although the exact origin of CSC is not clear, these cells express stem cell markers similar to normal stem cells of the tissue of origin and are capable of self-renewal as well as of giving rise to more differentiated derivatives of multiple lineages. These cells are also less sensitive to therapy and irradiation and have been coupled to tumor cell dormancy and cancer relapse (1, 2, 31). Hence, the cancer stem cell model proposes that only a subpopulation of the cells in a tumor, i.e. the tumor stem cells, actually possess the ability to regenerate new tumors, since these are the actual drivers of cancers through their self-renewal capacities (30, 32).

1.2 THE MYC ONCOPROTEINS MEDIATE MALIGNANCY IN CANCER

The *MYC* (*v-myc* avian myelocytomatosis viral oncogene homolog) oncogenes encode pleiotropic basic helix-loop-helic leucine zipper (HLHLZip) transcription factors (c-MYC, MYCN and L-MYC), which are central in cancer by contributing to the genesis and aggressiveness of the majority of all human tumors (33, 34). The MYC proteins (here referring to c-MYC and MYCN) form heterodimers with a partner protein called MAX (see *Figure 3*), and the MYC/MAX complexes consequently bind to regulatory sequences (termed E-boxes) of DNA to trans-activate or repress gene expression in cooperation with multiple other factors (8, 35).
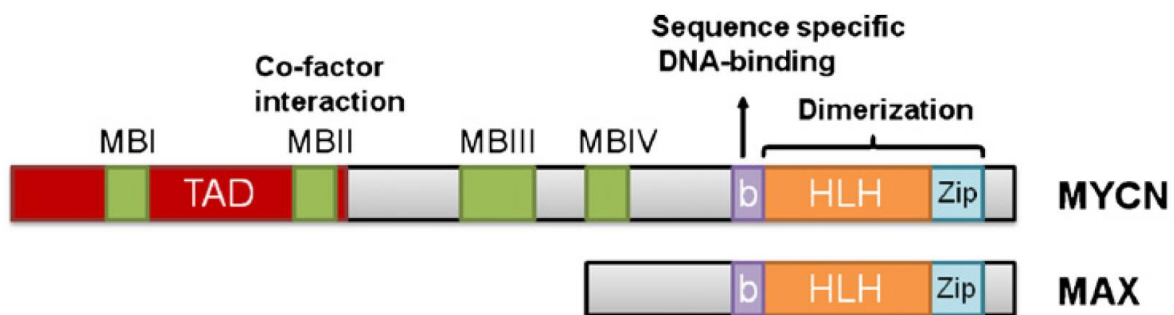
Figure 3. The protein structure of the MYCN and MAX proteins. The MYC *proteins contain several domains that are important for their normal functions as well as for their transformation abilities: the caboxy-terminal domain of* MYC *is responsible for the dimerization with the* MAX *protein (helix-loop-helix, HLH and leucine zipper, Zip), and for binding to regulatory sequences of DNA (basic region, b). The amino-terminal transcriptional transactivation domain (TAD) confers activation of gene transcription.* MYC *homology boxes (MB) are regions responsible for interacting with other proteins. MBIV contains the nuclear localization signal, responsible for subcellular localization to the nucleus. From Westermark, U, (2011), with permission from the publisher (36).*

While the role of L-MYC is less well understood, MYCN and to an even higher extent c-MYC, are implicated in mediating malignancy in up to 70 % of all human cancers (8). The target genes of c-MYC and MYCN are largely overlapping, and MYCN can even substitute for c-MYC expression in murine development (37). A major difference between the two proteins is their respective expression patterns, as c-MYC is normally ubiquitously expressed in all proliferating cells whereas the expression of MYCN is restricted to specific tissues during development (8, 38, 39). The MYC proteins are important for normal development (40-45) and cell physiology and the expression of MYC is normally tightly regulated, guaranteeing that its levels are kept low and is only induced upon cell cycle entry. Tumor suppressor-mediated negative feedback loops (such as p53), ensure that MYC expression is held in check, and their inactivation is often needed in order for MYC to reach its fully malignant potential (8).

1.2.1 MYC expression is elevated through multiple mechanisms in cancer

In cancer, the *MYC* genes are found deregulated by mutations, gene amplification and chromosomal translocations as well as by common polymorphisms in regulatory regions close to MYC (8, 35, 46). In common for these events is that it all leads to constitutively high expression of MYC, and consequently to aberrant regulation of its downstream genomic targets. This in turn causes uncontrolled proliferation which is uncoupled from growth-factor stimulation (47). Except from genetic alterations, MYC can also be up-regulated caused by enhanced translation or due to changes in its protein stability as well as in response to deregulated upstream signaling events which leads to elevated MYC transcription. In this regard, most key mitogenic signals in cells converge on MYC to carry out their growth promoting functions, including receptor tyrosine kinase signals like RAS/ERK/MAPK and PI3/AKT, as well as other important ligand-membrane receptor signals such as TGF-β and Notch. In addition, defects in the WNT/APC signaling pathway brings about MYC activation (8). Consequently MYC is believed to play important roles for the initiation and maintenance of cancer even in cases where no obvious genetic alterations in *MYC* exist. Importantly, this idea is supported *in vivo* by the finding that knock down of endogenous MYC expression, by expression of a dominant negative inhibitor of MYC, systemically in all cells in the adult mouse, is sufficient to cause tumor regression even if the tumors are induced by another oncogene (48, 49).

Except from its role in gene regulation, MYC also directly controls the expression of a number of small non-coding RNAs called microRNAs, with important consequences for the transformation process (8, 50, 51), and MYC has also been linked to other cellular processes like DNA replication and mRNA cap-dependent translation (8, 52, 53).

1.2.2 Which are the tumor promoting mediators downstream of MYC?

A large interest and focus during the last decades has been to map the downstream transcriptional program of MYC in cancer in the search for a common core set of target genes. Hence, the common goal has been to understand how MYC confers malignancy in

tumors. Intriguingly however, this work has revealed that MYC is able to directly bind to the promoters of ~15 % of all known genes, and drives transcription mediated by all three RNA polymerases; in fact, MYC has been coupled to almost all aspects of tumorigenesis (8, 54). Furthermore, the overlap between the "MYC target gene signatures" identified in different cell systems, which could explain MYC´s biological output in cancer, is surprisingly low (47, 55). Nevertheless, a main reason why MYC is implicated in so many cancers is believed to relate to its crucial role in regulating the cellular machinery for cell mass expansion and proliferation, which is a prerequisite capability in tumors (8, 56). Studies from lower organisms like flies to multiple mammalian systems and tumor models have identified MYC to regulate genes involved in organellar biogenesis, in particular ribosomal and mitochondrial genes. Chromatin immunopricipitation (ChiP) sequencing (ChiP-seq) and gene expression studies have identified core sets of MYC targets induced by serum stimulation that include genes involved in nucleotide metabolism, ribosomal biogenesis, RNA processing, and DNA replication (8, 57). Also, studies designed to identify tissue independent MYC targets have shown enrichment for ribosomal genes, again showing that MYC´s primary function may be to promote biomass accumulation (8, 56, 58). These findings hence confirm MYC to control cell growth and cell cycle regulation, and provide a strong link to its regulation of energy metabolism (8, 35, 54, 59-63).

Another essential aspect is that the MYC proteins play crucial roles in the regulation of pluripotency and self-renewal of normal and cancer stem cells (8, 64, 65) and have been reported to drive an embryonic stem cell (ESC) program which is also induced in various cancers (66). MYC is furthermore important for the recruitment and maintenance of the tumor microenvironment (49, 67) and for inducing EMT and metastasis (68). Systemic inhibition (in all tissues) of endogenous MYC leads to collapse of the tumor environment including the neo-vasculature, leading to tumor regression, again demonstrating the importance of the tumor associated stroma for tumorigenesis (49, 67). It also suggests that this function of MYC is critical for its tumor promoting ability (69).

1.2.3 How does MYC regulate gene transcription?

Based on the collected knowledge gained from genome wide association studies of MYC transcription, it is generally believed that MYC functions as a general transcription factor since it binds to ~15 % of all promoters in proliferating cells and have widespread types of targets. It has been suggested that upon elevated levels of MYC, its presumably constitutive expression leads to occupancy of non-canonical E-boxes, normally bound by other transcription factors. Consequently this could bring about alterations in genes that are not regulated by MYC during physiological conditions (8). In this regard, studies have shown that different threshold levels of MYC determine the biological outcome (70, 71). Additional mechanism to how MYC may regulate transcription relate to its demonstrated ability to directly influence global chromatin structure (72), and to bind to actively transcribed genes and function to mediate RNA polymerase II pause release, which consequently speeds up the transcriptional elongation process (73). Recently, new important insights into the function of

MYC have been reported that may lead to considerable refinement of the "MYC target gene concept" (47, 55). It was shown both in tumor cells and in physiological conditions and stem cells that, instead of activating a new set of specific target genes, elevated c-MYC levels causes an increased, or amplified expression of already actively transcribed genes (47, 55). Hence, higher levels of c-MYC leads to its binding to the same set of genes as already bound, although at elevated levels compared to when c-MYC is expressed at low levels. This increase in c-MYC's gene occupancy (involving both the core promoters and enhancers of the same genes), consequently leads to overall elevated levels of all the mRNA transcripts already actively transcribed in the cell (47). Consequently this newly proposed role of MYC as a universal amplifier of already "predetermined" gene expression (74), could help explaining the little degree of overlap generally seen in MYC-induced gene expression between different cell types. It could also explain how MYC can control such a great number of diverse cellular processes (55). Nevertheless, more research is needed to fully elucidate how MYC affects e.g. the stoichiometry of amplified mRNAs and its role in transcriptional repression (75).

1.2.4 Targeting MYC as cancer therapy

The concept of oncogene addiction proposes that although cancers contain endless numbers of genetic lesions, some tumors are still dependent on one single, or a few, dominant transforming oncogenes for their survival. Consequently, the targeted inactivation of the same oncogene(s) is sufficient to halt or revert tumor progression (20, 76). This theory has been supported by a large number of pre-clinical studies as well as by therapeutic applications of some cancers (76, 77).

MYC is a well-known example of this notion (78), although so far no therapies against MYC exist (79). Multiple *in vitro* studies as well as transgenic mouse models have confirmed that deregulated MYC expression is sufficient to induce different types of cancer (8, 33, 34). MYC is also important for cancer maintenance since in conditional *MYC* mouse models, withdrawal of ectopic MYC expression is sufficient to cause tumor regression mediated by apoptosis, senescence and/or differentiation, as shown in multiple different tumor models (8, 34, 80, 81). As mentioned above, it was demonstrated that systemic inhibition (in all tissues) of endogenous MYC in adult mice causes regression of tumors induced by other oncogenes while sparing normal cells and tissues, suggesting that MYC-directed therapies could be fairly well tolerated (48, 49). These findings, together with the realization that MYC is as an upstream regulator of numerous oncogenic events, implicate MYC as an attractive target in many types of tumors (33, 82). Importantly, the fact that MYC expression is deregulated in the majority of cancers suggest that therapeutic targeting of MYC and/or its key oncogenic functions could have wide-ranging application to the treatment of cancer (82, 83).

