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METABOLISM AND NEURAL DIFFERENTIATION IN CHILDHOOD NEUROBLASTOMA

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Metabolism and neural differentiation in childhood neuroblastoma

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To enduring guys and girls

ABSTRACT

Neuroblastoma is the most common and aggressive extracranial solid tumor during childhood. *MYCN*-amplification is found in approximately 25 % of all neuroblastoma cases, and is defined as high-risk disease. Development of novel therapeutic approaches focused on MYCN targeting are essential for increased survival these children. The MYC family of oncoproteins consists of transcriptional factors involved in many normal cellular processes. Abnormal expression of MYC is associated with 70 % of human cancers and correlates with an aggressive undifferentiated phenotype, chemotherapy resistance and poor clinical prognosis.

Targeting MYCN by small molecular weight molecules remains a challenge. In **paper I** we established that one known c-MYC targeting compound, the small chemical molecule 10058-F4, is also a potent MYCN inhibitor. 10058-F4 treatment increased cell death and neuronal differentiation in *MYCN*-amplified neuroblastoma cells and prolonged survival in mice. Interestingly, we found that MYCN inhibition resulted in changes in expression of metabolic proteins, in accumulation of intracellular lipid droplets and demonstrated that this is due to mitochondrial dysfunction. Our data reported in **paper I** strongly suggests that MYCN regulated metabolic processes may contribute to the aggressiveness of neuroblastoma.

In **paper II** we applied several approaches to further investigate the MYCN-mediated metabolic alterations in neuroblastoma. The combination of mass spectrometry based proteomics and transcriptome data analysis highlighted key metabolic enzymes involved in energetic pathways of cancer cells. The functional metabolic measurements supported the data analysis and demonstrated that MYCN not only enhanced the glycolytic capacity of neuroblastoma cells, but also increased mitochondrial respiration. The data presented in **paper II** suggests that *MYCN*-amplification is associated with a high-energetic metabolic phenotype. Importantly, we demonstrated that targeting of fatty acid oxidation resulted in potentiated neuronal differentiation, decreased viability of *MYCN*-amplified neuroblastoma as well as decreased tumor burden *in vivo* in a neuroblastoma xenograft model.

Our previous findings highlighted an important role of fatty acid metabolism in *MYCN*-amplified neuroblastoma. In **paper III** we used specific inhibitors and demonstrated that targeting of *de novo* fatty acid synthesis in *MYCN*-amplified neuroblastoma cells resulted in increased mitochondrial dysfunction and glycolytic flux. In addition, we observed that MYCN downregulation and neuronal differentiation are consequences of inhibiting *de novo* synthesis of fatty acids in neuroblastoma cells.

In **paper IV** we demonstrated that the miR-17~92 cluster, which is upregulated by MYCN, suppresses neuronal differentiation via targeting of the nuclear hormone receptor family in neuroblastoma. Importantly, we showed that MYCN inhibition leads to increased expression of the glucocorticoid receptor, which is accompanied by decreased levels of members of the miR-17~92 clusters and elevated expression of the neural differentiation markers TrkA, SCG2 and TH. Furthermore, increased GR expression followed after MYCN downregulation and decreased tumor burden was observed in a pre-clinical NB model following combined MYC inhibition and activation of glucocorticoid signaling.

Together the data generated in our laboratory and included in the present thesis demonstrates that targeting of MYCN and MYCN-controlled metabolic processes may provide an attractive basis for development of novel therapeutic approaches for childhood neuroblastoma.

LIST OF SCIENTIFIC PAPERS

I. 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 cell.
Proceeding of the National Academy of Science (PNAS), 2013, vol. 110, pp. 10258-10263

II. **Ganna Oliynyk**, Johanna Dzieran, María Victoria Ruiz-Pérez, Hanna Zirath, Taner Arslan, Henrik Johansson, Erik Fredlund, Janne Lehtiö, and Marie Arsenian Henriksson.
Fatty acid-dependent oxidative phosphorylation is the major source of energy production in *MYCN*-amplified neuroblastoma cells.
Manuscript, 2015.

III. María Victoria Ruiz-Pérez, **Ganna Oliynyk,** Lourdes Sainero Alcolado, and Marie Arsenian-Henriksson.
Induction of neural differentiation in childhood neuroblastoma upon inhibition of *de novo* fatty acid synthesis.
Manuscript, 2017.

IV. Diogo Ribeiro*, Marcus D.R. Klarqvist, Ulrica K. Westermark, **Ganna Oliynyk**, Johanna Dzieran, Anna Kock, Carolina Savatier Banares, Falk Hertwig, John Inge Johnsen, Matthias Fischer, Per Kogner, Jakob Lovén and Marie Arsenian Henriksson*.
Regulation of nuclear hormone receptors by MYCN driven miRNAs impacts neural differentiation and survival in neuroblastoma patients.
Cell Reports, 2016, vol 16, pp. 979-993
*Corresponding authors.

Publications not included in the present thesis

Ochs MJ, Ossipova E, **Oliynyk G**, Steinhilber D, Suess B, Jakobsson PJ. Mass spectrometry-based proteomics identifies UPF1 as a critical gene expression regulator in MonoMac 6 cells. *J. Proteome Res*, 2013, vol 6, pp.2622-9

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Wang H, Teriete P, Hu A, Raveendra-Panickar D, Pendelton K, Lazo JS, Eiseman J, Holien T, Misund K, **Oliynyk G**, Arsenian-Henriksson M, Cosford ND, Sundan A, Prochownik EV. Direct inhibition of c-MYC-MAX heterodimers by celastrol and celastrol-inspired triterpenoids. *Oncotarget*, 2015, vol 32, pp. 32380-95

CONTENTS

LIST OF ABBREVIATIONS

2-DG	2-Deoxy-D-glucose
3-BrPA	3-Bromopyruvic acid
ACC	acetyl-CoA carboxylase
ALK	anaplastic lymphoma kinase
AMPK	5' AMP-activated protein kinase
ATP	adenosine triphosphate
BAX	bcl-2-like protein 4
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra-large
BET	bromodomain and extraterminal domain
BPTES	bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide
BRD4	bromodomain-containing protein 4
BSA	bovine serum albumin
BUD31	protein BUD31 homolog
CDKs	cyclin-dependent kinases
CK-1	checkpoint kinase 1
CKI	cyclin dependent kinase inhibitors
CPT1	carnitine palmitoyltransferase I
DCA	dichloroacetic acid DCA
DNA	deoxyribonucleic acid
ETC	electron transport chain
FAO	fatty acid oxidation
FASN	fatty acid synthase
GLS	glutaminase
GLUT1	glucose transporter 1
GR	glucocorticoid receptor
HATs	histone acetyl-transferases
HK2	hexokinase 2
HLH	helix-loop-helix
INRG	international neuroblastoma risk group
iPS	induced pluripotent stem cells
KLF4	kruppel-like factor 4
LDHA	lactate dehydrogenase A
MAX	myc-associated factor X
miRNA	microRNA
MIZ-1	MYC-interacting zinc finger protein
MNA	*MYCN*-amplified
MNT	Max-binding protein
mRNA	messenger RNA
MXD	MAX dimerization protein 1

MYC	v-myc avian myelocytomatosis viral oncogene homolog
MYCN	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NB	neuroblastoma
NHRs	nuclear hormone receptors
NLS	nuclear localization sequence
NMNA	non *MYCN*-amplified
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NPM	nucleophosmin
OCT3/4	octamer-binding transcription factor
OXPHOS	oxidative phosphorylation
PDK1	pyruvate dehydrogenase kinase 1
PET	positron emission tomography PET
PTEN	phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
RAS	protein superfamily of small GTPases
RB	retinoblastoma protein
SCG2	Secretogranin II
SID	mSin3-interaction domain SID
SOX2	SRY (sex determining region Y)-box 2
TAD	terminal transactivation domain
TNF-α	tumor necrosis factor
TOFA	5-(Tetradecyloxy)-2-furoic acid
TrkA	tyrosine receptor kinase A
TrkB	tropomyosin receptor kinase B
Zip	leucine zipper

1 INTRODUCTION

1.1 CANCER

Cancer is a very complex disease comprised of more than 200 types of tumors. It is characterized by uncontrolled cell growth and unlimited metastasis to different types of tissues and organs, which is a major cause of death from cancer[1]. The development of the disease involves the dysfunction of numerous biological systems and processes including the immune system, metabolism, DNA repair, cell proliferation and cell death. Cancer is a key health problem and the leading cause of death worldwide[2,3].

1.1.1 Oncogenes and tumor suppressors

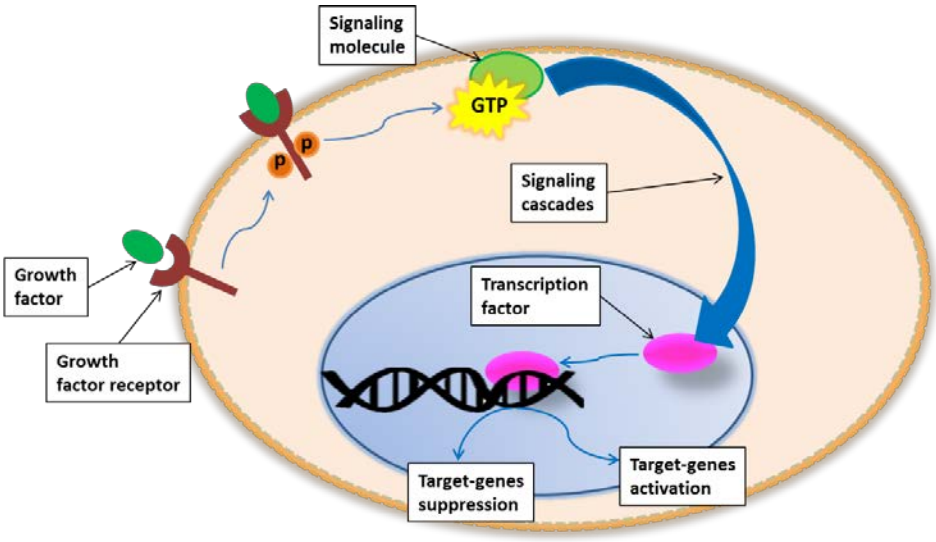


Figure 1. A schematic overview of proto-oncoproteins in cell-growth regulation. Proto-oncoproteins promote the cell cycle and are involved in different signal cascades, which regulate cellular proliferation and differentiation.

Proto-oncogenes are involved in regulation and control of cell growth, differentiation and apoptosis in normal cells. The proto-oncogenes which display abnormal expression level or have gain-of-function mutations and are able to promote cancerogenesis are termed oncogenes. They encode transcription factors, signal transducers and chromatin remodelers[4]. The functions of oncogenes and their products are very important for cell transformation, cancer cell progression and tumor biomass production[5,6] (**Figure 1**). Cytoplasmic proto-oncogenes encode growth factors, growth-factor receptors and different protein kinases. For instance, *RAS* family encodes G proteins. Nuclear proto-oncogenes encode transcriptional factors, such as *MYC* family.

Tumor suppressor genes are another group of genes which are involved in the control of the cell cycle, DNA repair, apoptosis as well as other cellular processes and have a crucial role in cancerogenesis. One copy of a tumor suppressor gene is enough to regulate cellular proliferation, but loss-of-function mutation of both alleles promotes cancer development. The *RB* (retinoblastoma susceptibility), *PTEN* (phosphatase and tensin homolog**)** and *p53* are examples of tumor suppressor genes important for control of cell proliferation[6].

1.1.2 Hallmarks of cancer

The network of processes involved in cancer initiation, development and progression was summarized and defined as the Hallmarks of cancer by Hanahan and Weinberg[7,8]. The highlighted processes are: (1) continuous proliferative signaling (cancer cells enhance their own growth), (2) deviation of response to growth suppressors (cancer cells resist anti-growth signaling), (3) resistance to programmed cell death/apoptosis, (4) limitless multiplication potential, (5) angiogenesis, (6) metastasis, (7) avoiding elimination by the immune system and (8) altered metabolism (**Figure 2**).

One of the crucial features of cancer is the ability to maintain constant proliferation. The process of cell growth is strictly controlled in normal tissues by signals from the microenvironment, which enhance or suppress cell proliferation. An increase of enhancer and/or deficiency of suppressors results in an uncontrolled growth rate in cancer cells. During the last decade many chemical compounds targeting specific genes and/or proteins involved in facilitating a particular hallmark of cancer have been developed and are in clinical trials or in clinical use (**Figure 2**).

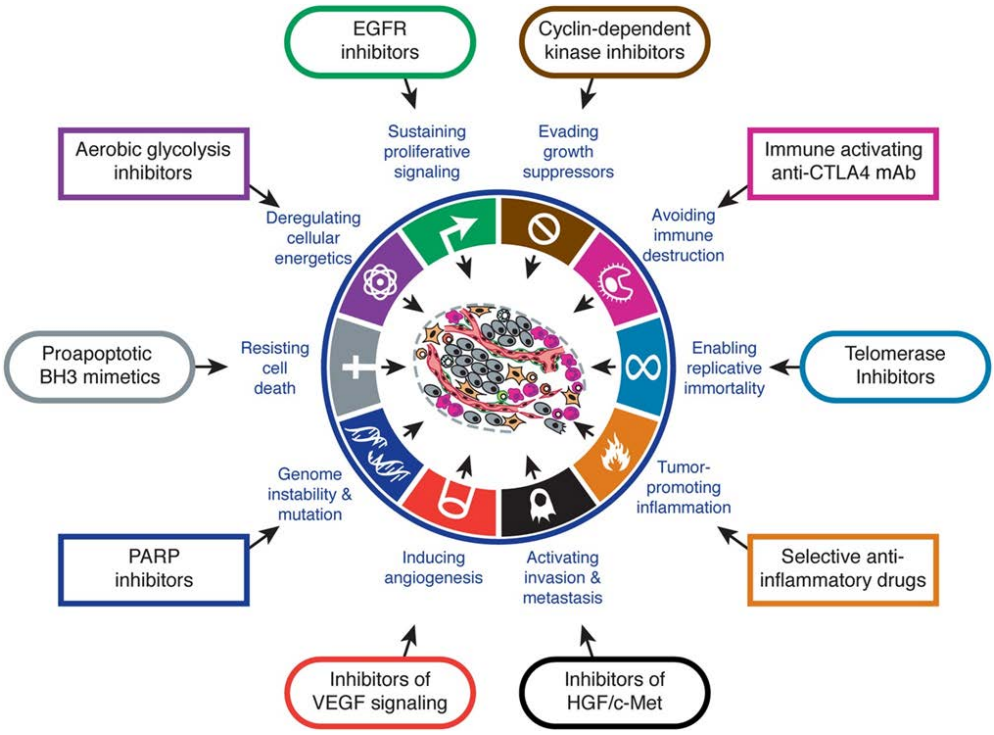


Figure 2. Therapeutic approaches of targeting of the Hallmarks of Cancer. Drugs and small chemical compounds, which target specific tumorigenic pathways and which approved for clinical trials are indicated. *(Reprinted from Hanahan and Weinberg, Cell 2011 with a kind permission from Elsevier).*

1.1.3 MicroRNAs in cancer

The microRNAs (miRNAs) were discovered in 1993 during studies on *Caenorhabditis elegans.* They are defined as small non-protein-coding RNAs that regulate protein expression by binding to matching sequences in the 3'-untranslated region messenger RNAs (mRNA), which results in the inhibition of mRNA stability[9]. Approximately 2,800 human miRNAs are available in public sources. In 2015, Rigoutsos' research group from Thomas Jefferson

University in Philadelphia identified 3,707 novel miRNAs, many of which are human-specific[10]. Around half of the known miRNAs are organized in clusters, including two or more members. MiRNA clusters have similar expression patterns and impact transcription of genes as polycistronic transcripts[11]. The study of the connection between miRNAs and their predicted targets is a very complex, since a single miRNA may affect nearly 100 mRNAs and each mRNA in turn may be targeted by several miRNAs[12].

MiRNAs are involved in a broad spectrum of biological processes, including carcinogenesis, through control of transcription and expression of mRNAs. At present, miRNAs targets in cancer have been associated with tumor proliferation, altered metabolism, metastasis, dysfunctional immune system, differentiation and angiogenesis[13,14]. There are several mechanisms of deregulation of miRNAs expression in human malignancies[15].

Altered expression of miRNAs correlates with genomic abnormalities such as amplification and translocation[16]. Epigenetic factors can also influence the level of miRNAs, and transcription factors can also induce oncogenic and/or inhibit tumor suppressive miRNAs[17-19]. Moreover, several miRNA families exhibit tumor suppressor functions; for instance, *miR-15* and *miR-16-1* and inhibit expression of anti-apoptotic proteins in different types of cancer[20].

MiRNAs play significant roles not only in cancer biology, but also in oncology. Cancer associated small non-coding RNAs can be detected in the biological fluids and used as biomarkers for disease diagnosis, monitoring and prognosis[21]. Moreover, it has been reported that the normalization of deregulated expression of miRNAs decreases tumor growth *in vivo*.[22] Re-expression of some miRNAs by treatment with small chemical molecules may be a possible basis for development of novel anti-cancer therapies.

1.1.4 Nuclear hormone receptors superfamily

The nuclear hormone receptor (NHR) superfamily includes 48 human and 49 mouse genes, including steroids, retinoids, thyroids and vitamin D_3[23,24]. The members of the superfamily are activated by numerus different ligands[25]. They bind to specific DNA sequences and are able to activate or suppress transcription. The activity of NHRs requires two steps: interaction with the corresponding hormone, followed by binding of the receptor dimers to DNA to regulate transcription. NHRs are involved in the regulation of development and differentiation of skin, the constant regulation of reproductive tissues and, most importantly, the members of the NHR family play a role in the regulation of neuronal differentiation[26].

1.1.5 Childhood malignancies versus adult cancers

Cancerogenesis is associated with old age and is more common in older individuals than in young[27]. However, cancer is the most common cause of death by disease during childhood (aged birth-14 years) worldwide. There are more than 150 cases per million in Europe each year[28]. In the majority of cases the cause of tumorigenesis is unknown. Quite often the types of tumors that develop in infants are different from the adult cancers. However, childhood cancers, as well as adult malignancies, may display deregulation of oncoproteins, such as MYC and loss of function of tumor suppressor genes, for instance p53 mutations. Pediatric malignancies are not associated with life style and/or impact of environment as many cancers in adults, but these factors may influences the children before the birth during pregnancy[29].

Generally, childhood tumors are a result of genetic alterations in cells, which occur very early, often before birth. But, according to a recent scientific report, 90 % of all human cancers are the results of errors in DNA replication[30]. Cancers in infants are characterized by an aggressive phenotype and are usually diagnosed at more advanced stages compared to adult disease. Pediatric and adult cancers differ not only in development, genetic background and diagnosis but also in therapeutic approaches, effects and consequences of therapy[31]. Chemotherapy, surgery and radiation therapy are the main therapeutic approached in pediatric oncology. Chemotherapy used in the treatment of cancers, targets cells with the highest growth rate and has more severe side effects in children than in adults, since their bodies are still actively developing. The majorities of side effects of anti-cancer treatments occur during or just after the therapy and go away in a short time. Unfortunately, anti-cancer therapy may lead to late side effects, such as delayed growth in children, cognitive problems as well as the formation of other types of cancer later on in life. Late side effects constitute a major problem in pediatric oncology[32].

1.2 NEUROBLASTOMA

Neuroblastoma (NB) is a rare embryonic neural tumor, but one of the most common and deadliest extracranial solid tumors of childhood. It is accounting for approximately 7 % of all pediatric malignancies and 15 % of childhood cancer deaths[33,34]. In Sweden 15-20 new cases are diagnosed per year. The majority of NB cases are diagnosed in infants under the age 5 years. Approximately 70% of all cases are patients with high-risk NB, which is usually characterized by genetic alterations and the metastasis. Only less than a half of these patients can be cured.

1.2.1 Origin of Neuroblastoma

Focusing on the cells of origin of neuroblastoma is important for understanding malignancy. The neural crest is present only during embryogenesis (**Figure 3**) and maturates to different cell types, such as glia, peripheral and enteric neurons, melanocytes, cells of craniofacial skeleton, adrenal medulla and Schwann cells[35]. The adrenal medulla is the inner part of adrenal gland and ganglion of the sympathetic nervous system. NB originates from primitive neuroepithelial cells of the neural crest (**Figure 3**), and can develop anywhere in the sympathetic nervous system. The majority of the primary tumors occurs in the adrenal glands, sympathetic ganglia and is commonly found in the abdomen and neck[36,37].

High expression of the proto-oncogene *MYC* during normal sympathoadrenal development is essential for neural crest migration and growth[38,39]. In differentiating sympathetic neurons, MYCN level decreases[40]. *In vivo* studies demonstrated that overexpressing *MYCN* in sympathoadrenal cells initiates neuroblastoma development[41]. *Th-MYCN* transgenic mice spontaneously develop neuroblastoma as a result of high expression of *MYCN*, controlled by the rat tyrosine hydroxylase[42].

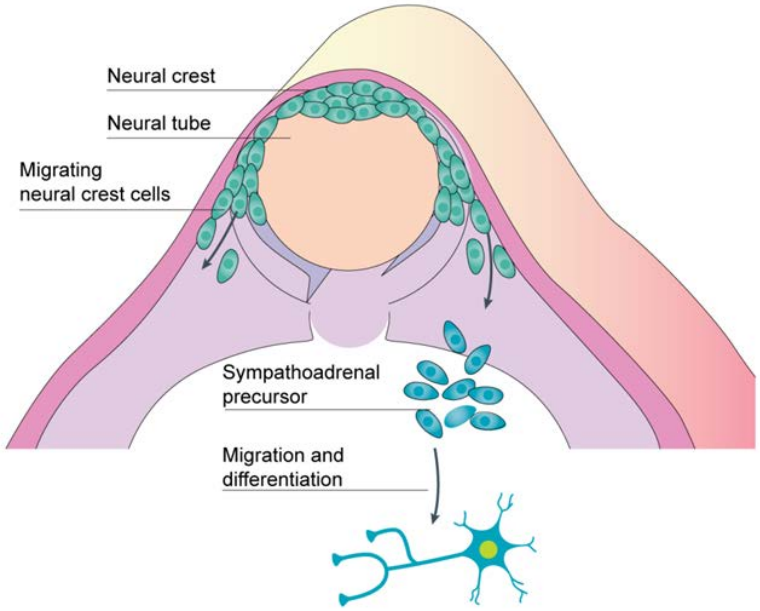


Figure 3. A schematic overview of neural crest development and migration. Migration of sympathoadrenal precursor from the neural crest and their specification depend on transmembrane signals. *(Adapted from Marshall, G. M. et al, Nat. Rev. Cancer 2014).*

1.2.2 Differentiation in neuroblastoma

Neuroblastoma tumors are characterized by three types of cells, based on their morphological and biochemical properties. N-type - neuroblastic cells malignant cells with neuronal properties; S-type – cells with non-neuronal phenotype; I-type – highly malignant cancer stem cells, are able to differentiate to S- or N- type[43]. Histology based on NB tumor cells sub-type is used in the clinic as an outcome prognosis and it has been shown that undifferentiated tumors with poor prognosis and tumors with neuron-like differentiated cells demonstrate favorable prognosis[44].

MYCN plays a major role in NB differentiation, it is critical for initiation of neural crest differentiation, but high MYCN expression associates with an undifferentiated phenotype in NB. Furthermore, high expression of MYCN correlates with enhanced expression of the tropomyosin receptor kinase B (TrkB) neurotrophin receptor, which associates with an aggressive NB phenotype. At the same time, *MYCN*-amplification suppresses the tyrosine receptor kinase A (TrkA) nerve growth factor receptor, which is marker for good prognosis in NB patients[45]. TrkA expression is critical during development of symphatethetic neurons.

Additionally, MYC enhances the level of the miR-17~92 cluster, which results in suppression of cellular differentiation via downregulation of the nuclear hormone receptor family[14,46]. Direct or indirect targeting of MYCN result in upregulation of estrogen receptor α, TrkA, glucocorticoid receptor and induced neural outgrowth of NB cells *in vitro* and decreased tumor burden *in vivo*.

The capacity of NB cells to differentiate along the sympathoadrenal linage is being used for the development of the novel therapeutic approaches in NB. All trans-retinoic acid (ATRA),

a derivative of vitamin A is used in the clinic and induces differentiation and prolongs survival of high-risk NB[47]. Importantly, it was demonstrated that ATRA also inhibits MYCN in *MYCN*-amplified NB, which potentiates neuronal differentiation. Furthermore, ATRA decreases the proliferation rate and tumor size *in vivo*[48].