1.2.5 How to target MYC in cancer?

To this end, several different strategies to inhibit MYC have been proposed, including targeting of its transcription or translation, its mRNA or protein stabilization, as well as its

dimerization to MAX or other chromatin-interacting factors important for its transcriptional function (33, 79). In this respect, large interest has been recently paid to the BET domain-containing transcriptional regulator, bromodomain containing 4 (BRD4), since it was found to play critical roles for MYC induced transcription, as well as for the transcription of the *MYC* gene itself. BRD4 recognizes and binds acetylated histones of chromatin. Its chemical inhibition by small molecules including JQ1 has been successful in inhibiting tumorigenesis in some pre-clinical models, including hematopoietic cancers and neuroblastoma (83-87). The molecule JQ1 binds to the domain of BRD4 which is important for its interaction with acetylated lysines in histones (79). These studies hence show that MYC inhibition, through the targeting of chromatin regulators, may be possible in some cancers.

Some reports have also suggested that some key MYC-induced pathways, or the fact that MYC overexpressing cells are dependent on nutrients like glucose or glutamine, can be explored for therapy (8, 88, 89). The important role of MYC in maintaining the tumor stroma (49, 67) is interesting, as targeting of the tumor microenvironment has been suggested as therapy (27, 90). There are furthermore reports pointing towards the possibility that inhibiting specific MYC-regulated microRNAs may be sufficient to prevent tumor progression (8, 50, 91). Moreover, targeting of specific pathways showing synthetic lethality with high MYC expression may be fruitful. The concept of synthetic lethality was originally defined in classical genetics as a state in which the mutation of either of two pro-survival genes, alone, promotes survival, whereas the simultaneous mutation of both genes leads to cell death. In cancer, this model has been adopted and is used as a conceptual basis for identification and development of cancer specific cytotoxic agents. Consequently, targeting a gene which is synthetic lethal with a cancer associated mutation or other cancer related aberrations, leads to cancer-specific cell death while sparing normal cells (92, 93). For instance, in relation to MYC, targeting of mitotic processes (Aurora kinases and CDK1) or a specific sumoylation mediating enzyme SAE1/2 has been shown to induce synthetic lethal therapeutic responses in mouse models (8, 79, 94-96).

The strategy of directly targeting MYC pharmacologically is extremely challenging for several reasons. Since MYC is a transcription factor it lacks the typical targetable clefts usually found in enzymes (82). Monomeric MYC in solution is a flexible, intrinsically disordered (ID) protein, which possesses only transient tertiary structure. Such proteins are commonly found among transcription factors, and it allows them to form weak but specific interactions with other proteins (such as MAX) via coupled folding and binding, required for their functions (97). In order for a small molecule to inhibit this binding the interaction must overcome the enthalpic and entropic changes associated with the favorable interaction of a partner protein. However, the "lock in" of a tertiary structure by selective molecules has been proposed to be possible, based on the finding that although the interacting domains of ID proteins are flat and featureless and hence hard to target, only a minor part of the dimer interface seems to be responsible for the protein-protein interaction. Consequently, targeting of these "hot spots" may be sufficient to prevent the proteins to interact properly (79, 82, 98-100). There are several examples of small compounds which have been shown to be

relatively potent inhibitors of protein-protein interactions (79). As an example, small molecules like the nutlins and RITA have been shown to interfere with the interaction between p53 and its suppressor MDM2, which leads to activation of wild type p53 (101, 102).

A number of small molecules identified in cellular screens have been documented to interfere with the c-MYC/MAX interaction and to induce selective growth inhibition in MYC overexpressing cells (103-105). Some of these molecules have furthermore been confirmed to bind directly to the basic helix-loop-helix leucine zipper (bHLHLZip) domain of c-MYC, which is responsible for its interaction with MAX (98, 100, 106, 107) (see *Figure 8*). Consequently these reports suggest that "direct" inhibition of MYC, through binding and consequent interference with the MYC/MAX dimerization, can be achieved, at least *in vitro*, using small molecules. Concerns however relate to the lack of drug-like structural properties, rapid turnover, and relatively low potency of currently known molecules (108). Importantly, we have showed for the first time that treatment with one of these small molecules, 10058-F4, is sufficient to induce prolonged survival *in vivo* using a transgenic tumor model of neuroblastoma (**Paper II**) (109). We have also characterized the direct binding to MYCN for several of the small molecules initially identified to bind to c-MYC, including 10058-F4 (**Paper IV**, Plos One, 2014, in press). Our results revealed that all molecules previously identified to bind to c-MYC, also bind to MYCN with equal affinities, and that the binding as such may be important for inducing a reduced MYCN/MAX dimerization and cell growth inhibition.

1.3 CANCER METABOLISM

Instead of being viewed as an indirect, secondary effect of proliferative signals, today the importance of an altered metabolism in tumors is evidently clear and is considered to be an emerging core hallmark of cancer, as those described by Hanahan and Weinberg (110-113). In fact, metabolic reprogramming including increased nutrition uptake and utilization occurs in direct response to oncogenic signaling, and is required to support anabolic growth of tumors (110, 113, 114). Hence the reprogramming of cellular metabolism may be a primary causal function selected for during tumorigenesis in response to activated oncogenes and inactivated tumor suppressors (110, 115).

1.3.1 Glycolysis versus mitochondrial metabolism

Traditionally, cancer cell metabolism has been defined based on Otto Warburg's groundbreaking work starting over 80 years ago when he demonstrated that normal non-proliferating cells mainly use mitochondrial oxidative phosphorylation (oxphos) to produce energy, whereas cancer cells, despite the presence of oxygen, show an increased glucose uptake and enhanced glycolytic capacity resulting in lactate production at the expense of mitochondrial respiration (112, 114, 116-118). However, although an elevated glycolysis is indeed observed in most rapidly growing cell lines and tumors (119), a more dynamic view of cancer bioenergetics has emerged during recent years. Specifically, it has become clear that

metabolic reprogramming in tumors is not simply a passive response to damaged mitochondria (110, 119). Instead, mitochondria are fundamental for anabolic reactions needed in order for the tumor to grow. Hence most tumor mitochondria are not defective in their ability to carry out their oxphos function, but instead the oxphos machinery is rewired away from ATP production to instead generate building blocks for anabolic reactions such as lipolysis and protein synthesis needed in the rapidly proliferating tumor cells (110, 119). Similarly, an increased glycolytic flux not only provides for a rapid source of ATP, but is also important for NADPH production for lipid synthesis and purimidine/ purine (nucleotide) metabolism via the pentose phosphate and folate pathways, respectively, as well as for amino acid metabolism (113, 115, 120).

1.3.2 "Metabolic plasticity": an escape mechanism in aggressive tumors?

It is important to note that large variability exist among cancer types as well as within specific tumor types, and the various bioenergetic types of tumors ranges from exclusively glycolytic to mainly oxphos-dependent (119). For instance, glioma cells display either a glycolytic- or oxphos dependent phenotype (121), and such metabolic heterogeneity has also been documented in diffuse large B cell lymphoma (122). It was also shown that breast cancer cells, compared to their normal counterparts originating from non-breast cancer tissue, show an elevated metabolic adaptation in response to hypoxia (condition in which the tumor cells are deprived of adequate oxygen supply) and glucose deprivation, mediated by an increased mitochondrial oxphos capacity (123). These findings suggest that switching between different energetic states can occur in cancer cells during tumor progression (119). The harsh conditions in the tumor environment, which is known to include both hypoxia and nutrient deprivation, may hence trigger metabolic adaptations of mitochondria in the tumor cells (123). In this regard, it has been shown that simply changing substrate availability can restore the defective mitochondrial function in tumor cells (124). Such adaptations may hence be an important survival mechanism for cancer cells in response to changes in the microenvironment and in their biosynthetic needs, and could promote tumor progression (119). The tumor microenvironment is an also important modulator of tumor cell metabolism through the "fueling" of anabolic growth of the cancer cells, mediated by the secretion of nutrients (such as fatty acids and lactate) from the CAFs and adipocytes of the tumor stroma or metastatic niches (125-127).

The concept of "metabolic plasticity" is important from a therapeutic point of view, since conventional cytotoxic drugs target rapidly dividing cancer cells which are likely to have high glycolytic rates, but presumably misses quiescent cells, which could be dependent on oxphos. Indeed, a recent study has highlighted the importance of an oxphos capacity in treatment-resistant cancer cells (128). The authors found that cancer cells which enter a state of dormancy in tumors due to tumor hypoxia and limited nutrition availability, and hence are not killed off by cytotoxic treatment, are sensitive to inhibition of mitochondrial oxphos. This finding suggests that the ability of some cancer cells to switch between different bioenergetics states could explain the re-growth of tumors after conventional treatment (128).

There are also interesting links between mitochondrial bioenergetic properties and cancer stem cell functions. In mouse embryonic stem cells, the level of mitochondrial oxphos capacity was shown to modulate both the differentiation and tumor formation capacity (129), and an induced dependency on oxphos has been documented during the transformation of human mesenchymal stem cells (130).

1.3.3 MYC is a key regulator of metabolism in cancer

As described earlier, MYC is a key mediator of metabolism by regulating the core machinery for biosynthetic reactions and cell growth (131) (see *Figure 4*). In fact, this function of MYC is important as MYC-driven cancer cells are addictive to various nutrients, and it has been suggested that such addicted state can be exploited for therapy (115).

MYC is a known inducer of aerobic glycolysis; many glycolytic enzymes have been described as both direct and indirect targets of MYC, including lactase dehydrogenase A (LDHA) and glucose transporters as well as hexokinase 2 (HK2), among others (46, 132, 133). There is a well-documented interplay between MYC and the hypoxia inducible factor 1 (HIF1) transcription factor in the regulation of glycolysis, suggesting that HIF1 regulates glycolysis under hypoxic conditions in collaboration with MYC, whereas MYC regulates the same genes under non-hypoxic conditions (46, 134).

In addition, MYC overexpressing cells have been shown to be dependent on glutamine for survival, referred to as glutamine addiction. This state is believed to evolve in some glycolytic tumors in response to the compensatory need for glutamine anapleurosis (replenishment) of the tricarboxylic acid cycle (TCA)/citric acid cycle intermediates, to maintain the activity of mitochondrial biosynthetic processes (131, 135, 136). Glutamine may also be important for maintenance of phospohlipid synthesis while the contribution from glucose decreases (136), as well as for providing a source of NADPH and nucleotide biosynthesis (137). MYC overexpression directly promotes glutaminolysis by upregulating transporters that enhance glutamine uptake, and by inducing the expression of the glutamine-to-glutamate catalyzing enzyme glutaminase (GLS) (131, 136). Furthermore, MYC has been shown to indirectly mediate glutaminolysis through suppression of the microRNA miR-23a/b, which negatively regulates GLS (138). Importantly, many different types of tumor cells in culture are highly addicted to glutamine (89, 139, 140), suggesting that this could relate to high MYC expressing tumors as well.