1.2.3 Neuroblastoma is a heterogeneous disease

Neuroblastoma is a highly heterogenic disease and thus can be found in different forms: from localized tumors, which can be treated by surgery to metastatic types with high resistance to therapy and poor prognosis. Also, NB includes a very special group – spontaneous regressing tumors[49,50]. The international neuroblastoma risk group (INRG) is a pre-treatment classification system. INRG includes 4 different group of cancer: very low-risk, low risk, intermediate-risk and high-risk. Very low-risk and low-risk groups include NB cases with localized primary tumors without *MYCN*-amplification and 11q chromosome aberration and can be curable with surgery and/or chemotherapy. Besides, this group includes patients with spontaneously regressing tumors. Intermediate-risk NB includes cases with tumors that spread to the lymph nodes, but with negative *MYCN* status and, cannot be treated by surgery alone, chemotherapy is essential. High risk NB is characterized by positive genetic markers of poor-prognosis, such as *MYCN*-amplification, 17q gain and 11q or 1p36 deletion, and *ALK* mutation. *MYCN*-amplification is found in 30% of high-risk NB and is associated with an undifferentiated phenotype and poor outcome. Approximately 2 % of NB patients have a family history of disease. Two thirds of these cases have *ALK* gene activation, which result in a high proliferation rate of cancer cells. The therapeutic strategy for high-risk NB is very intense and includes several types of treatment[51].

1.2.4 Neuroblastoma and energy metabolism

Metabolic pathways are dysregulated in the majority of human cancers. The number of studies focused on the investigation of mechanisms behind metabolic alterations in adult cancers progressively increase, however in contrary very little information about the energetic pathways in pediatric malignancies, including NB, is available.

Only few studies dedicated to metabolic processes in NB have been published. In 2012 Kusunoki's research group from Mie University, Japan demonstrated that elevated expression of GLUT1 is associated with poor prognosis in patients and that glycolysis inhibitors may be used as a possible treatment option for NB[52]. Unfortunately, the difference between *MYCN*-amplified (MNA) and non *MYCN*-amplified (NMNA) NB was not investigated. Only 5 from 47 tumor samples were MNA and only NMNA NB cells used for *in vitro* assays. In 2010 B. Kofler's research group from the University Hospital Salzburg, Austria studied 14 NB samples derived from NMNA undifferentiated tumors and observed a low mitochondrial respiratory capacity, but intact mitochondrial architecture in these tumors[53]. Several early reports also demonstrated intact mitochondrial mass and architecture in neuroblastoma cells[54].

A recent study demonstrated high level of glucose uptake in low and intermediate risk groups of NB using positron emission tomography (PET). PET is an imaging technology for monitoring cancer progression, based on uptake of labeled glucose by tumor cells. However, the application of PET in high-risk NB was not successful[55]. High-risk NB associated with

MYCN-amplification, which may promote mitochondrial respiration. Increased respiratory capacity may explain the significant lower glucose uptake by *MYCN*-amplified NB tumors.

1.2.5 Therapeutic approaches in neuroblastoma

The therapeutic strategy in NB is a very challenging task, which depends on the risk classification of disease. Patients with tumors that spontaneously regress do not have severe symptoms and do not require therapy, but they are examined frequently and in the case of tumor progression treatment is initiated immediately[56]. Patients with localized primary tumors are usually treated by operation alone, and in some cases surgery following chemotherapy, which is applied to shrink the tumor size. The combination of restrained chemotherapy doses and surgery is used for intermediate-risk NB patients. The children diagnosed with high-risk NB go through intense therapy, which includes combination of several chemotherapeutic drugs, radiation therapy, surgery, treatment with cis-retinoic acid and autologous stem cell transplantation[56,57]. However, despite this, only half of the patients are cured from high-risk NB. Also, patients suffer from drug toxicity and late side effects, following the therapy. Therefore, the development of new therapeutic strategy based on molecular and genetic characteristics is essential.

1.3 THE MYC ONCOPROTEIN FAMILY

The *MYC* (*v-myc* avian myelocytomatosis viral oncogene homolog) gene family is composed of three known and genetically distinct members: *c-MYC*, *L*-MYC and *MYCN*. They encode nuclear phosphoproteins of similar size (**Figure 4**). The MYC proteins (c-MYC, MYCN and MYCL) contain a C-terminal domain consisting of a basic region that can bind to DNA and a helix-loop-helix (HLH)-leucine zipper (Zip) domain, which enables interaction with another HLH-Zip protein, MAX. All members of the MYC family and some associated proteins (e.g. MAX, MXD, MNT) are transcription factors (**Figure 4**) albeit with weak activity. MYC regulates expression of approximately 15% of all human genes through binding to the CACGTG Enhancer Box sequences (E-boxes). Upon MYC-MAX binding, the complex recruits histone acetyl-transferases (HATs), which increase histone acetylation and thereby open up the overall chromatin structure. This allows various transcription factors to interact with their respective target genes[37]. MYC is also able to bind to vertebrate CpG islands (CGIs), which also are sites of transcriptional initiation[58]. Importantly, recently several scientific reports demonstrated the ability of MYC to amplify gene expression via accumulation in the promoter regions of active genes[59,60]. Moreover, in 2010 it was shown that MYC plays an important role in the RNA polymerase II mediated promoter-proximal pausing[61].

The associated proteins MAX network transcriptional repressor (MNT) and MAX gene-associated protein (MGA) are members of MXD protein family (**Figure 4**) and able to form heterodimers with MAX. Dimerization of MAX with MNT or MGA results in suppression of transcription of MYC target genes[62].

Importantly, MYC is able to act not only as a transcriptional activator, but also as a transcriptional suppressor. The MYC-interacting zinc finger protein (MIZ-1) binds to initiator elements in the core promoter and activates genes transcription via recruiting p300 and NPM.

Binding of MYC to MIZ-1 results in the re-localization of MIZ-1 within the nucleus and loss of both co-factors p300 and NPM[63].

c-MYC and MYCN share same biological processes, including the dimerization with MAX and binding to the same specific sites in DNA, but their pattern of expression differs, which could be the major reason for the biological differences between these proteins. c-MYC is usually expressed in all proliferating cells, but MYCN is expressed only during development processes and its expression is tissue-specific[38,64].

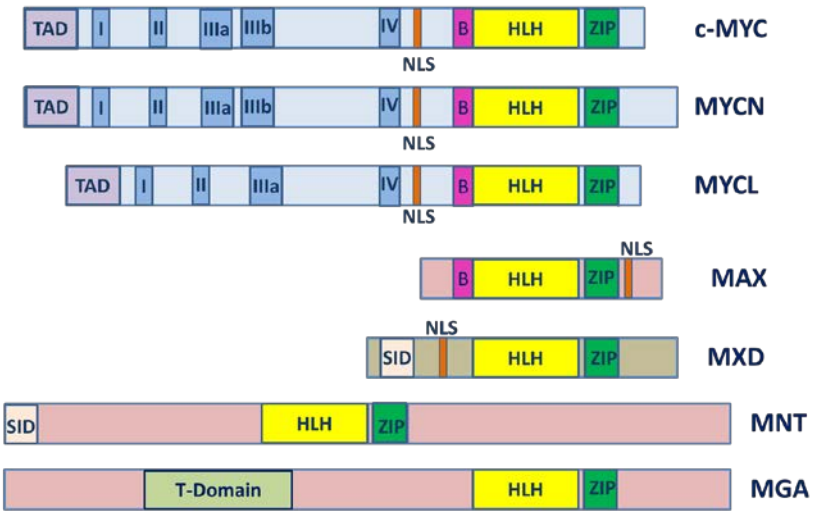


Figure 4. A schematic overview of the structure of MYC proto-oncoprotein family and associated proteins. The MYC proteins contain several preserved domains. Two conserved regions MYC-box I and II are in N-terminal transactivation domain (TAD) and three MYC-boxes in the central domain. Also, the central domain includes a nuclear localization sequence (NLS). The C-terminal region includes a basic helix-loop-helix zipper domain (bHLHZip) and sequence specific DNA-binding basic region (B). The associated proteins also contain a bHLHZip domain, basic region and NLS. mSin3-interaction domain (SID) and MGA contains T-box DNA binding domain (T-domain).

Taken together, the knowledge about MYC demonstrates its importance for many different normal cellular processes such as proliferation, apoptosis and differentiation[65](**Figure 5**). Several functions of MYC are discussed below.

1.3.1 MYC role in the control of cell cycle

The machinery of cell cycle is synchronized and highly controlled. The cell cycle incudes three states, which divided on different phases: resting state - G_0 phase (quiescent); interphase state - G_1, S (synthesis) and G_2 phases; division state – mitosis phase. Also, the restriction point (R-point) in G_1-phase. Cells respond to mitogenic growth signaling in G_1-phase until R-point, after which cells continue to proliferate or stay in G_0-phase in non-proliferative stage. Each phase during the proliferation is strictly controlled in order to avoid DNA damage[66,67]. The progression of cell cycle through the checkpoints depends on cyclins and cyclin-dependent kinases (CDKs). The expression of CDKs is stable, while cyclins are synthesized and degraded on demand. In the initiation of the G_1-phase, in response to mitogenic signals, the levels of cyclins D1, D2 and D3 are increased in order to activate CDK4 and CDK6. The

cell growth rate increases in G_1 phase, in order to facilitate DNA synthesis in S phase the expression of transcription factors is promoted by cyclin-CDK complexes. In addition, cyclin-CDK complexes reduce the level of S phase inhibitors via ubiquitination followed by proteasome degradation[66]. During S phase cyclin-CDK complexes phosphorylate the proteins involved in replication, which is essential for cell cycle progression. For instance, the cyclin D-CDK4 complex enables the activation of the E2F transcription factor via phosphorylation of retinoblastoma susceptibility (RB) protein[68,69]. Active E2F initiates transcription of *cyclin A* and *cyclin E*. The cyclin E-CDK2 complex promotes cell progression to S phase and initiates transition between G_2 phase and mitosis. In the division state cyclin-CDK complexes are crucial for promoting of mitogenic signals[66]. In the end of mitosis the cyclin B-CDK1 complex dephosphorylates the RB protein, which is critical for cells to exit the division state[66]. Cyclin dependent kinase inhibitors (CKI) are negative regulators control cell cycle during each phase and able to stop its progression if DNA damage occur.

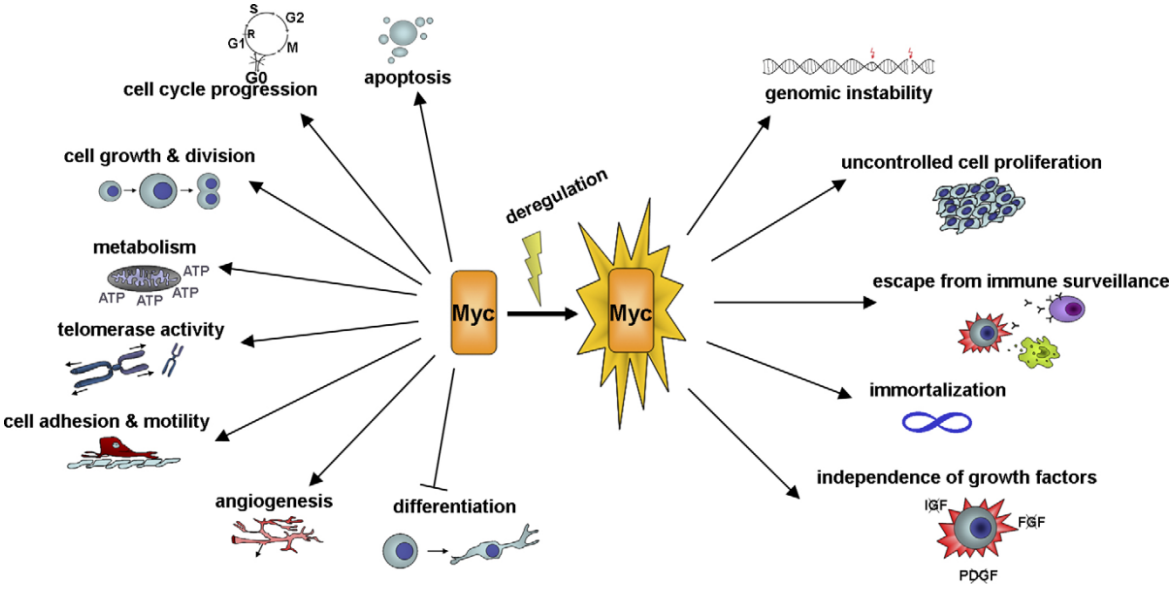


Figure 5. MYC controlled cellular processes. MYC involved in normal cellular processes, as well as has a great importance in cancerogenesis. *(Reprinted from Vita and Henriksson, Seminars in Cancer Biology 2006, with a kind permission from Elsevier).*

MYC is expressed in all states of the cell cycle. Elevated expression of *MYC* promotes quiescent cells to enter cell cycle, while downregulation of *MYC* leads to slow down or stop of cell cycle. MYC impacts cell cycle progression via several different mechanisms. It directly regulates transcription and induces expression of cell cycle related genes, including *cyclins*, *CDKs* and *E2F* genes encoding the E2F transcription factors. Also, via stimulation of the CDK activating kinase, MYC promotes activity of cyclin-CDK complexes. Furthermore, MYC is able to inhibit CKIs, such as p27 and p21, by blocking their transcription. Importantly, MYC upregulates genes encoding the proteins, which are crucial for DNA replication, such as cell division cycle 6 (CDC6), chromatin licensing and DNA replication factor 1 (CDT1) as well as cell division cycle 45 (CDC45)[70].

The regulation of cell cycle progression is one of the most important MYC functions in normal as well as in cancers cells. MYC overexpression in malignant cell promotes cell

growth, leading to doubled size and mass, via regulation of cell division, protein synthesis and mitochondrial biogenesis.

1.3.2 MYC triggered apoptosis

MYC not only controls the cell cycle and cellular growth, but is also a potent promoter of programmed cell death - apoptosis. In 1987, Spandidos' research group from the University Medical School, Edinburgh, UK demonstrated that expression of *MYC* and *RAS* in rat fibroblasts result in an increased apoptosis compared to cells with *RAS* expression alone[71]. In the early 1990s several research reports showed that abnormal MYC expression enhances the sensitivity of premalignant cell to apoptosis[72,73]. During proliferation cells require growth promoting and survival signals. Cell growth promoted by abnormal expression of MYC upon withdrawal of survival signals leads to activation of apoptosis[73]. Importantly, in 1994 MYC overexpression was shown to activate the tumor suppressor p53. The activation of p53 results in the promotion of pro-apoptotic proteins, such as BAX, NOXA and PUMA, and p21 [74]. MYC targets include the members of BCL-2 family, which is characterized by both pro- and anti-apoptotic functions and are localized to the mitochondria. BCL-2 and BCL-X_L inhibit the pro-apoptotic protein BAX. MYC is able to inhibit expression of BCL-2 and BCL-X_L also, via the promotion of association of BID with mitochondria, MYC interferes with anti-apoptotic properties of those proteins[75]. Furthermore, MYC activates cell death pathways in response to tumor necrosis factor (TNF-α). TNF-α induces both pro- and anti-apoptotic signals in the cells. Ectopic expression of MYC inhibits anti-apoptotic signaling mediated by TNF-α[76]

1.3.3 Impact of MYC on cancer cell differentiation

MYC oncogenes are involved in many cellular processes, including control of differentiation. The members of the MYC family are known to be downregulated during differentiation processes. Nonetheless, recent studies established that MYC proteins also support differentiation in neuronal cells and skin stem cells[77].

Over the last three decades it has been reported that abnormal MYC expression inhibits cellular differentiation in the broad spectrum of transformed and primary cell lines. Moreover, MYC downregulation is associated with terminal differentiation in cell culture. There are several reports suggesting that expression of the MYC dimerization partner MAX is reduced during differentiation, leading to decreased expression and activity of MYC[78,79]. MYC inhibits differentiation not only *in vitro* but also *in vivo.* Animal studies in mice with conditional MYC alleles indicate that the ability to suppress differentiation is crucial for MYC-regulated carcinogenesis. Deactivation of MYC results in tumor regression and differentiation of cancer cells in transgenic mouse models[80,81]. Interesting, in hepatocytes the transforming activity of MYC depends on the developmental stage of the cells. In a murine model of hepatocarcinoma, MYC activation in the mature liver cells induces only cell growth and DNA synthesis, however overexpression in embryonic hepatocytes leads to cancer initiation[82].

The mechanisms by which MYC regulates proliferation are relatively well-know, in contrast to the mechanisms of MYC-controlled differentiation. One of the most accepted hypotheses is that MYC alters cell cycle progression to maintain proliferation, which results in decreased

differentiation. MYC as a transcription factor decreases expression of numerous differentiation-associated genes (*TrkA*, *SCG2*) suggesting that MYC may impair differentiation independently of cell cycle arrest through alterations in the transcriptome. Furthermore, it is well known that MYC targets miRNAs clusters including the miR-17~92 cluster, which in turn inhibits cellular differentiation[14,46]. However, more detailed information about MYC-mediated differentiation and its role in human oncogenesis needs be studied.

1.3.4 MYC is a factor of pluripotency

MYC is one of four transcription factors with the ability to reprogram human somatic cells into induced pluripotent stem cells (iPS), which may be used as a unique approach in transplantation therapy[83,84]. *In vivo* studies showed that iPS cells generated from mouse fibroblasts by retroviral induction of four genes (*OCT3/4*, *SOX2*, *MYC* and *KLF4*) demonstrate great similarity to embryonic stem cells. Unfortunately, 20% of the second generation of mice derived from an iPS cell line developed tumors, which is associated with high MYC expression, while at the same time the level of the other three factors remained low[85]. However, several scientific reports demonstrated that MYC is important, but not essential for cell reprograming, while crucial for iPS cells tumorigenicity[86]. Nevertheless, the novel function of MYC may give a clue about its role in control of cancer stem cells[81]. Furthermore, gene set enriched in cancer stem cells are also associated with aggressive and undifferentiated phenotype of MYC-driven tumors[87,88]. On the contrary, a study demonstrated that the differentiation of bone marrow hematopoietic stem cells requires an increased MYC expression[89]. MYC's role in control of cell stemness and tumor initiation has to be investigated further.

1.3.5 Cancer metabolism and MYC

MYC proteins are involved in regulating nucleotide biosynthesis, ribosome and mitochondrial biogenesis and metabolism[90]. MYC overexpressing tumors are mostly aggressive and characterized by high proliferation rate, which requires increased energy production it targets genes involved in different metabolic pathways, which results in enhanced ATP production and increased levels of substrates essential for cancer cell growth. In human Burkitt's lymphoma, glucose consumption, lactate production and glutamine uptake are induced by c-MYC[91]. MYC enhances expression of key glycolytic enzymes, such as the glucose transporter 1 (GLUT1), hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA), which lead to increased glucose consumption and lactate production. MYC promotes the conversion of glucose to pyruvate and elevates expression of pyruvate dehydrogenase kinase 1 (PDK1) to supply oxidative phosphorylation (OXPHOS) with pyruvate derived acetyl-CoA[91,92].

The activation of both c-MYC and MYCN promotes glutamine metabolism via up-regulation of glutamine transporters[93,94]. Moreover, MYC promotes expression of mitochondrial glutaminase (GLS), the first enzyme in the glutaminolysis pathway, by suppressing miRNAs that target GLS. Some MYC overexpressing cell lines are characterized by glutamine-depending proliferation, its removal initiates apoptosis[93,95].

MYC enhances OXPHOS and aerobic glycolysis to stimulate cell cycle progression. Furthermore, increased mitochondrial biogenesis and production of lactate and pyruvate associated with high MYC expression is essential for the rapid cell cycle entry[96]. In conclusion, MYC targets include the genes involved in the main energy metabolism processes (OXPHOS, glycolysis and glutaminolysis) to support cell proliferation.

1.3.6 The mechanisms of deregulation of MYC expression in cancer

Low levels of *MYC* expression is observed during normal proliferation processes[97], at the same time alterations in *MYC* expression is found in approximately 70% of human cancers. Deregulation of the *MYC* genes is a consequence of several processes: chromosomal translocations, amplifications, single nucleotide polymorphisms in regulatory elements and mutations in upstream signaling pathways. Chromosomal translocation mainly occurs in leukemia and Burkitt's lymphomas[98,99] while amplifications occur in solid tumors. Enhanced expression of oncogenes results in overexpression of MYC proteins and abnormal activation of its downstream targets. Proliferation of cancer cells with high MYC level is independent of growth-factor promotion, which leads to uncontrolled proliferation, one of the hallmarks of cancer. Furthermore, enhanced MYC expression facilitates abnormalities in chromatin structure, metabolic processes, ribosomal biogenesis, cell death and angiogenesis[7,98,100,101].

The crucial role of the MYC oncogene may be explained by its impact as a transcription factor on a huge set of genes involved in carcinogenesis[102]. Targets of MYC or "MYC signatures" have been identified in a broad range of human malignancies[103-105]. Importantly, these MYC signatures involved in different processes display low overlap, making it difficult to generalize MYC's oncogenic properties based on one set of genes[106,107]. MYC functions not only as a classical transcription factor regulating gene expression though the recruitment of the transcription apparatus to promoters, but also by stimulating transcription elongation in cancer cells[61,108,109].

Amplification of the *MYCN* oncogene has been found in approximately 25% of NB cases and it is used in the clinic as a genetic marker for poor outcome[110]. Like c-MYC, MYCN directly activates genes that limit proliferation and increase apoptosis.

1.3.7 Targeting MYC in cancer

MYC oncogene activation is one of the most common hallmarks of cancer cells. High level of MYC expression is involved in cancer initiation and progression, and is often associated with an aggressive tumor phenotype and chemotherapy resistance. These factors make MYC an attractive therapeutic target. Unfortunately, MYC does not display enzymatic activity, in addition the structure of the protein lacks any pocket suitable for small molecule inhibitors. There are however several strategies for MYC targeting. Direct inhibitors interrupt MYC-MAX dimerization and prevent DNA binding as well as increased MYC protein degradation, while indirect inhibitors target transcriptional initiation shown to be specific for MYC[111,112] (**Figure 6**).

The activity of MYC requires its interaction with its protein partner MAX[112], interruption of MYC-MAX dimerization is direct approach to target MYC functions. During the last decades many small chemical compounds were designed to inhibit MYC-MAX binding[113]. Two

examples of these compounds are 10058-F4 and 10074-G5 that are able disrupt MYC-MAX complex and induce cancer cell death and differentiation *in vitro*[114]. The small molecule KJ-Pyr-9 was identified as a MYC inhibitor in the screen of pyridine library. KJ-Pyr-9 prevent MYC-MAX complex formation in the cells, very importantly, it has a striking effect on cancer cell growth *in vivo*[115]. Nevertheless, inhibition MYC activity through interruption MYC-MAX heterodimerization requires further studies to improve specificity and effectiveness (**Figure 6 A**).

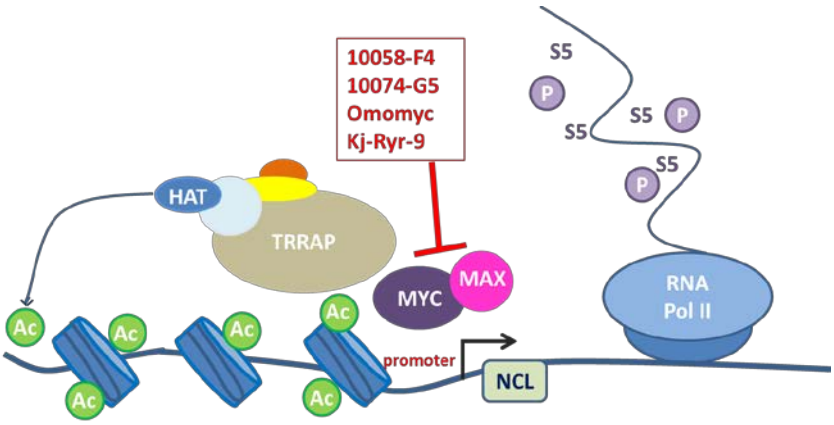
One of the effective strategies to disrupt MYC-MAX dimerization is the use of a small (90 amino acids) protein, Omomyc. It is a dominant negative molecule derived from the bHLHZip region of MYC; it is able to form homodimers with MYC to prevent its DNA binding and initiating transcriptional activation. At the same time the functions of MYC which do not require E-box binding remain unaffected[116,117] (**Figure 6 A**). The studies in double transgenic animals with overexpressed *MYC* and conditionally expressed *Omomyc* demonstrate the capacity of Omomyc to affect cancer cells selectively. Systemic expression of Omomyc significantly affects proliferation rate of rapidly dividing cells, while at the same time cells death did not increase in normal cells[118].

The small molecule JQ1 was identified in a molecular screen. It prevents transcriptional elongation through the binding to acetyl lysine binding site of BET proteins, which result in dislocation of BRD4 from chromatin[119]. Several studies reported the efficiency of JQ1 *in vitro* and *in vivo* in human malignancies such as triple negative breast cancer, acute myeloid leukemia, multiple myeloma and neuroblastoma[120-123]. The anti-tumorigenic effect of JQ1 is strongly associated with MYC downregulation and its targets[98,124,125]. JQ1 affects MYC through inhibition of BRD4 one of the members of BET family, involved in regulation of *MYC* transcription[126] (**Figure 6 B**). The new generation bromodomain inhibitor I-BET has been reported to increase cell death in neuroblastoma cells *in vitro* and to reduce tumor burden *in vivo* in mouse models[123,127]. Members of the BET (bromodomain and extraterminal domain) protein family bind to acetylated lysines on histones in order to recruit the essential elements for the transcriptional elongation[119].