Interestingly, in addition to promoting glycolysis and glutaminolysis, a large number of studies demonstrate that one of MYC's main functions is to regulate mitochondrial biogenesis and function through direct gene activation of a large number of nuclearly encoded mitochondrial proteins, as well as through activation of other factors which promote mitochondrial biogenesis (141-143). In fact, MYC has been demonstrated to be crucial for the regulation of mitochondrial mass, structure and dynamics (141, 144). For instance, *myc-/-* rat fibroblasts have fewer and smaller mitochondria with less visible cristae, as well as reduced membrane polarization compared to wild type *myc+/+* expressing cells, whereas the

mitochondrial mass, length and cristae pattern was slowly restored (over weeks) following activation of ectopic myc (144). Chronic depletion of myc in rat fibroblasts furthermore leads to a reduced oxphos capacity (144, 145), whereas induction of MYC in pre-B cells leads to enhanced oxygen consumption rates (141, 143). Interestingly, it was shown that a high MYC-induced proliferation rate is clearly linked to an increased mitochondrial function. In myc negative cells, which show dramatically reduced proliferation rates, proliferation was demonstrated to depend primarily on glycolysis, while myc positive cells used both glycolysis and oxidative phosphorylation for maximal proliferation (145).

As already mentioned, in addition to glycolysis and mitochondrial metabolism, MYC directly regulates ribosomal biogenesis and RNA processing and consequently causes enhanced protein synthesis (131). Moreover, MYC is an important regulator of nucleotide biogenesis, and this function of MYC is critical for the orchestration of DNA replication (46, 131, 146).
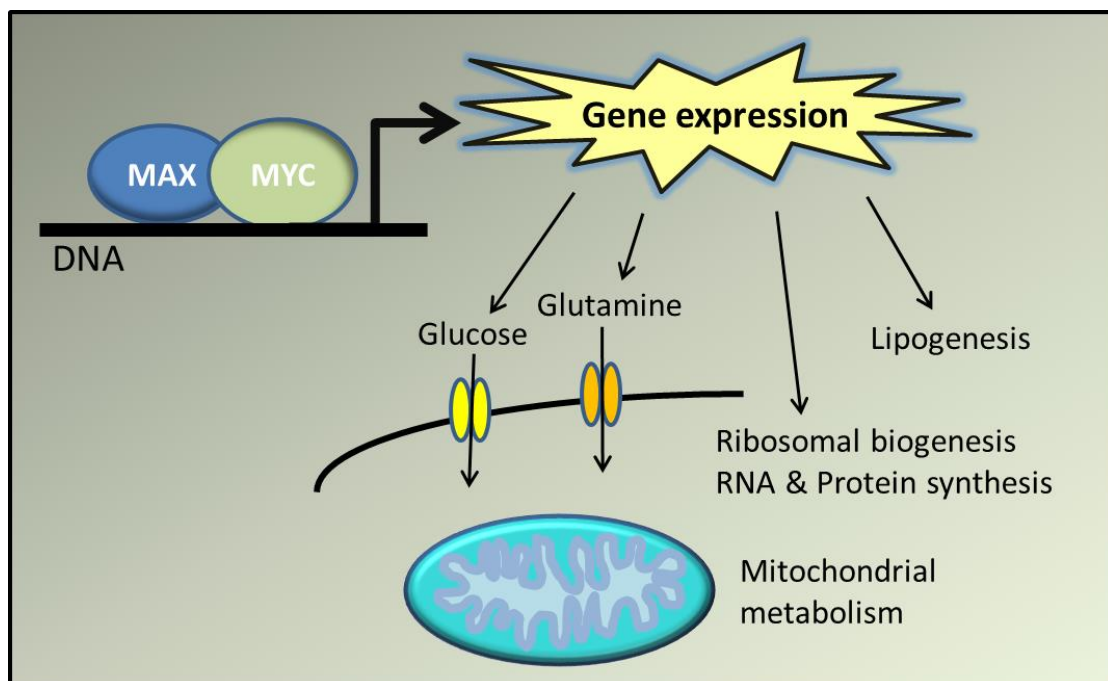
Figure 4. MYC and metabolic alterations go hand in hand in cancer. MYC is linked to all key aspects of metabolic reprogramming in cancer, comprising the induction of aerobic glycolysis, enhanced glutaminolysis and lipogenesis, as well as up-regulation of mitochondrial metabolism. In addition, one of MYC's major functions in cancer involves the positive regulation of ribosomal biogenesis and function and consequently protein production. Together, these MYC-induced metabolic alterations lead to rapid cell mass expansion and hence to tumor growth.

1.3.4 MYC regulates lipid metabolism

Relatively few studies have addressed the link between MYC signaling and the regulation of lipid metabolism in cancer. However, Morrish *et al* demonstrated that high-myc expressing

rat fibroblasts show a 2-fold increased glucose-derived *de novo* synthesis of palmitate, compared to fibroblasts with chronically depleted *myc* (147). Some reports have also addressed the cancer cell usage of fatty acid oxidation (FAO), also referred to as β-oxidation, in relation to MYC. Fatty acid β-oxidation was found to either increase after induction of MYC in FL5.12 pro-B cells (148), whereas it was found decreased in high MYC-expressing p493-6 B cells compared to when MYC was turned off, as measured by the conversion of ^{14}C-labeled oleic acid to ^{14}C-labeled CO_2 (149). Similarly, MYC induction in myocardiocytes was shown to decrease the rate of FAO, while glycolysis and mitochondrial biogenesis was induced (150). We have found that NB cells with high MYCN expression show a dramatically enhanced capacity to oxidize exogenously added fatty acids (palmitate), compared to when MYCN was downregulated (**Paper III**). Furthermore, we have demonstrated that lipid droplets accumulate upon MYCN inhibition in NB cells, and experiments using inhibitors against FAO and lipolysis suggested that impairment of FAO is involved in this process (**Paper II**) (109). The different outcomes on the utilization of fatty acids in response to MYC signaling obtained in different cell systems is not surprising as the response to MYC signaling on metabolism is likely to be both cell and context dependent (8, 151).

1.4   NEUROBLASTOMA

Neuroblastoma (NB) is a rare pediatric cancer usually affecting very young children, under five years of age. Tumors are found in sympathetic nervous tissue along the spinal cord; most often in the adrenal glands (see *Figure 5*). NB originates from a population of cells in the developing embryo called the neural crest. Neural crest cells give rise to a number of different cell lineages during development, whereof those forming the sympathetic nervous system are the precursors of NB (152-155) (see *Figure 6*). Although NB is a rare disease, it is the most common extracranial (non-brain) solid tumor type in children and the most commonly diagnosed cancer in infants (children under 1 year of age). NB is also one of the most deadly cancers in children (20). In Sweden, there are approximately 15-20 new cases diagnosed per year (156), whereas, for instance in the US, 710 children are estimated to be diagnosed with NB in 2014, representing 7 % or all childhood cancers (157). NB is an extremely heterogeneous disease, ranging from differentiated tumors which are highly curable and which can even sometimes spontaneously regress, to rapidly growing metastatic tumors, which are refractory to treatment (153, 158).

At the time of diagnosis over 60 % of NB patients over the age of 1 have metastases and show unfavorable outcomes (154, 159). Activation of the *MYCN* oncogene through amplification (MNA) is the most common genetic lesion (15~25 % of all NB) and is a hallmark of advanced disease and poor prognosis (152, 153, 158). Despite intensive treatment, which includes a combination of high dose chemotherapy, surgery, myoablative chemotherapy and hematopoietic stem cell transplantation (36), MNA is associated with a survival rate of only 15-35 % (36). Importantly, high activity of the MYC signaling pathway is predictive of high risk and stage of disease independently from *MYCN* amplification

(MNA) (160), and overexpression of the human MYCN protein in sympathoadrenal cells driven by the rat tyrosine hydroxylase (*th*) promoter leads to NB development in mice (161) as well as in zebrafish when co-expressed with activated anaplastic lymphoma receptor tyrosine kinase (ALK) (162).
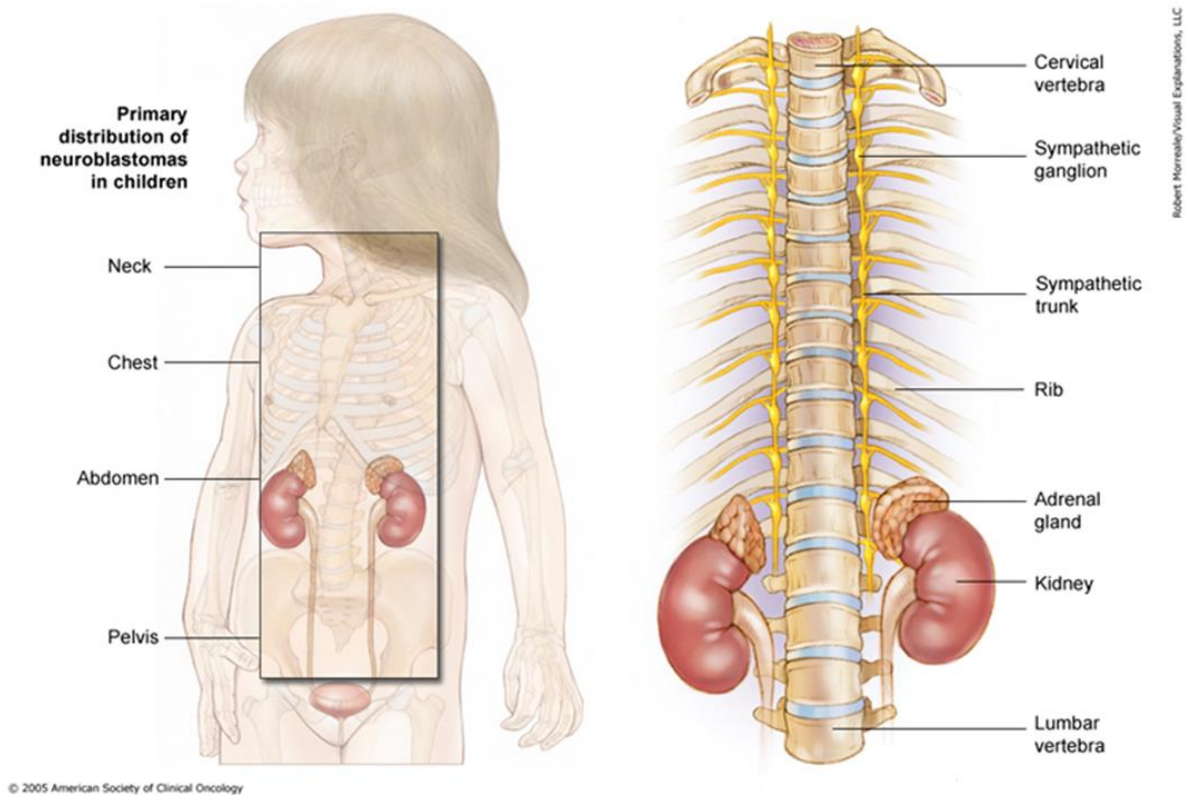
Figure 5. Distribution of neuroblastomas. Neuroblastoma develops in sympathetic neuronal tissue. Tumors most frequently occur in the adrenal gland or along the sympathetic ganglia in the abdomen, but can also arise in sympathetic nervous tissue in the neck, chest and pelvis regions. Illustration from: American Society of Clinical Oncology (163), with permission from the publisher.

1.4.1 Genetic alterations in neuroblastoma

In contrast to some cancers in adults, NB tumors show few recurrent somatic mutations (164) although a few important examples exist, including activating mutations in the chromatin remodeling ATP dependent helicase: α-thalassaemia/mental retardation syndrome X-linked (*ATRX*), and in other chromatin remodeling genes, as well as genes in the RAC-RHO pathway, among others (154). Most NB tumors develop sporadically, but there are also rare cases of familial NB (< 2 % of all NB cases) in which important predisposing single nucleotide polymorphisms (SNPs) adjacent to specific genes have been identified, including activating mutations in the receptor tyrosine kinase ALK, and deactivating mutations in the transcription factor paired-like homeo-box 2b (PHOX2B), which normally plays important roles for the development of the neural crest-derived sympathoadrenal neuronal lineage (152, 154, 165). The receptor tyrosine kinase ALK is important since it is one of the most common oncogenic driver-lesions found in both familial and sporadic NB, where *ALK* can be both

mutated and amplified. It is normally expressed in neural crest cells of the sympathoadrenal lineage during development, where it is believed to regulate the fine-tuning of differentiation and proliferative signals (154).

Several research reports have also uncovered important epigenetic alterations and differential expression of microRNAs, which play significant roles for the disease (51, 154, 166-168). These microRNAs can be used as biomarkers and may have potential as therapeutic targets (169). In contrast to the low mutation frequency found in NB (164) chromosomal aberrations are instead common, where segmental gains and losses (including gain of chromosome 17q, and 1p, and loss of 11q) are found in high risk tumors whereas whole chromosomal gains are generally present in low risk tumors (153, 154). Prognostic factors predicting a favorable outcome include young age < 18 months, a differentiated histology and high expression of the neurotrophic tyrosine kinase receptor TrkA (encoded by the *NTRK1* gene) (152-154, 158).
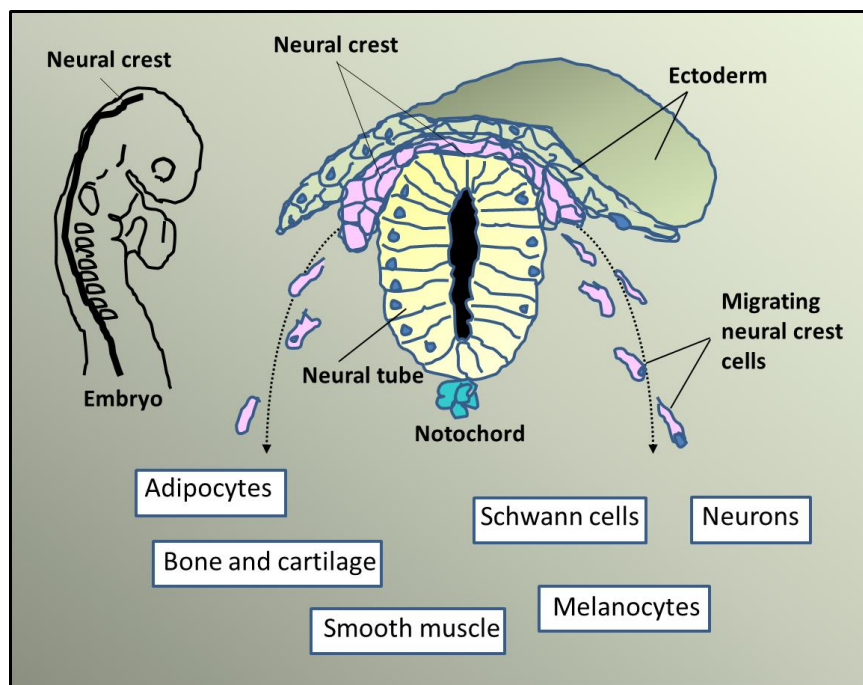
Figure 6. Neuroblastoma is a cancer derived from cells of the neural crest during development. The neural crest is a transient population of multipotent stem cells in the developing embryo of vertebrates, which become invasive (through EMT) and delaminate from the roof plate of the neural tube and migrate to the periphery of the embryo. Along the migration route, the neural crest cells encounter specific signaling cues, leading to the activation of transcriptional programs that in turn mediate differentiation. The neural crest cells give rise to various cell types, including: peripheral and enteric (of the gut) neurons and glia, melanocytes, smooth muscle cells, and cells of the adrenal medulla and craniofacial skeleton. NB is widely accepted to be derived from the neural crest cells of the sympatoadrenal lineage, however, the mechanisms and timing of the initiating cancerogenous events remain unknown.

1.4.2   Differentiation capacities of neuroblastoma cells and tumors

Studies of NB cells in culture have revealed their fascinating physiology involving the capability of differentiation along the sympathoadrenal lineage (154). It was discovered early on that vitamin A derivatives (retinoids) induces differentiation *in vitro*, and these findings were translated to the clinic where 13-cic-retinoic acid is still included as a standard treatment for the targeting of minimal residual disease in high risk patients (36, 154, 170). Interestingly, MYCN down regulation precedes differentiation induced by retinoic acid, and direct genetic down regulation of *MYCN* also leads to neuronal differentiation *in vitro* (36). The degree of tumor differentiation is used in the Shimada histology grading system where a differentiated histology including high presence of Schwannian stromal content (a type of glial cells) is predictive of low risk disease (154), and the expression of differentiation markers can furthermore predict stage and outcome in patients (160). The Schwann cells, developmentally derived from the neural crest, are reported to influence the clinical behavior of NB and to confer a favorable prognostic impact that correlates with low MYCN/high TrkA expression. Despite conflicting reports regarding the origin of Schwann cells in NB tumors, identical genetic composition in neuroblasts and Schwann cells from the same tumors indicate the presence of a common self-renewing pluripotent tumor cell in NB. Schwann cell abundance in NB tumors may hence reflect the consequence of a more differentiated tumor state (171). This conclusion is supported by the identification of cells characteristic of neural crest stem cells both *in vitro* and in NB tumors. Some NB cell lines can be induced to differentiate along specific neural crest lineages including neuronal and Schwann cell-like cell types (172, 173). NB cells can also be enriched as non-adherent tumor neurospheres in culture with stem cell like properties (174). In this regard, MYCN may be important since it has been shown that MYCN induces the expression of pluripotency genes in NB cells (64).

1.4.3   Little is known about the role of tumor energy metabolism in neuroblastoma

Except from our previous report (109) (**Paper II**), only a few studies have investigated the role of tumor metabolism in NB cells. In the non-*MYCN* amplified (NMNA) human NB cell line SH-SY-5Y, both the oxphos capacity and glycolysis was found to increase in response to ATRA treatment (175). However, this study did not take into account the role of MYCN in the modulation of mitochondrial metabolism. Presumably, the outcome on metabolism by ATRA may differ in NB cell lines with MNA, since down regulation of MYCN is well-known to precede differentiation by ATRA (36). Interestingly, a marked increase in total lipid levels (including cholesterol, phospholipids, free fatty acids and cholesterol esters) was documented in NB cells in response to differentiation (176). Similarly we have seen that ATRA treatment of MNA NB cells, except from causing dramatically reduced MYCN levels and leading to differentiation, also results in lipid accumulation (unpublished observations). The most convincing report on the role of glycolysis in NB showed that, in tissues from 47 patients, elevated mRNA and protein expression of the glucose transporter GLUT1 correlated with a poor clinical outcome (177). Treatment with the glycolysis inhibitor 3-bromopyruvate (3BrPA) furthermore suppressed proliferation of NB cells with high GLUT1 gene expression

compared to those with low expression (177). However, the connection to MNA was not investigated.

2 AIMS

The overall aims of this thesis were to investigate different strategies of targeting MYC in cancer cells and to gain insights into how MYCN reprograms tumor cell metabolism in childhood neuroblastoma. This thesis comprises four papers with the specific aims to:

Paper I. Identify cytotoxic drugs showing selectivity towards MYC overexpressing tumor cells.

Paper II. Analyze the effects of a conventional c-MYC binding small molecule, 10058-F4 towards MYCN overexpressing NB, as well as *in vivo* in the *TH-MYCN* transgenic mouse model and in a cell xenograft model of NB.

Paper III. Investigate how MYCN impacts on tumor cell metabolism in aggressive NB by using the combined information obtained from quantitative proteomics, gene expression analysis of patient data, and functional analysis using NB cells with conditional MYCN expression.

Paper IV. Characterize the binding of 10058-F4 and other structurally related and unrelated small molecules to MYCN using surface plasmon resonance (SPR), and assess their biological effects in NB cells.

3 RESULTS AND DISCUSSION

3.1 PAPER I. IDENTIFICATION OF CYTOTOXIC DRUGS THAT SELECTIVELY TARGET TUMOR CELLS WITH MYC OVEREXPRESSION

Elevated levels of c-MYC can be found in a wide range of human malignancies and is readily correlated with tumor aggressiveness and an adverse outcome in patients (33). In Burkitt's lymphoma (BL), a cancer of immune cells called B-cells, c-MYC is constitutively expressed due to the translocation of *c-MYC* to one of the immunoglobulin (Ig) loci, and these tumors grow rapidly and are fatal if left untreated (178-180). BL exists in three variants: the endemic (malaria associated) type present in sub-Saharan Africa (sometimes referred to as the lymphoma belt), which often affects young children and adolescents, the less common sporadic type present in the rest of the world, and the AIDS-related BL. In all three types of BL, the *c-MYC* translocation is the chief driver event in the disease (179). Except from BL, *c-MYC* translocation is also frequent in other hematological malignancies including lymphoblastic and myeloid leukemia (33, 181). As already discussed (on page 14), amplification of the *MYCN* gene is highly correlated to aggressive disease and poor clinical outcome in NB, and the survival rate of these patients is approximately 30 % despite intensive treatment (165, 182, 183). Amplification of *MYCN* is also observed in other cancers, including glioma, medulloblastoma, rhabdomyosarcoma and small cell lung cancer (33). In spite of important efficacy of chemotherapeutics in the treatment of many cancers, concerns relate to the lack of selectivity for the tumor cells, resulting in severe side effects and to the development of chemotherapy resistance in some patients (6, 184). For these reasons, identification of anti-cancer drugs and compounds showing selectivity against deregulated MYC expression could be important for treatment optimization of a broad range of cancers by providing for more effective treatments with fewer side effects (33, 185, 186).