A recent report highlighted another approach to target MYC indirectly. The cyclin-dependent kinases have a crucial role in regulation of transcription initiation and elongation. The CDK7 subunit of TFIIH enhances transcriptional initiation, elongation and pause relies through phosphorylation of carboxy-terminal domain of RNA polymerase II[128]. Specific inhibition of CDK7 results in significant impact on the transcription of the cell-cycle regulators, including MYC[111,129] (**Figure 6 B**). The novel covalent compound THZ1 was discovered and characterized as a specific CDK7 inhibitor[130]. Importantly, THZ1 selectively inhibits growth of *MYCN*-amplified NB cells and demonstrates high efficacy in NB mouse models[111]

Recently it was demonstrated that inhibition of Aurora kinase A (Aurora-A) is an effective approach of indirect MYC targeting. Aurora kinase is very important for mitotic processes and is expressed in many human cancers. Aurora-A forms a complex with MYC and prevents its degradation. Downregulation of Aurora kinase A results in MYC degradation and cell death[131-133]. One of the potent Aurora-A inhibitors is small molecule alisertib, which demonstrates anti-tumorigenic activity in different types of human malignancies

A



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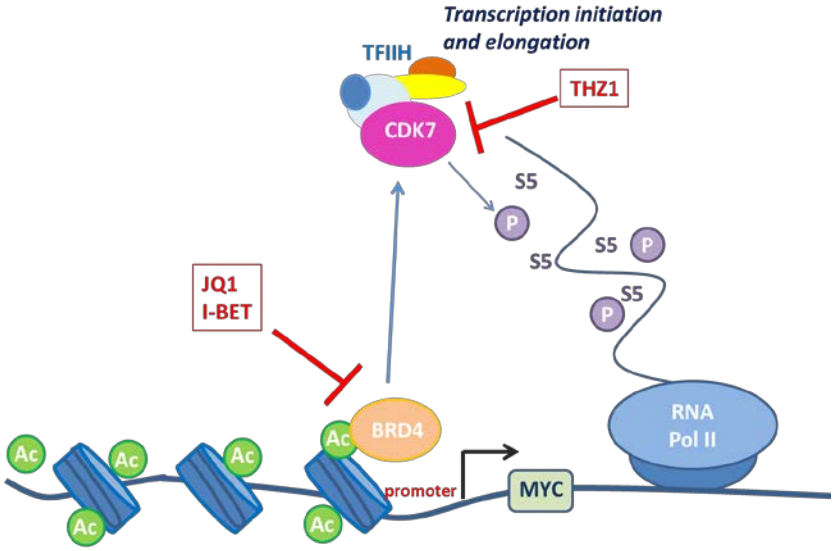

Figure 6. A schematic overview of approaches MYC targeting in cancer. (A) Examples of direct strategies to interfere with MYC-MAX dimerization. **(B)** Examples of indirect MYC targeting via regulation of its transcription.

Importantly, alisertib has been shown to effective in decreasing proliferation of NB cells both *in vitro* and *in vivo*[134].

Synthetic lethality is a definition used for increase cell death as consequence of the combined alteration in the expression two or more genes, whereas deregulation of only one gene will not have the same effect. The analysis of the transcriptome of MYC-overexpressing tumors identified genes involved in synthetic lethal relationship with MYC, such as the AMPK-related kinase 5, BUD31, LDHA and the checkpoint kinase 1 (CK-1)[135-137]. Targeting synthetic lethal interactions of MYC is a promising approach in anti-cancer therapy.

1.4 CANCER METABOLISM

Cancer metabolism applies to all modifications in cellular metabolic pathways, which is altered in cancer cells compared to the majority of normal cells. Metabolic changes are one of

the emerging hallmarks of cancer and involve modifications in glycolysis, OXPHOS and the ability of cancer cells to reprogram metabolic processes in order to adapt to high energy demand conditions[7].

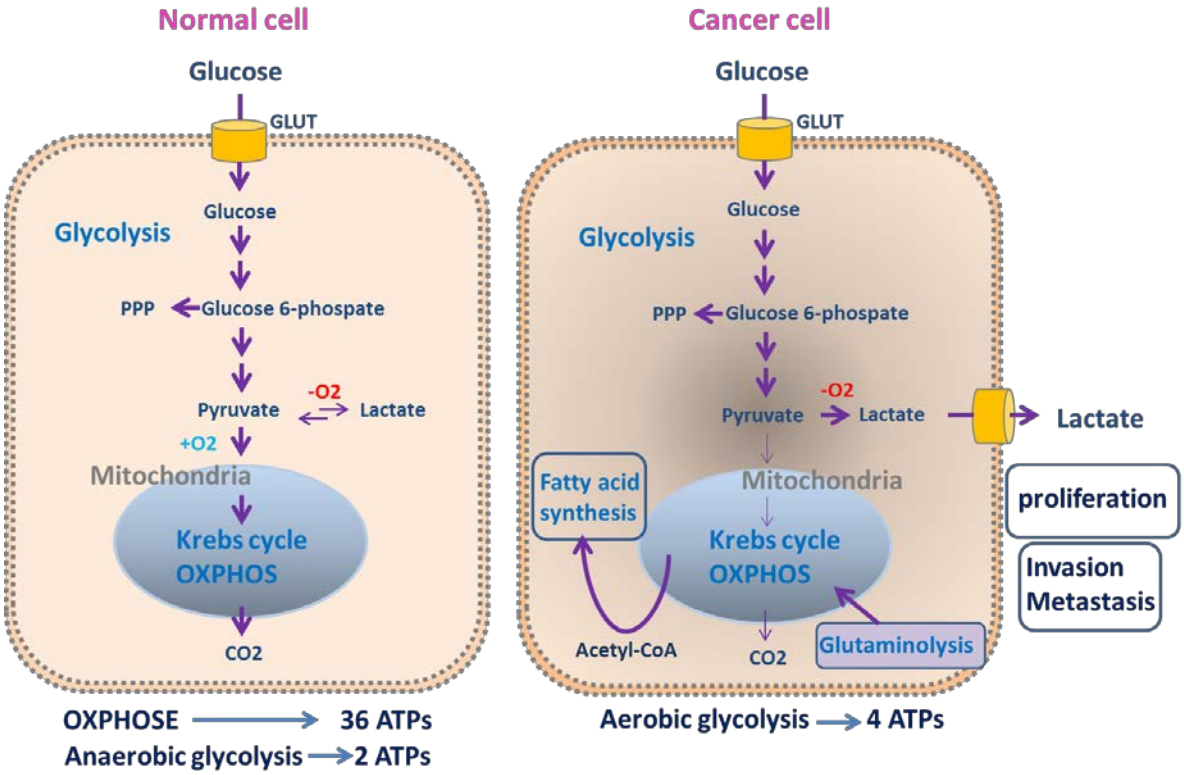

Figure 7. Difference in metabolic pathways between normal and cancer cells. Normal cells predominantly breakdown glucose to pyruvate, which is oxidized to CO_2 via the Krebs cycle and mitochondrial respiration, generating 36 ATP molecules per glucose molecule and 2 ATP molecules via anaerobic glycolysis. Many cancer cells metabolize glucose to lactate, producing 4 ATP molecules per molecule of glucose.

1.4.1 Aerobic glycolysis versus oxidative phosphorylation

Energy production in normal cells depends on mitochondrial oxidative phosphorylation, which generates 16 times more adenosine triphosphate (ATP) than glycolysis. Nonetheless, cancer cells exhibit increased uptake of glucose for aerobic glycolysis. This phenomena is known as the Warburg effect, and was first described in the 1920s by Otto Warburg[138]. The idea was initially based on a hypothesis that mitochondrial dysfunction is common for cancer cells. However, during the past decades, numerous scientific reports demonstrated that in a majority of cancers, mitochondrial respiration is intact[139-142]. Cancer includes more than 200 types of diseases, which differ by the cell of origin, localization of the primary tumors, mutations, age of patients and metastasis. Quite often, the single tumor consists of cell populations characterized by different metabolic phenotypes, depending on the stage and the microenvironment[143].

Recent studies proposed the idea that the Warburg phenotype is a not the result of decreased mitochondrial respiration, but rather of enhanced glycolysis and that inhibition of the glycolytic function may induce OXPHOS. Cancer cells have a spare respiratory capacity, which may compensate ATP production upon inhibition of aerobic glycolysis.

Functional connection between OXPHOS and glycolysis has been established in many types of human cancers. For instance, inhibition of LDHA, a member of aerobic glycolysis pathway, in cancers cells result in enhanced OXPHOS[140]. Malignant astrocytoma cells exhibit a glycolytic phenotype; however upon glucose deprivation a condition that demonstrates a progressively elevated mitochondrial respiration[144]

Recent studies highlighted a novel hypothesis - the reverse Warburg effect. The classic Warburg effect is the observation that upon hypoxic conditions enhanced glucose uptake result in increased level of lactate released to cytoplasm. The reverse Warburg effect facilitates cancer cell proliferation via high aerobic glycolysis where lactate is converted to pyruvate to support ATP production via OXPHOS in cancer associated macrophages [145,146]. These results indicate that the connection between mitochondrial OXPHOS and glycolysis are cooperative rather, than competitive.

1.4.2 Metabolic adaptation is a critical factor for cancer aggressiveness

Metabolic reprogramming is a critical step in cancerogenesis, important not only for cancer cell proliferation and biomass synthesis, but also for cell adaptation to the low oxygen level and energy demand condition, which is associated with low levels of nutrients. Indeed, one of the hallmarks of cancer is metabolic switching to aerobic glycolysis, via overexpressing of glucose transporters and increased level of glucose fermented to lactate[147]. In addition, there are more and more evidence about intact and fully functional mitochondria in cancer[148]. Recent studies demonstrate that some cancer cells exhibit metabolic plasticity, where OXPHOS and aerobic glycolysis may be reversible[149,150]. Also, different types of the same cancer can be characterized by dissimilar energy metabolism phenotypes[139,151]. Furthermore, the same cancer cells display different metabolic profile depending on the level of energetic substrates. For example, short term glucose deprivation, results in elevated mitochondrial respiratory capacity in breast cancer cells[152]. The study of melanoma cells *in vivo* highlighted that metabolic alterations in primary tumors and metastasis differ[153]. Many solid human cancers exhibit a high glycolytic rate, but even then, the majority of ATP (around 30 molecules) are produced via OXPHOS[154,155].

A deeper understanding of the metabolic plasticity of cancer cells is essential for developing new targeting anti-cancer therapy and it can meet great challenges, since the majority of novel drug targets were identified during well controlled experiments.

1.4.3 Fatty acid biosynthesis (Lipogenesis)

Fatty acids (FA) include several molecule types, which differ in lengths and level of saturation of the hydrocarbon chains. The main components of biological membranes are cholesterol, phospholipids and glycolipids. FAs form the lipophilic tails of phospholipids and glycolipids. Many type of cancer cells display an enhanced rate of *de novo* lipid synthesis, which requires high level of FAs. *De novo* FA biosynthesis, or lipogenesis, in the mature mammalian organism delimited to the several organs, such as liver, the lactating breast and adipose tissue[156]. Elevated rate of FA synthesis in cancer cells may be a consequence of energy demand due to high proliferation rate and/or decreased dietary or serum derived lipids in the tumor microenvironment. Besides, the activation of FA synthesis can be a result of genomic alterations in cancer cells.

Lipogenesis plays a very important role during cancerogenesis. FA synthesis not only provides the majority of the building blocks for cell growth and serves as a fuel for mitochondrial energy production, but is also involved in the regulation of signaling pathways related to cell proliferation and metastasis. Oxidation of FAs produces the double the amount of energy compered to glucose oxidation. Few scientific reports demonstrate that FA β-oxidation is essential for the survival of cancer cells upon impaired glycolysis[157,158]. Targeting *de novo* FA synthesis and oxidation may be used for developing of a promising anti-cancer therapeutic strategy.

1.4.4 Targeting cancer metabolism

Abnormal metabolic pathways depend highly on available nutrition and are attracted in order to target proteins involved in glucose transport (GLUT1 and GLUT4) and glycolytic enzymes (HK2, PKM2 and LDHA); in glutaminolysis (GLS); in fatty acid biosynthesis (FASN) and fatty acid oxidation (CPT1); the complexes of mitochondrial respiration and APT production. Targeting cancer metabolism is one of the most important and novel approach in the clinic, a few examples of interfering with energy production pathways in cancer is illustrated in (**Figure 8**) and discussed below.

The members of the glucose transporter protein family (GLUT) are responsible for glucose transport through the plasma membrane and are often found upregulated in many human malignancies[159,160]. Targeting two members of the family, GLUT1 and GLUT4, have been successful in *in vitro* studies. A total block of GLUT1 expression by the small molecule WZB117 reduces glucose uptake, reduces cellular proliferation and increases the sensitivity of the breast cancer cell to radiotherapy[161]. Ritonavir, a protease inhibitor, targets GLUT4 and decreases viability of glucose dependent multiple myeloma cells[162]. Treatment with 2-DG, an inhibitor of the key glycolytic enzyme HK2 results in cell cycle arrest and significant apoptosis in various human cancers[163,164]. Two more inhibitors of HK2 activity, LND and 3-BrPA have been shown to have potent potential as anti-cancers agents[165-167]. LDHA is a member of the glycolytic pathway and catalases pyruvate conversion to lactate to facilitate cancers cell's proliferation. Inhibition of LDHA has been proven as a promising strategy for hereditary leiomyomatosis and renal cell cancer[168,169].

Glutaminolysis is one of three main fuel sources for mitochondrial respiration and a precursor for lipid synthesis[170]. Also, many cancer cells exhibit "glutamine addiction" and its withdrawal results in cell death. GLS is an enzyme essential for glutamine conversion to glutamate found to be upregulated in cancers cells. The small molecule BPTES is a GLS inhibitor which reduces proliferation rate in cancer cells, for instance in glioblastoma cell lines and decreases tumor progression in renal cell cancer and breast carcinomas *in vivo*[171,172].

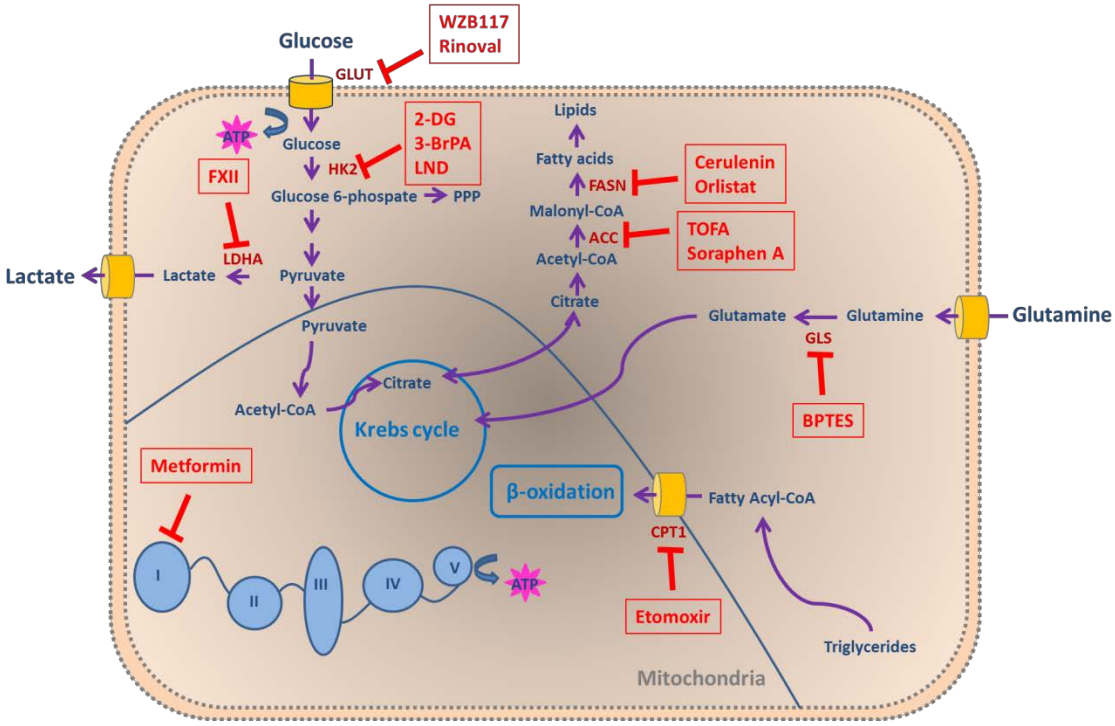

Figure 8.A schematic overview of metabolic pathways in cancer. The red boxes indicate examples of chemical compounds, which target key enzymes and interfere with the energetic activity of cancer cells.

The mitochondrial respiratory chain includes several complexes, which facilitate electron transport by oxidation-reducing reactions essential for ATP production. Targeting the complexes of the electron transport chain (ETC) leads to a slowdown in biomass growth of proliferative cells. Metformin decreases liver glucose production and is associated with increased insulin sensitivity and weight loss and, importantly it is the most commonly used anti-diabetic drug worldwide. Recently, metformin was identified as potent inhibitor of mitochondrial respiration and tumor growth suppressor via inhibition of complex I in the ETC[173,174].

Elevated levels of lipogenesis and overexpression of fatty acid synthase (FASN) are found in different types of cancers[175,176]. Lipid *de novo* synthesis is essential for the production of building blocks in rapidly proliferating cells and as a source of mitochondrial fuel for ATP generation[177]. Cerulenin is organic compound isolated from *Cephalosporium caerulens* which inhibits FASN activity with anti-tumorigenic effect[178,179]. Treatment with another FASN inhibitor orlistat, an anti-obesity drug, leads to a promising outcome of reduced proliferation and decreased angiogenesis in cancer cells[180,181]. Inhibition of Acetyl-CoA carboxylase (ACC), a key enzyme of fatty acid synthesis pathway, by the small molecule TOFA results in decreased proliferation of ovarian tumors *in vivo*[181]. Also, targeting of ACC by the antifungal polyketide soraphen A results in reduced ability to form spheres and decreased population of cancer stem cells in breast cancer cell lines[182]. Recently it has been shown that inhibition of β-oxidation results in selective growth inhibition of *MYC*-overexpressing triple-negative breast cancer cells *in vivo*[183].

Overall these results highlight that targeting cancer metabolic pathways is a promising anti-tumorigenic therapeutic strategy.

2 AIMS

The overall aim of this thesis was to target MYCN and study the biological consequences of altered MYCN expression in NB using a combination of different methodological approaches.

The specific aims of the individual papers constituted the present thesis:

I. To target MYCN-MAX interaction with small chemical molecules previously shown to inhibit MYC-MAX heterodimerization and to analyze the biological consequenses of MYCN inhibition in NB cells.

II. To investigate the impact of MYCN on energy metabolism in NB using a combination of proteomics, transcriptomics and functional data analysis, as well as targeting of a neuroblastoma xenograft model *in vivo*.

III. To explore how the *de novo* synthesis of FAs is connected to cell differentiation in NB cells.

IV. To study the impact of crosstalk between NHRs and the MYC pathway activity during NB pathogenesis and diffrentiation.

3 RESULTS AND DISCUSSION

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

The MYC transcription factors are associated with a broad range of human cancers. MYC is also an essential factor for initiation and progression of tumorigenesis[98]. The high-risk NB group is characterised by *MYCN*-amplification and very poor prognosis. Also, MYCN is known to inhibit neuronal differentiation and promote the proliferation rate of cancer cells. MYCN activity requires its interaction with MAX in the same manner as for c-MYC. Targeting MYC is a solid basis for developing a novel anti-cancer therapeutic approach. During the last decades, many small molecular weight chemical compounds have been identified as direct c-MYC inhibitors, targeting c-MYC-MAX dimerization[184,185]. In contrast, the direct inhibition of MYCN is not well studied.

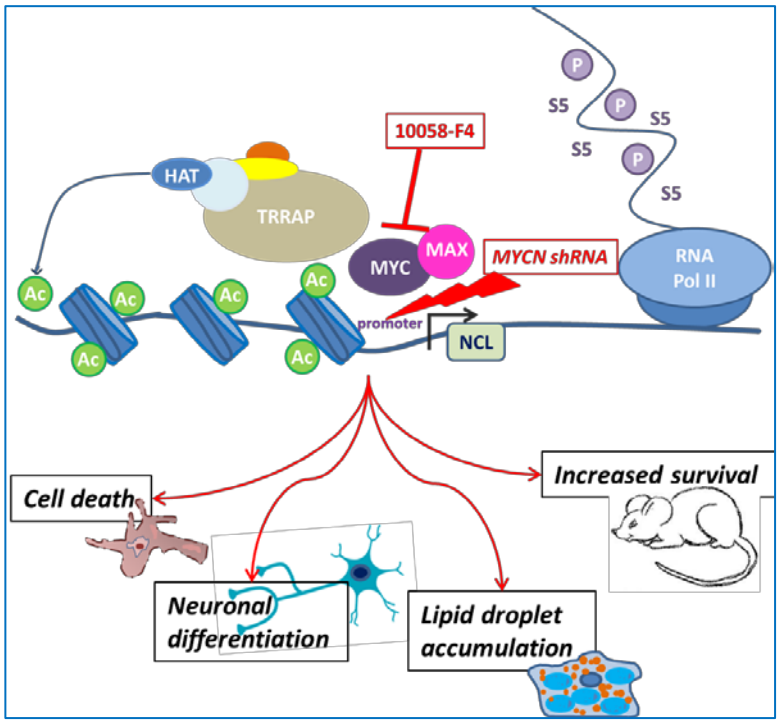

Figure 9. A graphic summary of paper I. MYCN downregulation in *MYCN*-amplified neuroblastoma cells by 10058-F4 and/or *MYCNshRNA* results in increased cell death, neuronal differentiation and lipid droplet accumulation; targeting MYCN *in vivo* prolongs survival.

The aim of **paper I** was to target the MYCN-MAX complex in *MYCN*-amplified neuroblastoma cells using a small c-MYC binding molecule 10058-F4 *in vivo* and *in vitro*. **Paper I** contains several original discoveries summarized in **Figure 9**. We found that the c-MYC inhibitor 10058-F4 is also potent against MYCN. This molecule induced cell death and reduced cell growth and migration in MNA NB cells. Furthermore, 10058-F4 treatment of transgenic *Th-MYCN* mice, which spontaneously develop NB, significantly prolonged survival. MYCN is also a known suppressor of cellular differentiation. We showed that 10058-F4 treatment is associated with neuronal differentiation, upregulation of the NGF receptor TrkA and that NGF treatment potentiates the 10058-F4-induced differentiation in MNA NB cells. Importantly, we found that chemical targeting of the MYCN-MAX complex or genetically-induced MYCN downregulation using *shMYCN* resulted in accumulation of

lipid droplets in MNA NB cells, suggesting that MYCN is an important regulator of metabolism.

To further analyze the biological consequences of 10058-F4 treatment in *MYCN*-amplified NB, we applied a quantitative proteomics approach using a SK-N-BE(2) NB cell line treated with 10058-F4. In parallel, we downregulated the expression of *MYCN* using short hairpin RNA (shRNA) and compared the proteome profiles after *MYCN* knock-down and after 10058-F4 treatment. By mass spectrometry based proteomics we demonstrated that MYCN as well as c-MYC are the top two transcription factors among the predicted targets of 10058-F4. Pathway analysis based on the identified proteins demonstrated that the biological effect of 10058-F4 treatment highly correlates with the outcome of genetic downregulation of MYCN using shRNA. According to Ingenuity prediction analysis, OXPHOS and β-oxidation were the most significantly affected canonical pathways. The detailed pathway analysis of the generated protein profile highlighted the possible link between MYCN-regulated metabolic processes, mitochondrial dysfunction and aggressiveness of NB.

Importantly, during our experiment with 10058-F4 and MNA NB cells we made an unexpected discovery, we found that cells accumulated intracellular lipid droplets. Using several different approaches of MYCN targeting, such as the small molecule JQ1 as well as *MYCN* specific *shRNA*, and several stable transduced cell lines we demonstrated that the lipid accumulation is a direct consequence of MYCN inhibition. The analysis of tumor tissue from 10058-F4 treated animals showed increased lipid accumulation also *in vivo*. Our data suggested that only inhibition of abnormal MYC expression results in lipid accumulation, since targeting of physiological MYC level in primary fibroblasts did not show this effect.

Furthermore, inhibitors against the major metabolic pathways showed that only targeting of OXPHOS and β-oxidation, but not of glycolysis-associated enzymes or fatty acid synthase, resulted in accumulation of lipid droplets. The targeting of ETC complexes and β-oxidation by specific inhibitors resulted in lipid droplet accumulation in NB cells, which supported our hypothesis that lipid accumulation is the result of decreased lipid oxidation. This idea also has been indirectly supported by our results demonstrating that inhibition of ACC, an enzyme involved in *de novo* FA synthesis, prevented lipid accumulation initiated by 10058-F4. However this result could be explained by promoted β-oxidation via increased levels of malonyl-CoA. In contrast, targeting of the fatty acid synthesis pathway via the inhibition of FASN by cerulenin did not prevent lipid droplet formation. Together our data suggests that sustained lipid synthesis and decreased proliferation rate are not major sources of intracellular lipid accumulation.

To estimate the impact of metabolic alterations on patient survival, we analyzed an mRNA data set generated from 251 NB patients. Importantly, several genes encoding the metabolic proteins downregulated by 10058-F4 correlated with increased survival in NB patients.