In this paper, our aim was to investigate the selectivity of known chemotherapeutic drugs and compounds towards MYC-overexpression in cancer cells. Hence, we screened a library of 80 conventional compounds to assess their ability to induce synthetic lethal interactions with MYC in BL and NB cells. Cell lines inducible for c-MYC (BL) and MYCN (NB) were used to assess the effect of the drugs on proliferation and apoptosis.

Our results revealed that only 25 % of the compounds showed specificity towards MYC overexpression in terms of mediating reduced cell growth (using 30 % difference as cut-off), where the majority did not discriminate between c-MYC and MYCN-overexpression. We found that MYC overexpression sensitizes cells to inhibition of specific cellular mechanisms or pathways, since, out of the positive hits, specific classes of drugs targeting certain cellular components could be delineated. Largely, these were compounds acting on microtubules (vinblastine (VBL), paclitaxel (TAX) and podophyllotoxin (POD)), topoisomerases (10-hydroxycamptothecin (OH-CPT), camptothesin (CPT), doxorubicin (DXR), etoposide (ETO) and daunorubicin)), and on the biosynthetic machinery of DNA, RNA and protein synthesis and turnover (anisomycin, gliotoxin, mitomycin C, MG132, methotrexate, cycloheximide, actinomycin D, heatshock protein 90 (HSP-90) inhibitors, and aphidicholin). In addition, the

histone deacetylase inhibitor trichostatin A (TSA), and the kinase inhibitor staurosporin were identified.

Importantly, we classified these compounds and drugs based on their major known function, however, many of these agents can also be divided into several subcategories. As an example, anisomycin is an antibiotic which inhibits protein- and partly DNA synthesis, but is also an activator of stress-activated kinases and MAP kinases (187).

Interestingly, although only a subset of the chemical agents used showed selectivity towards MYC-overexpressing tumor cells, these actually belong to the same classes of drugs as currently used for the treatment of both NB and BL, including DNA alkylating agents, microtubule-targeting agents and topoisomerase inhibitors, antibiotics and the dihydrofolate reductase DHFR inhibitor methotrexate (in the case of BL) (33, 184, 188). In common for these chemotherapeutic regiments is that these are directed against mechanism important in highly proliferating cells. Nevertheless, these same pathways are also known to be integrally linked with elevated MYC signaling in cancer (8). An interesting example is the folate antagonist methotrexate which is one of the earliest clinically used chemotherapeutics (189), as it has been shown that one of the few genes which can partially rescue the *c-myc* knockout phenotype in rat embryonic fibroblasts, is a mitochondrial enzyme of folate synthesis, (120), and c-MYC directly induces folate metabolism (145). Folate is important for purine and consequently DNA synthesis (189, 190), and folate deficiency is linked with impaired DNA methylation and has been shown to result in gene expression changes of fatty acid metabolism in mice (191). Consequently, some of the currently used treatments for NB and BL as well for other types of cancers are already directed against MYC overexpression, although non-intentionally, since these drugs are used due to their effectiveness in treating patients, without taking into account their specific actions towards MYC. Hence, this notion highlights the use of chemical screens as a potent strategy to identify new compounds active in a MYC overexpressing setting. This is important since, although the identified MYC-related pathways in our screen are already susceptible for treatment, this study also shows the need for new, more selective inhibitors, as currently used drugs are associated with cytotoxicity and drug resistance (6, 192, 193).

Theoretically, the observed MYC selectivity of identified hits on growth inhibition and apoptosis could be due to effects on MYC itself or its functions, referring to the concept on oncogene addiction discussed earlier (see page 8) (20, 76-78). The MYC selectivity could also be related to synthetic lethal interactions upon inhibition of certain pathways in a MYC overexpressing setting, as also mentioned before (see page 9). To understand the mechanisms behind the MYC selectivity of our identified hits, the specific action towards MYC for some of these drugs was consequently analyzed in more detail. We found that treatment with the topoisomerase inhibitors CPT, OH-CPT and DXR, and the histone deacetylase inhibitor TSA led to reduced c-MYC levels, while no effect was observed by the topoisomerase II inhibitor ETO. Importantly, the rapid c-MYC downregulation was demonstrated to precede CPT- and OH-CPT-induced apoptosis, showing that the reduced c-MYC levels did not happen as an

indirect response to cell death. Although all three microtubule-targeting agents induced apoptosis to a significantly higher extent in MYC overexpressing cells, none of these drugs affected the c-MYC levels. These chemotherapeutic drugs act by disrupting the dynamics of microtubules during mitotic spindle formation, which causes mitotic arrest followed by apoptosis (194, 195). Interestingly, synthetic lethal interactions between MYC and components of mitotic processes, including Aurora kinases and CDK1, have been shown before (95, 96). There are also interesting links between tubulin dynamics and mitochondrial voltage dependent anion channels (VDAC) in the regulation of respiration (196, 197). To analyze effects on c-MYC/MAX DNA binding we performed electrophoretic mobility shift assays (EMSAs) on cell extracts treated with the following eight drugs: CPT, OH-CPT, DXR, ETO, TAX, POD, VBL or TSA. None of these agents were effective in disrupting the c-MYC/MAX DNA binding, except from DXR, which caused a reduction in the amount of c-MYC/MAX as well as MNT/MAX bound to the E-box containing oligonucleotide probe, while the DNA binding by upstream-stimulatory factor 1 (USF) remained unaffected. Hence, DXR may act by disturbing the transcriptional activity of MYC.

Taken together, these data provide clues concerning which pathways are sensitive for inhibition in MYC overexpressing tumor cells. These insights can be used when designing and developing novel drugs directed against MYC for cancer therapy.

3.2   PAPER II. MYC INHIBITION INDUCES METABOLIC CHANGES LEADING TO ACCUMULATION OF LIPID DROPLETS IN TUMOR CELLS

The MYC oncoproteins are among the most attractive targets in cancer due to their critical roles in mediating aggressiveness in the majority of human tumors (79). The fact that many different tumor cell types are in particular need of elevated MYC expression for their survival and tumor progression (8, 34, 80, 81), and since inhibition of MYC in normal cells is fairly well tolerated in mice (48, 49), it highly supports MYC as an important target for a wide range of cancers. In NB, elevated MYCN signaling is not only important for mediating aggressiveness in the MNA tumors but can also be implemented to predict high risk and stage of the disease in patients with NMNA tumors, who were initially classified as intermediate risk (160). In MNA NB cells, down regulation of MYCN leads to apoptosis as well as to neuronal differentiation (36, 170).

A number of c-MYC selective small molecules have been identified in cellular screens, and some of these have been demonstrated to bind to c-MYC, to disrupt its protein-protein interaction with MAX, and induce c-MYC selective growth inhibition (98, 103, 104, 106, 198, 199). However, all of these studies have been focused on the c-MYC protein, and the only two previously published investigations of possible *in vivo* effects showed lack of anti-tumor responses and rapid metabolic degradation of the molecules (108, 200). These reports hence support the general conception that protein-protein interactions are nearly "undruggable", at least *in vivo,* as discussed before (see page 9).

The small molecule 10058-F4 shows efficacy in NB both in vitro and in vivo

In this paper, we made the important discovery that a previously identified small c-MYC-binding molecule, 10058-F4, shows efficacy against MYCN in NB cells in addition to its previously documented effects against c-MYC in other tumor cell lines. This molecule was originally identified as a disruptor of c-MYC/MAX dimerization, leading to c-MYC-selective cell growth inhibition and apoptosis *in vitro* (104, 198, 199), but its potency against MYCN had previously not been investigated, nor had it been shown that *in vivo* effects can be obtained by a small MYC-binding molecule. Here, we revealed for the first time that 10058-F4 treatment was sufficient to elicit anti-tumorigenic effects in mouse models of NB, where the survival of *MYCN*-transgenic mice treated with 10058-F4 was significantly prolonged. This well-established MYCN-driven NB model highly resembles high risk NB in humans (161). We also applied 10058-F4 treatment in a cell xenograft model of NB using an aggressive, MNA and *p53*-mutated cell line, SK-N-BE(2), derived from a tumor after relapse (201). Here, the effects were less striking, although we did see a significant tumor growth delay, and we also detected more cleaved caspase-3 staining in treated tumor sections, indicating apoptosis-induction by 10058-F4.

Hitherto, no attempts of using high throughput proteomic analysis in tumor cells in response to treatment with small MYC-binding molecules have been documented. Here, using proteomics and gene ontology (GO) predictions, we found that the biological outcome after treatment with 10058-F4 for 20 hours was highly similar as compared to genetic down

regulation using shRNA against *MYCN* in NB cells. Ingenuity analysis predictions furthermore showed that c-MYC and MYCN were the two most significantly affected transcription factors in response to both treatments. The overall patterns of affected proteins in response to both treatments involved striking changes in ribosomal proteins and other factors related to protein synthetic processes. In terms of MYC inhibition, this biological outcome is expected given the important role of c-MYC in ribosomal biogenesis and function (56, 58, 61). Similarly, a large fraction of the affected proteins were involved in mitochondrial respiratory- and oxphos-related pathways. Although, c-MYC is a well-known regulator of mitochondrial metabolism (131, 141, 143), the possible implication of an MYCN- mediated elevated mitochondrial metabolism for tumor aggressiveness in NB had previously not been shown.

Surprisingly, despite the described similarities between the two treatments, in terms of affected biological pathways and processes, there was little overlap when looking at the exact protein identities (not shown). One explanation for this discrepancy could be due to the respective mechanisms of MYC-inhibition, since sh*MYCN* treatment for 20 hours leads to MYCN protein down regulation, while 10048-F4-treatment at this short time-point was shown to decrease the MYCN/MAX interaction in cells but not to cause MYCN down regulation until after ~48 hours. It is also important to note that the binding-selectivity of 10058-F4 towards MYC has never been thoroughly investigated. Although it was shown in the original report that 10058-F4 caused a more robust inhibition of the c-MYC/MAX association compared to other protein-protein interactions of the bHLH-ZIP, HLH or ZIP family of proteins analyzed, still, it did cause some degree of inhibition of the interaction between other proteins (104). It is hence very probable that this molecule binds to additional proteins, which hence could influence on the biological outcome in cells.