Our study demonstrates that the c-MYC inhibitor 10058-F4 in addition targets the MYCN-MAX dimer and induces MYCN downregulation. Cell cycle arrest, increased apoptosis and differentiation are other outcomes of 10058-F4-induced MYCN inhibition *in vitro*. Furthermore, 10058-F4 treatment prolonged the survival of transgenic *Th-MYCN* mice and delayed tumor growth in a NB xenograft mouse model.

Our findings reported in **paper I** demonstrate that direct targeting of MYCN activity is a solid basis for developing of novel promising therapeutic approaches. Also for the first time we show a strong connection between *MYCN*-amplification and altered mitochondrial respiration, as well as lipid metabolism.

3.2 PAPER II. FATTY ACID-DEPENDENT OXIDATIVE PHOSPHORYLATION IS THE MAJOR SOURCE OF ENERGY PRODUCTION IN *MYCN*-AMPLIFIED NEUROBLASTOMA CELLS

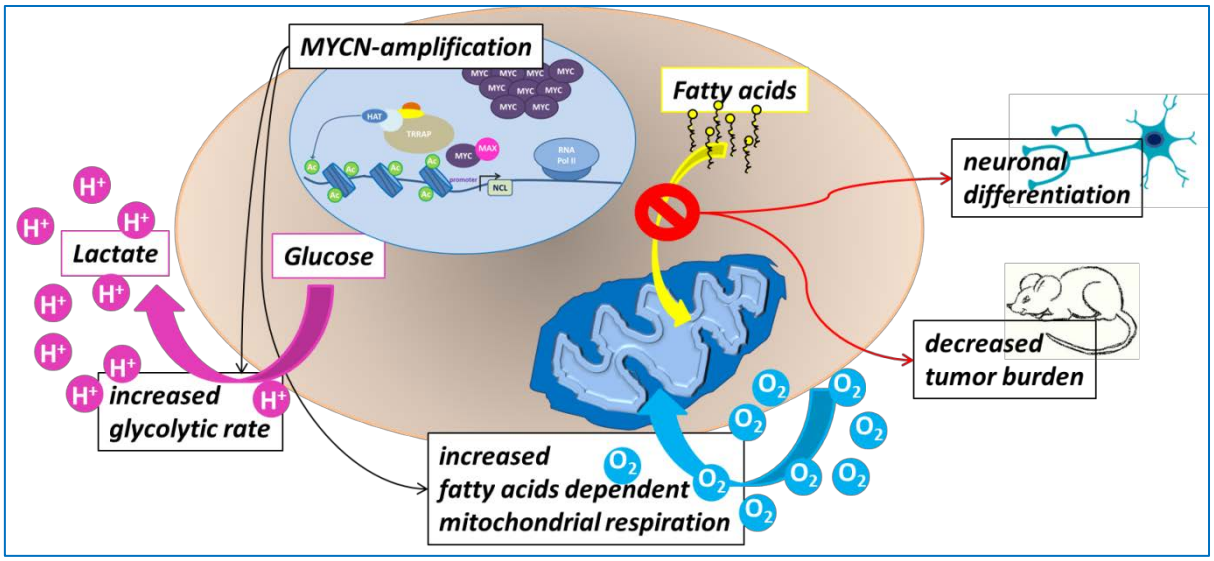

Figure 10. A graphic abstract of paper II. MNA NB cells characterised by enhanced aerobic glycolysis and fatty acid dependent OXPHOS. Inhibition of β-oxidation potentiates neuronal differentiation in NB *in vitro* and reduces tumour burden *in vivo.*

In **paper I** our findings indicated a strong connection between MYCN and metabolic alterations in MNA NB cells. To expand on these previous discoveries, we used a stably transduced MNA NB cell line SK-N-BE(2), (BE(2)*shMYCN*), where MYCN expression was downregulated by doxycycline treatment for 24 and 48 hours. Next we applied a mass spectrometry based high-resolution quantitative proteomics approach, which allowed us to identify more than six thousand proteins[186,187]. We compared the quantified protein profile generated from MNA NB cells with a gene expression data set of 649patients. The analysis of protein and gene expression highlighted changes in key enzymes involved in glycolysis (e.g. hexokinase 2, HK2; aldolase C, ALDOC, LDHA); β-oxidation (e.g. hydroxyacyl CoA dehydrogenase, HADH) in *MYCN*-amplified NB. We found significantly higher levels in *MCYN*-amplified cell lines, compared to NMNA cells, of components of the Krebs cycle (e.g. isocitrate dehydrogenase, IDH2, citrate synthase, CS, oxoglutarate dehydrogenase-like, OGDHL), which are essential for cancer progression and are associated with poor patient outcome in *MYCN*-amplified NB.

Gene set enrichment analysis of the protein profile of BE(2)*shMYCN* cells showed that a large number of proteins which were overexpressed in MNA NB cells are associated with mitochondrial respiration (e.g. NDUFS1, SDHB, COX5B, ATP5F2) and mitochondrial translation processes (e.g. mitochondrial ribosomal protein S28, MRPS28, mitochondrial ribosomal protein S27, MRPS27, glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1, QRSL1). Importantly, high expression of these proteins and genes correlated with poor prognosis in NB patients. MYCN has been shown to enhance the metabolic activity of mitochondria[188]. We demonstrated that the proteins involved in the mitochondrial biogenesis (e. g. mitochondrial fission process 1, MTFP1 and including optic atrophy 1, OPA1), which correlated with poor prognosis in NB patients, were upregulated in MNA NB cells.

To functionally access the metabolic alteration indicated by data analysis we applied Agilent Seahorse XF technology, which provide the information about main energy parameters and the metabolic phenotype of the cells. Importantly, the metabolic profiling performed using an extracellular flux analyzer showed that MNA NB cells display both enhanced glycolytic and respiratory capacity compared to NMNA NB cells. Also, in response to high energy demand conditions, such as inhibition of mitochondrial ATP production and/or uncoupling of mitochondrial respiration, MNA cells significantly increased the glycolytic rate and the mitochondrial capacity. Thus, our functional data demonstrates that *MYCN*-amplified NB cells are characterized by a high energetic metabolic phenotype.

In addition, we found that MYCN stimulates exogenous fatty acid oxidation (FAO) in NB cells, an important metabolic pathway in many types of cancer cells[189,190]. Based on this observation and our data analysis we hypothesized that OXPHOS is the main source of fuel in high energetic NB cells. There are three main fuels of mitochondrial respiration: glucose, glutamine and fatty acids. We measured the oxygen consumption rate (OCR) in BE(2)*shMYCN* cells in the presence and absence of specific metabolic inhibitors. UK5099, which blocks glucose oxidation, via inhibition of mitochondrial pyruvate carrier (MPC), etomoxir, which inhibits β-oxidation of FA via targeting of CPT1, and BPTES which inhibits CSL1 and suppresses glutaminolysis. The drop in OCR in live cells upon acute etomoxir treatment suggests that 75 % of mitochondrial respiratory capacity or OXPHOS is fatty acid dependent in BE(2)*shMYCN* cells.

We next analyzed the level of the key enzyme of β-oxidation, *CPT1* in a neuroblastoma patient data set and found that high expression of *CPT1* does not depend of *MYCN* expression level. However, high expression of *CPT1* correlates with reduced survival of NB patients with high *MYCN* level specifically. Additionally, the obtained data shows that CPT1 inhibition decreases survival of MNA NB cells, but has non-significant impact on the viability of NMNA NB cells. Furthermore, etomoxir treatment potentiated neural differentiation in MNA NB cells and reduced tumor burden *in vivo*.

During cancerogenesis, cells reprogram their metabolism in order to sustain a high proliferation rate. However, very little is known about the metabolic phenotype of MNA NB. Understanding of MYCN regulated metabolic processes in NB could provide essential information for development of novel therapeutic strategies.

Taken together, our findings (**Fig. 10**) provide novel information about MYCN-mediated high-energetic metabolic phenotype in NB, which can be a possible mechanism of cancer cell survival during NB pathogenesis. We further highlight the crucial role of FAO in aggressiveness of MNA NB cells. For the first time, we showed that MNA NB cells display fatty acid dependent OXPHOS and that inhibition of fatty acid β-oxidation specifically targets MNA NB cells. Most importantly, our *in vivo* study demonstrated reduced tumor burden in a MNA NB xenograft mouse model upon etomoxir treatment.

In summary, our data suggest that inhibition of fatty acid oxidation in *MYCN*-amplified neuroblastoma may be used for the improvement of therapeutic strategies in one the most aggressive childhood cancers.

3.3 PAPER III. INDUCTION OF NEURAL DIFFERENTIATION IN NEUROBLASTOMA UPON INHIBITION OF *DE NOVO* FATTY ACID SYNTHESIS

In **paper I** we demonstrated that lipid metabolism could be linked to the induction of differentiation of NB cells upon MYCN inhibition. The aim of **paper III** was to study how the *de novo* synthesis of fatty acids is connected to cell differentiation in NB cells. We found that this inhibition resulted in several biological effects, such as downregulation of MYCN, mitochondrial dysfunction and neural differentiation in neuroblastoma cells.

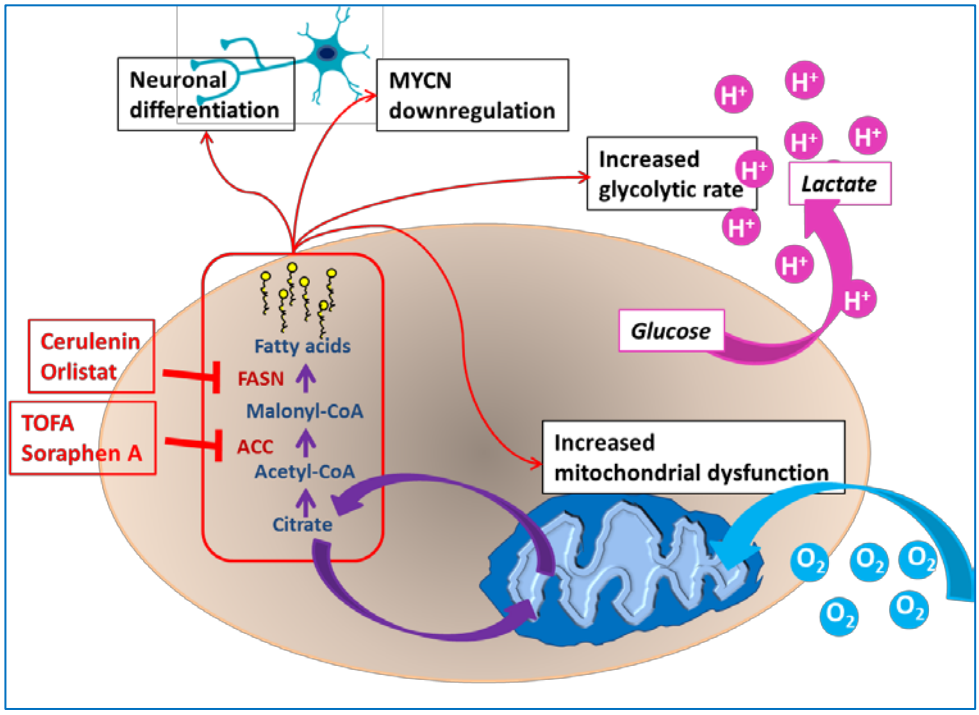

Figure 11. Graphic abstract of paper III. Targeting of *de novo* synthesis of fatty acids in neuroblastoma cells initiates neural differentiation, leads to MYCN downregulation, increased mitochondrial dysfunction and glycolytic capacity of NB cells.

Reduced overall survival of neuroblastoma patients correlated with high expression of the two main enzymes involved in the fatty acid synthesis pathway, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). Therefore, we used four different chemical compound targeting fatty acid synthesis; cerulenin and orlistat inhibiting FASN and TOFA and soraphen A inhibiting ACC. All four small molecules reduced neuroblastoma cell viability. In addition, treatment during 7 days resulted in MYCN downregulation and induction of neurite outgrowth in MNA NB cells. Furthermore, we showed that targeting fatty acid synthesis initiated neurite outgrowth not only in several MNA NB cell lines, but also in non-*MYCN*-amplified NB cells. Inhibition of fatty acid synthesis leads to deprivation of intracellular fatty acids. To investigate whether the observed differentiation was a consequence of reduced levels of intracellular fatty acids, we treated SK-N-BE(2) cells with TOFA or cerulenin in the presence of BSA-coupled palmitate or BSA alone as a control. We found that exogenously added palmitate or oleate did not prevent compound-induced differentiation or MYCN inhibition in SK-N-BE(2) cells. Furthermore, our results indicated that BSA-coupled palmitate or oleate were able to induce neurite outgrowth by itself. Next,

we demonstrated that 7 days lack of either glucose or glutamine in the cell culture medium did not result in differentiation in SK-N-BE(2) cells and we thus concluded that not all nutritional stress conditions induce neural differentiation.