Based on our results in NB cells, we proposed that 10058-F4 may bind to MYCN (similarly as shown for c-MYC) leading to decreased MYCN/MAX interaction, which in turn causes MYCN protein destabilization, ultimately resulting in its degradation, as supported by proteasome inhibition experiments. Importantly, we also detected an overall, although slight, decrease in MYCN protein levels in tumors from transgenic mice when comparing five control-treated, to five 10058-F4-treated tumors, as analyzed by western blot (unpublished results). However, the animal experiment was primarily designed to assess the overall survival in response to 10058-F4-treatment, and the tumors were hence grown to a size that warranted sacrifice of the animals. Consequently, this experiment was not optimal for analyzing possible biological differences in the tumors, as such changes may be detected at a much earlier time-point, before the tumors reach their maximal sizes, especially when considering the documented poor potency of 10058-F4 (108).

Importantly, *in vitro*, 10058-F4 induced cell growth inhibition and apoptosis to a higher extent in MNA NB cells compared to NMNA cells. Treatment also caused morphological differentiation which, was potentiated by nerve growth factor (NGF), indicating involvement of functional TrkA receptor signaling (170). Indeed, we detected increased *TrkA* mRNA as

well as protein expression levels in MNA NB cells upon 10058-F4 treatment. High expression of TrkA is the most reliable marker known to predict a favorable outcome in NB patients, and its expression correlates inversely with amplification of *MYCN*. (36, 153, 170). Consequently these results indicate that MYC-directed therapies may have potential in terms of mediating differentiation in NB.

Together our data showed that 10058-F4 targets MYCN-overexpressing NB cells and elicit *in vivo* efficacy in NB tumors *in vivo*. Hence, these results highlight the potential of developing small molecules directed against MYCN in the management of MNA NB.

New findings of possible clinical relevance on mitochondrial metabolism in high risk NB

While investigating the actions of 10058-F4 in NB cells, we also made the unexpected discovery that treatment resulted in accumulation of cytoplasmic lipid droplets, as revealed by Oil red O staining and electron microscopy. This effect occurred in all NB cell lines tested, as well as in a breast cancer cell line, which is known to possess elevated c-MYC levels (202). Interestingly, treatment of normal non-transformed dermal and lung fibroblasts did not result in accumulating lipid droplets, indicating that this effect is related to MYC-inhibition in cancer cells and not in normal cells. Using sh*MYCN* treatment of NB cells, we confirmed that lipids accumulated in direct response to MYCN down regulation. Likewise, treatment with the BRD4 inhibitor JQ1 (mentioned at page 9) led to MYCN protein degradation and lipid accumulation. Lipid droplet formation furthermore applies to c-MYC inhibition as well, as shown in experiments using isogenic rat embryonic fibroblasts with different *myc* expression status in combination with 10058-F4 treatment. Importantly, lipid accumulation was not simply an effect of cell growth inhibition since out of three cytotoxic drugs tested, only cisplatin (CIS) induced lipid accumulation while it did not impact on the cell cycle profile at the concentration used. In contrast, CPT and ETO induced cell growth arrest in the absence of lipid droplet formation.

In an attempt to identify the metabolic explanation behind this lipid-phenomenon we analyzed our proteomic data in search for metabolic enzymes differentially regulated by 10058-F4. Since mapping of enzymatic alterations revealed that several metabolic pathways were affected, we next used inhibitors of key enzymes of these pathways to analyze if their inhibition would lead to lipid accumulation, similar as to after MYCN-down regulation. We found that inhibition of β-oxidation (using etomoxir and thioridazine) as well as of complexes of the respiratory chain (using oligomycin, rotenone, 2-thenoyltrifluoroacetone (TTFA), Antimycin-A) led to lipid accumulation, while the glycolysis inhibitor 3BrPA or glycolysis/oxphos-inhibitor dichloroacetic acid (DCA) did not. The fact that inhibition of lipid synthesis, using an inhibitor of FASN, cerulenin, did not prevent lipid accumulation by 10058-F4, furthermore suggested that a continued lipolysis was not likely to be responsible for the observed lipid response. Instead, based on our collected data and the fact that FAO-inhibition has been shown to result in lipid accumulation in non-adipose cells (203-205), the most apparent explanation is that MYCN inhibition leads to mitochondrial dysfunction, and these changes in turn limits the process of FAO whereby excess lipids accumulate outside of

mitochondria. The link between β-oxidation and respiration is well-established, considering that β-oxidation generates electron donors in the form of FADH$_2$ and NADH and these molecules are shuffled into the respiratory chain (206). In support of our theory, we found that the mitochondria in response to 10058-F4 treatment were structurally altered, with less elaborate cristae. This phenotype demonstrates an overall reduced protein content in the mitochondria and further proves that mitochondrial dysfunction occurs upon MYCN inhibition.

Importantly, we found that high expression of the majority of identified mitochondria-related enzymes, including those of the respiratory chain, the citric acid cycle and β-oxidation, correlates with poor survival in NB patients. Hence, it indicates that these pathways are important for mediating tumor aggressiveness.

Together these findings demonstrate that MYCN is linked to an elevated mitochondrial metabolism important for the aggressiveness of MNA NB tumors. This data also suggested that high MYCN-expressing cells use fatty acids as an energy source.
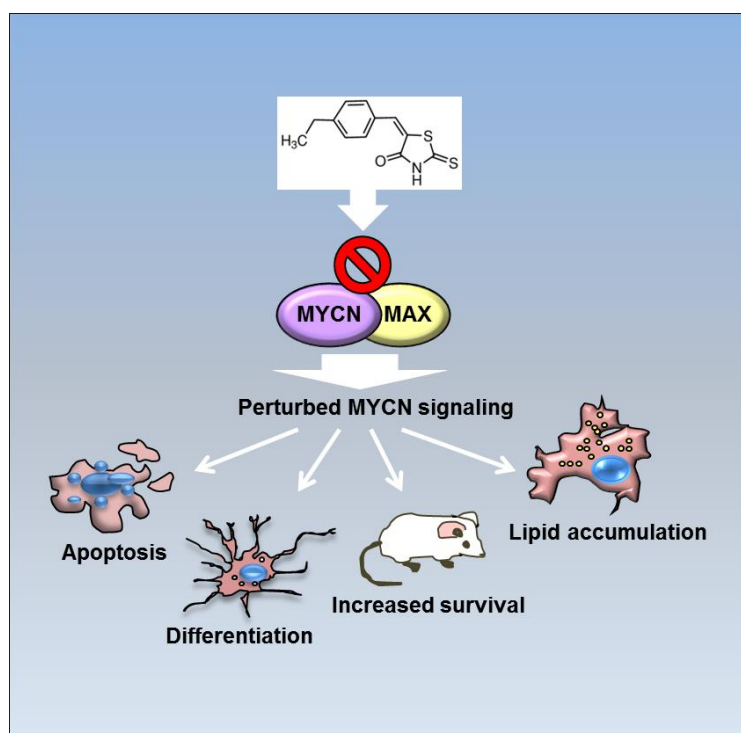
Figure 7. Effects of the small molecule 10058-F4 in neuroblastoma cells and mouse models. Treatment with 10058-F4 resulted in decreased MYCN/MAX dimerization, and induced apoptosis and cell growth inhibition in MNA NB cells compared to NMNA cells. It also caused NB cell differentiation including upregulation of TrkA expression levels, and treatment in vivo led to prolonged survival of TH-MYCN transgenic mice. We also found that 10058-F4 induced lipid accumulation in NB cells. Importantly, lipid droplet formation occurred in direct response to MYCN inhibition, caused by mitochondrial dysfunction and possibly involving interference with fatty acid oxidation. Illustration from: Zirath H and Arsenian Henriksson M, Onkologi i Sverige, (2013) (207), with permission from the publisher.

3.3 PAPER III. MYCN MEDIATES METABOLIC PLASTICITY IN CHILDHOOD NEUROBLASTOMA

Metabolic remodeling is a key feature of advanced cancers by providing growth and survival advantages (113, 114). The perturbed metabolism in tumors allows for accumulation of biosynthetic building blocks such as nucleic acids, lipids and proteins, needed for anabolic reactions in the rapidly proliferating cancer cells. (208). It is also important for tumor cell adaptations in response to changes in the tumor environment (112, 114).

Despite the aggressive behavior of MNA NB tumors, the connection between MYCN-signaling and tumorigenic cell metabolism is largely unexplored (see page 17). Therefore, elucidating the cellular and molecular mechanism underlying MYCN-dependent tumor metabolism may have substantial clinical benefit since it could reveal targetable bioenergetic pathways crucial for tumor survival.

In this paper, we have investigated in greater detail as compared to in **Paper II** (109), the direct outcome of MYCN-expression on NB metabolism by using the combined information obtained from high resolution proteomics, mRNA expression profiling of clinical tumor samples, as well as functional assays using anti-*MYCN* sh-RNA in NB cells (209).

MYCN *is a master inducer of metabolic capacities in NB*

Our analysis of protein and gene expression alterations associated with MYCN highlighted glycolytic enzymes (e.g. hexokinase 2, HK2 and GLUT1), as well as enzymes involved in lipolysis (e.g. fatty acid synthase, FASN) and β-oxidation (e.g. hydroxyacyl-CoA dehydrogenase, HADH) to play functional roles in the disease, since their high expression correlated to reduced patient survival. Evidently, MYCN mediates an overall activated mitochondrial metabolism, based on the observed elevated expression of components of the respiratory chain, the citric acid cycle, and of detoxification systems of reactive oxygen species (ROS). Overall, this MYCN-linked mitochondrial signature appears highly important for tumor aggressiveness since the majority of corresponding transcripts correlate to an adverse outcome in patients when expressed at high levels. Consequently, MNA tumors may have greater demands for mitochondrial functions including high ROS detoxification capacities for their cell survival.

Interestingly, we could functionally demonstrate that MYCN expression not simply confers either an induced glycolytic or respiratory capacity in NB cells, but instead persistently mediates an enhanced metabolic pathway flux depending on nutrition availability. We showed for the first time that MYCN directly induces glycolysis, as well as an elevated respiratory capacity in NB cells. In addition, MYCN was wound to positively regulate the oxidation of exogenous fatty acids. Hence, this is to our knowledge the first time it is demonstrated that MYC, or any singe onco-protein, regulates all key aspects of metabolism in the same cell system. We therefore hypothesize that MYCN may confer metabolic flexibility or "plasticity" in NB cells which could be an important mechanism for tumor aggressiveness.

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Potentially important roles of lipid metabolism in NB

Lipid metabolism is an emerging field in cancer research that has received less attention than the Warburg effect and the role of mitochondrial respiratory processes, and glutamine metabolism (208, 210). Hence, it was interesting to note that MYCN regulates important lipid metabolic pathways in NB cells and tumors, including FAO-related enzymes and lipolytic pathways, the latter which included an elevated expression of *FASN* and enzymes involved in ketone and cholesterol metabolism (not shown).

Rapidly proliferating tumor cells are in great demand of fatty acids for synthesizing new cell membranes (which are mainly composed of phospholipids), and for use as signaling molecules (208). In fact, a highly recognized feature in cancer is an elevated *de novo* fatty acid synthesis mediated by the enzyme FASN, which is linked with tumor aggressiveness and poor survival (211). We found that *FASN* is expressed at high levels in MNA tumors at the mRNA level and that its high expression correlates with poor survival. Surprisingly however, in NB cells, MYCN-down regulation led to increased FASN protein levels. Consequently it would be interesting to assess the FASN levels in tumor samples using immunohistochemistry (IHC), to further evaluate the potential role of FASN in NB.

Several recent reports have demonstrated that FAO is an important pro-tumorigenic feature in some cancers (208, 212, 213). In prostate cancer, FAO is a major bioenergetic pathway (214), and was shown to involve the oxidation of exogenous fatty acids (215). In our study, the expression of several enzymes involved in β-oxidation was differentially regulated by MYCN both at the mRNA and/ or at the protein level. We found that high expression of enzymes regulating the last steps of fatty acid oxidation correlates with a poor outcome (e.g. HADH), whereas those involved in the first steps are generally inversely associated with reduced survival. These differences could relate to an altered preference for specific fatty acid substrates in benign as compared to in malignant NB tumors, mediated by specific FAO enzymes. Anti-tumor effects such as decreased proliferation have been documented in NB cells in response to treatment with different fatty acid substrates other than palmitate (216, 217). In contrast, we showed here that high MYCN-expressing SK-N-BE(2) cells possess an elevated capacity to oxidize exogenously added palmitate compared to after MYCN downregulation, whereas endogenous FAO seemed not to be actively used in these cells based on the lack of effect by etomoxir on the oxygen consumption rates (OCR). Etomoxir is an inhibitor of one of the rate limiting enzymes of fatty acid β-oxidation, the fatty acid transporter carnitine palmitoyltransferase (CPT1) (205). Interestingly, high expression of the brain specific variant of this enzyme, CPT1c, has been documented in a large number of tumors including NB, where it is suggested to promote tumor cell growth and survival (218). In NB, high *CPT1c* expression correlates with poor survival independently from MNA or an elevated MYC signaling, suggesting that it mediates aggressiveness in NMNA tumors (unpublished observations).

In ovarian cancer, it was shown that adipocytes in the microenvironment of the omentum causes preferential homing of metastatic tumor cells to these areas by providing for nutrients

in the form of fatty acids (125). It is hence tempting to speculate that a similar mechanism could relate to NB tumors as well. As an organ specialized in steroid hormone synthesis and secretion, the adrenal gland requires fuel in the form of neutral fat, fatty acids, and fatty acyl esters of cholesterol. These precursor molecules are stored as lipid droplets in steroid synthesizing cells and are as such an important component in the adrenal physiology (219).

Together our results consequently further support a potentially important role of fatty acid β-oxidation in MNA NB, as suggested in **Paper II** (109). It also proposes that MYCN confers "metabolic plasticity" in NB, which could represent an important mechanism for cancer cell adaptation and survival during tumor progression (119, 129, 130), and could also have implications in cancer treatment resistance (128).

3.4 PAPER IV. TARGETING OF THE MYCN PROTEIN WITH SMALL MOLECULE C-MYC INHIBITORS

Due to the difficulties inherent with disrupting protein-protein interactions (discussed on page 9-10) which are common among transcription factors (e.g. the MYC/MAX dimerization), their specific inhibition using small organic molecules is widely considered to be a difficult task (220). However, several small molecules, especially those identified in Edward Prochownik´s group, have shown interesting potential (73, 82, 104), whereof some were demonstrated to bind directly to the bHLHZip domain of c-MYC (98, 100, 106, 107). Out of these, 10058-F4 is by far the most studied c-MYC-binding molecule, and it has been shown to cause anti-cancerous effects in different tumors cell lines *in vitro* (103-105) and is the first MYC-binding molecule shown to cause anti-tumorigenic effects *in vivo* (**Paper II**) (109).

Our findings in **Paper II** (109) suggested targeting of MYCN by the small molecule 10058-F4 and also revealed *in vivo* effects by this compound (109). Encouraged by these previous findings, here we wanted to confirm the direct binding of 10058-F4 to the bHLHLZip domain of MYCN, by using the highly sensitive surface plasmon resonance (SPR) method (221). In addition to 10058-F4, we also included three of its previously characterized structural analogs (#474, #764 and the non-c-MYC binder 10058-F4(7RH)) (105), a structurally unrelated compound (10074-G5) shown to bind to a distinct region of the bHLHLZip domain of c-MYC (105), as well as an 10058-F4-derived metabolite, C-*m/z* 232 (108) (see *Figure 8*), in order to compare the MYCN-binding affinities (if present) and biological effects (growth inhibition, apoptosis, neurite outgrowth, and lipid accumulation) of these molecules in MNA NB cells. We also assessed whether these molecules could decrease the MYCN/MAX interaction, using proximity ligation assays (PLAs).

We found that all molecules previously shown to bind c-MYC (10058-F4, #474, #764, 10074-G5) as well as the metabolite C-*m/z* 232 (for which the binding has never been tested before) also bind to MYCN. Although our data indicated binding by #764 to both MYC proteins, we could not determine its K_D-value, and hence its binding affinity. Interestingly, the binding affinities for the other molecules correlated positively with the IC_{50} values obtained from growth inhibition assays, suggesting that the binding to MYC *per se* is important for the growth inhibition capacity of these small compounds. While keeping in mind that standard errors, especially in case of #474 and 10075-G5, were quite large, the order of the compounds showing the lowest to the highest affinities to MYCN was as follows: C-*m/z* 232, 10058-F4, #474 and 10074-G5.

Interestingly, only 10058-F4, #474, #764 and 10074-G5, decreased the interaction between MYCN and MAX (after treatment for 6 hours in MNA NB cells), as assessed by proximity ligation assays (PLAs). Hence, it suggests that the affinity of the binding to MYCN is important for causing a reduced interaction between MYCN and MAX by these small molecules, which in turn could be responsible the observed anti-proliferative effects seen in NB cells. In addition, these same molecules (10058-F4, #474, #764 and 10074-G5) showed higher levels of apoptosis-induction in high MYCN-expressing Tet21N cells as compared to

after MYCN down regulation by doxycycline, whereas the metabolite (C-*m/z* 232), which showed the lowest MYCN binding affinity, did not induce apoptosis in either setting.

To test our previous assumption (mentioned in the discussion on **Paper II** on page 25) that 10058-F4, by binding to MYCN and preventing its interaction with MAX, eventually leads to MYCN protein destabilization and proteasomal degradation, we assessed the effects on MYCN protein levels after the molecule-treatments. Surprisingly, only 10058-F4 and the structurally unrelated compound 10074-G5 caused down regulation of MYCN protein levels. Interestingly, these two molecules were also the most potent in inducing both neurite outgrowths and lipid accumulation in MNA NB cells.

Consequently, two MYCN-binding molecules (10058-F4 and 10074-G5), decreased the MYCN/MAX interaction, caused reduced MYCN levels, elicited enhanced growth suppression and apoptosis induction in high MYCN-expressing NB cells, and were potent in inducing neurite outgrowth and lipid accumulation. In contrast, the MYCN-binding molecule #474 did not lead to decreased MYCN levels, although it did cause reduced MYCN/MAX dimerization and showed MYCN-selective effects on growth suppression and apoptosis. Interestingly, #474-treatment did not induce morphological differentiation or lipid droplet accumulation in MNA NB cells. The metabolite C-*m/z* 232, showed the weakest binding affinity and was not sufficient to reduce the association between MYCN and MAX, or causing reduced MYCN levels. It was also inefficient in reducing cell growth, and did not induce apoptosis. It furthermore only led to lipid droplet accumulation at very high concentrations, indicating that this effect was an unspecific response (222). Nevertheless, it did induce some morphological differentiation in terms of longer neurite outgrowths, although this effect was much less striking as compared to 10058-F4 and 10074-G5.

These results suggest that the decreased MYCN/MAX interaction alone can be sufficient to cause MYCN-selective biological responses in MNA NB cells. However, conceptually, an induced apoptosis in NB cells with high MYCN levels could also reflect unspecific targeting of other mechanisms selective in cells with higher proliferation rates (192, 193). Similarly, lipid droplet accumulation by C-*m/z* 232 may also occur as a stress response upon mitochondrial dysfunction, mediated by other mechanism not directly related to MYC-inhibition (222).

Taken together, these results confirm that the small molecule 10058-F4 as well as several other c-MYC-targeting compounds binds directly to the MYCN oncoprotein in addition to c-MYC. It also suggests that the binding, as such, to MYC, is responsible for causing interference with the MYCN/MAX dimerization and for causing growth inhibition *in vitro*.

Figure 8. Illustration shows the secondary structure of the bHLHZip domain of MYC where the aligned amino acids of human MYCN and c-MYC are shown below. The small molecules analyzed in this paper are indicated under their reported (10058-F4, #474 and #764, 10074-G5) (93) or assumed (C-m/z 232) binding sites. From **Paper IV** (Mueller I, 2014, Plos One, in press), with permission from the publisher.

4 CONCLUSIONS AND FUTURE DIRECTIONS

One main reason for the improved overall outcome of NB, as well as of BL patients over the last decades, is due to intensified treatments, including the use of chemotherapeutic drugs (158, 223) Nevertheless, regarding NB, still today, children affected with high risk disease have a poor survival, especially those whose tumors are *MYCN* amplified or show chromosome 11q deletion (158). Hence, considering the poor prognosis of these children, new therapeutic approaches are urgently needed.

Investigating different strategies of inhibiting MYC in cancer is important since it could provide for new insights for future novel cancer therapies that could have potential against a broad array of human tumors, including pediatric malignancies.

By analyzing the MYC-selective effects of 80 chemotherapeutic drugs and compounds in **Paper I** (195), we found that MYC-overexpressing cells are dependent on a restricted number of yet specific signaling pathways for their survival. Importantly, among the identified hits were drug classes acting against topoisomerases, microtubules, and the cellular biosynthetic machinery of DNA, RNA and protein synthesis and turnover, all inherently associated with MYC in cancer cell growth (8). These pathways may be particularly vulnerable for targeting in MYC-deregulated tumors and provide hints towards future treatment optimization. Unfortunately, chemotherapy often causes severe side effects including acute and long term toxicities, and in this sense children are particularly vulnerable (154, 165, 223). In fact, treatment resistance of recurring NB is the major cause of failure of treatment and for death (165). Consequently, future studies that take into account the mechanisms behind tumor drug resistance are especially important in order to find new treatment strategies against recurring high risk childhood cancers (6). The fact that MYC is highly associated with a "stemness" phenotype in cancer (8, 64-66), which also relate to MYCN in NB (172-174), is important, since CSCs are believed to be responsible for tumor cell dormancy and drug resistance in cancer (1, 2, 31, 224). Hence, this specific feature of MNA NB cells could be applied when designing new chemical screens, in order to enrich for molecules which target cells with characteristics of tumor initiating cells. This concept was shown to be successful in terms of identifying CSC-selective compounds in breast cancer, using a high throughput screen on breast cancer cells that had been induced to go through EMT (224).

Inhibiting MYC's function by the action of selective small molecules which bind to the MYC protein and consequently interfere with its binding to MAX, represents a challenging yet attractive goal in cancer research (79). Our findings in **Paper II** (109) and **Paper IV**, that a small molecule, 10058-F4, binds directly to MYCN, reduces its dimerization to MAX, and was sufficient to elicit anti-tumorigenic events *in vivo,* is important as it provides proof of principle of "direct MYC targeting" that may strengthen the idea of developing small molecules directed against MYCN as a treatment for pediatric cancer. Applying new chemistry and further validation of the selectivity of both new and existing MYC-binding compounds may hopefully lead to more effective inhibitors with therapeutic potential.

Importantly, our study (**Paper I**) (195) supports the use of chemical screens as a powerful approach to identify compounds active in a MYC overexpressing setting, and hence future screens may lead to the discovery of novel compounds with clinical potential.

There are also some interesting examples of already existing targeted strategies, which are aimed at inhibiting signaling pathways involved in deregulating the expression of MYCN. These include inhibition of the Aurora A kinase, as well as BET bromodomain inhibitors, and these approaches have shown potential in pre-clinical studies (86, 94, 154, 225). Importantly, while using 10058-F4 as a research tool we identified novel biological functions of MYCN that could have important clinical implications. We found (in **Paper II** (109) and **Paper III**) that MYCN induces profound metabolic reprogramming in NB cells, indicative of an enhanced metabolic plasticity in MNA NB. Our data hence provide important suggestions for future therapeutic interventions by pointing towards selective targetable metabolic biomarkers and/or bioenergetics functions in MNA NB, including an elevated ROS detoxification system and a deregulated lipid metabolism. It has been shown for other cancers that LC-MS metabolomic-based methods can be used to identify new metabolic biomarkers and possible therapeutic targets involved in conferring tumor aggressiveness (226). Hence it would be interesting to use this approach in NB to compare with our findings from quantitative proteomics and gene expression profiling.

Understanding the key differences of mitochondria involved in mediating tumor aggressiveness in MNA NB may be important as several mitochondrial targeting anticancer agents (mitocans) already exist (227). One interesting example of an approved mitocan is metformin: a widely used anti-diabetic drug that has received renewed attention, since it was revealed from population studies that it also possesses antineoplastic activity. Metformin usage is associated both with reduced cancer risk and/or improved prognosis in cancer patients (228, 229). It is believed to work through the inhibition of the complex I of the respiratory chain (208, 228), but has also been shown to activate the AMP-activated kinase (AMPK) both *in vitro* and *in vivo* (230, 231). AMPK is known to play key roles as a sensor of cellular energy homeostasis, and shows tumor suppressor functions in many types of cancers (231, 232). In NB we have shown that MYCN regulates the expression of several subunits of complex I of the respiratory chain, and that high expression of these components correlates with reduced survival (**Paper II and III**) (109). Furthermore, low mRNA expression of the alpha catalytic subunits of *AMPK* is correlated with poor overall survival in NB (unpublished observations). Consequently it would be interesting to investigate the effect of metformin in pre-clinical models of NB.

The discovery that MNA NB tumors and cells possess an induced expression of antioxidant mediating enzymes (including GSH-regulators and peroxiredoxins) is particularly interesting, as changes in ROS detoxification capacities has been coupled to chemotherapy resistance in cancer stem cells (233), and elevated ROS levels is linked to MYC-mediated genomic instability (8). Targeting of cancer specific antioxidant systems could hence provide a means of inducing selective toxicity in tumor cells by causing intolerable high levels of ROS, which

could lead to cell death (227). There are several examples of drugs and natural compound acting against ROS detoxification systems in cancer (227). For instance, the natural compound β-phenylethyl isothiocyanate (PEITC) which is found in cruciferous vegetables, was shown to effectively inhibit the GSH antioxidant system in transformed ovarian epithelial cells, and this consequently led to preferential killing of the transformed cells mediated by severe accumulation of ROS (234).

Also, the seemingly important role of fatty acid metabolism in NB is interesting and should be investigated further, since several pre-clinical inhibitors of fatty acid metabolism exist, whereof some are already approved for clinical use (208), including orlistat (which targets FASN) (235) and ranolazine (which targets CPT1) (236). Also, two drugs used for antianginal medication, trimetazidine and ranolazine, targets β-oxidation by inhibiting the acetyl-CoA acyltransferase 2 (ACAA2) enzyme. We found that high mRNA expression of *ACAA2* is correlated with poor outcome in NB (**Paper III**), and consequently it would be interesting to assess whether MNA NB cells are sensitive to these drugs.

Our finding that MYCN induces the capacity of oxidation of exogenous fatty acids in NB fits well with our parallel identified link between MYCN and an elevated oxphos- and ROS scavenger system, as β-oxidation of long chain fatty acids has been shown to result in high levels of ROS production (237, 238). Also, it has been demonstrated that prostate cancer cells, in which FAO is used as a primary source of energy (214), possesses a high antioxidant production required for cancer cell survival by maintaining the cellular redox balance (215). Likewise, a distinct oxphos tumor subset was been identified in diffuse large B cell lymphoma with an elevated capacity of using FAO, and these cells show high levels of antioxidant scavenger systems. The perturbation of FAO and glutathione synthesis furthermore proved selectively toxic to this tumor subset (122).

In conclusion, together our findings on small MYC-binding molecules, and on metabolic alterations associated with MYCN in NB, may be of importance for the development of new treatment options for high risk NB patients.

5 PUPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är ett samlingsnamn för en grupp på över hundra olika sjukdomar som både kan vara godartade (benigna) eller elakartade (maligna), och kan drabba alla slags celltyper. Gemensamt för cancersjukdomarna är att celler börjar växa på ett okontrollerat sätt och inkräktar på kroppens normala hälsotillstånd. Många typer av cancrar är idag inte livshotande utan går att bota. Aggressiv cancer som växer in i omkringliggande vävnad och sprider sig och bildar dottertumörer (metastaser) på anda ställen i kroppen innebär däremot ofta en försämrad chans till överlevnad hos patienten.

Cancer uppkommer till följd av skador på cellers genetiska material, dvs. på DNA:t som finns i varje cell. Skadorna kan vara medärvda eller uppkomma till följd av olika livsstilsfaktorer som exponering för skadliga ämnen eller strålning, men sker även ofta helt slumpartat. Risken för att få cancer ökar dock med stigande ålder. I DNA finns koder (i form av gener) som ger upphov till proteiner vilka i sin tur utgör grunden för cellens maskinerier och signaleringssystem. Man kan därför säga att de genetiska skadorna leder till felsignalering i cellen. Skadorna måste dock träffa specifika gener, och vara av sådan karaktär att de ger cellen överlevnadsfördelar vilket gör att den kan dela sig obehindrat samt undkomma de kontrollsystem som normalt sätt ser till att eliminera felaktiga celler. Under tidens gång ansamlas fler och fler skador i de felaktiga cellerna och en slags "micro-evolution" pågår där vissa celler dör medan andra blir mer aggressiva. Ibland kan det ta årtionden för en tumör att utvecklas medan cancerbildning kan ske snabbt i andra fall. Det är extra svårt att förstå varför barn får cancer. Cancer hos vuxna och barn skiljer sig åt i vissa avseenden. Till exempel så vet man att barncancer generellt sett ofta utvecklas till följd av att något gått fel under fosterutvecklingen, snarare än att de utsatts för olika riskfaktorer.

Neuroblastom är en typ av barncancer som uppstår från de celler som under fosterutvecklingen bildar det yttre sympatiska nervsystemet. Tumörerna hittas oftast i en av binjurarna eller i nervvävnad längs med ryggraden. Neuroblastom är en ovanlig sjukdom med ca 15-20 nya fall per år i Sverige, och drabbar oftast små barn under fem år. Det finns flera olika undergrupper av cancern som skiljer sig från väldigt godartade till extremt aggressiva, och prognosen varierar därför stort. Trots att sjukdomen är ovanlig så tillhör neuroblastom en av de dödligaste typerna av barncancer, och det är därför som forskningen kring neuroblastom är så viktig. Något som är en tydlig indikation på aggressivitet hos sjukdomen är då genen *MYCN* återfinns i för många kopior, genom s.k. gen-amplifikation. Detta betyder att för mycket MYCN-protein bildas, eller "uttrycks" i cellerna vilket i sig fungerar som en gaspedal för snabb celltillväxt och aggressivitet. Överlevnaden för patienter som drabbas av denna typ av neuroblastom är endast 15-30 %. MYCN tillhör en grupp proteiner som innefattar c-MYC, MYCN och L-MYC. MYC proteinerna är så kallade transkriptionsfaktorer, vilket betyder att de binder till DNA och reglerar gener och därmed specifika proteiner, vilka i sin tur påverkar cellen. MYC proteinerna är viktiga för cellers normala funktioner och för att en normal fosterutveckling ska kunna ske. I ett stort antal cancertyper så återfinns däremot MYC (främst c-MYC och MYCN) i för stor mängd. Detta

leder till allvarliga felsignaler som gör att celler växer alltför fort och utvecklar ytterligare egenskaper som leder till aggressivitet hos tumörceller.

Den här avhandlingen baseras på arbeten där vi undersökt olika strategier för att selektivt ta död på cancerceller som innehåller för mycket MYC protein, med fokus på barncancern neuroblastom. Vi har även utforskat en av de bakomliggande mekanismerna till hur MYCN proteinet ger upphov till aggressivitet i neuroblastom, nämligen genom omprogrammering av cellmetabolismen.

Våra upptäckter ger ledtrådar till hur man kan optimera behandling mot MYC-överuttryckande cancerceller, samt ger indikationer på att det i framtiden kan gå att utveckla behandlingsmetoder baserade på små molekyler som slår direkt mot MYC i cancer. Våra studier uppmärksammar även betydelsen av cellmetabolismen i aggressiv neuroblastom, där speciellt en överaktiverad mitokondriemetabolism är starkt kopplad till aggressivitet i tumörer. Våra resultat kan därför komma att vara av betydelse för utvecklandet av nya behandlingsmetoder mot cancerspecifik metabolism.

För en mer utförlig populärvetenskaplig beskrivning av arbete nr II (Paper II), se: *Zirath, H., och Arsenian Henriksson, M. Onkologi i Sverige, (2013) (207).*

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