Previously we demonstrated that MYCN inhibition leads to enhanced mitochondrial dysfunction in MNA NB cells. To investigate the functional consequences we performed extracellular flux assays to study the effect of targeting of fatty acid synthesis and found that inhibition of ACC reduced mitochondrial respiration in MNA NB cells. Besides, our results indicated increased glycolytic capacity in NB cells upon inhibition of *de novo* synthesis of FA.

Anti-cancer therapy is associated with severe side effects, with are consequences of the high toxicity for non-transformed cells. Thus therapeutic approaches based on induction of cellular differentiation are very promising. After embryonic development, only adipose tissue, liver and the lactating mammary gland display lipogenesis, this may reduce the negative impact of FA synthesis on targeting of non-cancer cells. Lipogenesis is a well-established hallmark associated with many different type of human tumors[7]. The analysis of a transcriptome data set demonstrated a correlation between high expression of two key enzymes of *de novo* FA synthesis, ACC and FASN, and reduced overall survival in NB patients. In **paper III** we showed increased neuronal like outgrowth, as well as an elevated expression of differentiation markers upon inhibition of *de novo* synthesis of fatty acids in both MNA and NMNA NB cells. Also, our results indicate that MYCN and c-MYC expressions were reduced in several NB cell lines in response to inhibition of lipogenesis. It is very well known that high MYCN expression is associated with a non-differentiated tumour phenotype, and that MYCN inhibition promotes neurite-outgrowth in NB. In contrast, according to our data c-MYC inhibition in NB is not always followed by neuronal differentiation. The reason for these differences is not known.

In **paper II** we showed that FA is the main source of energy in NB cells. Therefore, increased neuronal differentiation may be the result of nutritional stress. **In paper III** we therefore studied the effect of glucose and/or glutamine withdrawal in NB cells and demonstrated that their deprivation did not initiate morphological changes in NB cells. Furthermore, the effect of FA synthesis inhibition was neither reversed by exogenously added palmitate nor by oleate. The findings reported in **paper III** indicate that inhibition of fatty acid synthesis in NB cells results in neuronal differentiation, but this effect was not the consequence of fatty acid withdrawal *per se*.

Previously we reported that MYCN downregulation in NB cells results not only in neuronal differentiation, but also in enhanced mitochondrial dysfunction. Moreover, the data presented in **paper II** highlighted that FAs are the main the source for mitochondrial energy production. In **paper III** we then demonstrated that neural differentiation of NB cells is connected not only with MYCN downregulation, but also with mitochondrial biogenesis (**Figure 11**). The functional assays supported our idea showing that targeting *de novo* synthesis of fatty acids *in vitro* reduces the mitochondrial respiratory capacity in NB.

Together, our study shows that targeting of the fatty acid synthesis pathway in NB may be the basis for development of novel therapeutic approaches for NB treatment.

3.4 PAPER IV. REGULATION OF NUCLEAR HORMONE RECEPTORS BY MYCN DRIVEN MIRNAS IMPACTS NEURAL DIFFERENTIATION AND SURVIVAL IN NEUROBLASTOMA PATIENTS

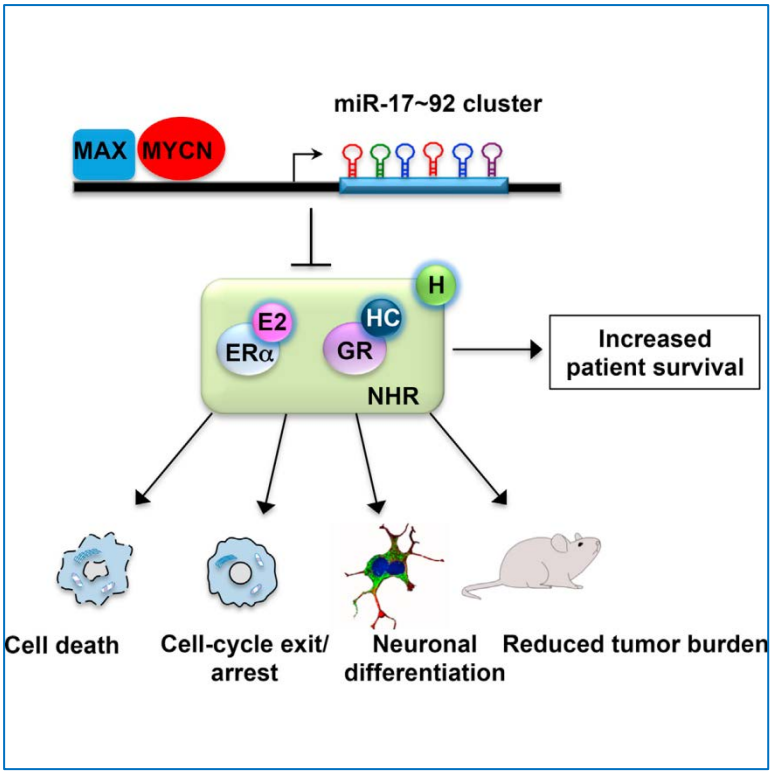

Figure 12. Graphical abstract of paper IV. MYCN-regulated miR-17~92 cluster suppresses neural differentiation via targeting of members of nuclear hormone receptors family (NHR). Combination of MYCN inhibition and glucocorticoid activation promote neural differentiation and reduce tumor burden. (*Reprinted from Ribeiro et al, Cell reports 2016 with a kind permission from Elsevier*).

We previously demonstrated that NB differentiation is inhibited by the MYCN-regulated miR-17~92 cluster through downregulation of estrogen receptor alpha[14]. Given that the NHR family has been associated with differentiation in cells of neural origin, we hypothesized that several other NHRs also could play a role in the development and/or progression of neuroblastoma. Thus, the aim of **Paper IV** was to investigate the regulation of NHRs in MYCN-driven NB. We first applied an *in silico* prediction analysis to identify miR-17~92 associated binding sites in the 3'-UTRs of all NHR family members. We found that miR-17~92 target sites are disproportionally enriched in the 3'-UTRs of the NHR superfamily. Furthermore, our analysis indicated that high expression of several NHRs correlated with low levels of MYCN and with increased survival in NB patients. Next, using RT-PCR and TaqMan based arrays we evaluated the expression level of NHR family members in a NB cell line stably expressing miR-17~92 cluster. We found that the genes encoding the glucocorticoid receptor (*GR*), peroxisome proliferator-activated receptor beta delta (*PPARD*), liver X receptor B (*LXRB*), and nuclear receptor related 1 (*NURR1*) were downregulated. Using luciferase reporter constructs containing the wildtype or mutated 3'-UTRs from the NHR-encoding genes; we verified that these are the direct targets of miR-17~92. We furthermore found that the nuclear receptor coactivator 1 (*NCOA1*) is a direct target of miR-18a, and thus that further NHRs may be targeted via NCOA1.

The analysis of one mRNA data set from 649 NB patients highlighted the association of high expression of NHRs with increased overall survival in NB patients. Besides, we demonstrated the reverse correlation between the genes encoding the NHRs and *MYCN* levels.

Since GR was the most downregulated member of the NHR family in MNA NB patients we focused on this protein. Analysis of cell lines where the MYCN or miR-17~92 expression could be genetically modulated revealed that GR expression in NB cells is suppressed by MYCN as well as by miR-17~92. Furthermore, we demonstrated that activation of GR signaling by its ligand dexamethasone (DEX) enhances neural differentiation and expression of differentiation markers in NB cells.

We next investigated the significance of MYCN-mediated GR regulation *in vivo*. *Th-MYCN* mice were treated with the small molecule MYC inhibitor 10058-F4 during 6 days. Inhibition of MYCN led to a two-fold increase in the number of GR positive cells compared to untreated tumors.

To study the functional role of GR in neuroblastoma, we treated SK-N-BE(2) MNA NB cells for 6 days with DEX, 10058-F4 and the combination of the MYC inhibitor and DEX (10058-F4+DEX), where ligand was added after 72 hours of 10058-F4 pretreatment. Incubation with 10058-F4 resulted in decreased MYCN expression and increased GR level in SK-N-BE(2) cells. We did not detect changes in MYCN expression upon DEX treatment alone, however the combination treatment potentiated the decrease in MYCN level initiated by 10058-F4. Also, DEX or combined 10058-F4+DEX treatment suppressed GR expression. Furthermore, 10058-F4 alone or in combination with DEX promoted expression of the differentiation marker TrkA. Moreover, combination treatment increased the level of the apoptosis marker cleaved poly (ADP-ribose) polymerase (PARP) and decreased the expression of proliferating cell nuclear anti-gene (PCNA).

To study the impact of GR on cellular differentiation in NB in another model, we applied our differentiation scheme to tumor-spheres cells derived from *Th-MYCN* mice. We obtained similar results as in SK-N-BE(2) cells. 10058-F4 treatment downregulated MYCN expression and increased GR levels while incubation of *Th-MYCN* cells with DEX resulted in MYCN decrease and combination treatment potentiated this effect. Furthermore, all treatments involved in our approach resulted in reduced expression of miR-17, miR-18a and miR-19a. We generated stably expressing GR MNA NB cell lines for further understanding of GR functions in NB pathogenesis. Activation of GR in these cells resulted in decreased MYCN levels as well as elevated expression of the TH neural differentiation marker.

Next, we employed our treatment scheme to *in vivo* experiments using a NB xenograft model using the highly aggressive SK-N-BE(2) cells. Indeed, MYCN inhibition followed by activation of GR signaling resulted in a significant reduction in tumor burden.

The data reported in **paper IV** highlighted the importance of the NHR family as targets of the miR-17~92 cluster for neuroblastoma pathogenesis. Our results demonstrate that increased expression of GR not only promoted neural differentiation, but also decreased MYCN expression in MNA NB cells. Furthermore, GR signaling resulted in upregulation of canonical differentiation markers in neuroblastoma.

Taken together our findings summarized in **Figure 12** reveal MYCN-mediated deregulation of NHRs through the miR-17~92 cluster as a critical factor in NB tumorigenesis. Importantly, these recent findings can contribute to the development of novel therapies for *MYCN*-amplified NB.

4 CONCLUSION AND OUTLOOK

MYCN-amplification is associated with an aggressive type of childhood NB and with poor outcome for patients. We are aiming to improve the biological understanding of this disease, which today is the major cause of death from cancer in children. Our recent results included in this thesis show that it is feasible and possible to directly target the MYCN-MAX dimer. In addition, these findings support the idea of developing an anti-cancer therapy based on targeting of MYCN-regulated metabolic processes essential for cancer cell survival and proliferation in *MYCN*-amplified NB cells.

Targeting of MYCN and understanding of the biological outcome of MYCN downregulation in NB is a very challenging goal in cancer research. We reported in **paper I** that a small chemical molecule, 10058-F4, previously identified as a c-MYC inhibitor also successfully targets MYCN-MAX dimerization, which in turn results in MYCN downregulation, increased neuronal differentiation *in vitro* and prolonged survival *in vivo*. We further demonstrated that is possible to achieve MYCN inhibition by treatment with a low-molecular weight compound. Besides, we detected intracellular lipid accumulation as a direct consequence of MYCN downregulation accompanied by mitochondrial dysfunction in *MYCN*-amplified NB cells. Together, our findings demonstrated the importance of MYCN-mediated metabolic processes in NB.

Paper II and **paper III** are logical continuations of **paper I.** Indeed, our data demonstrates that the aggressiveness of *MYCN*-amplified NB may be driven by a high-energetic metabolic phenotype in this type of cancer. In **paper II** we reported that MYCN not only maintains a high glycolysis rate, but also enhances OXPHOS in MNA NB. Using analysis of transcriptome data, we demonstrated that high expression of key metabolic enzymes correlated with *MYCN*-amplification in NB patients. Furthermore, we showed intact mitochondria and energy production via OXPHOS is critical for MNA NB survival. Furthermore, in **paper II** we established that mitochondrial respiration is highly dependent on fatty acids in MNA NB cells. Importantly, our data suggests that targeting of the main source of mitochondrial respiration - fatty acid oxidation - results in enhanced neuronal differentiation *in vitro* and reduced tumor burden *in vivo*.

Our data clearly demonstrates that MYCN-mediated metabolic alterations are one of the most prominent features of MNA NB cells. In **paper III** we focused on the investigating the possible connection between the metabolic and differentiation processes in these cells. Our results showed that inhibition of *de novo* synthesis of fatty acids resulted in mitochondrial dysfunction and in neuronal outgrowth in NB cells. Moreover, we observed MYCN inhibition upon reduced lipogenesis.

In **paper IV** we demonstrated that MYCN suppresses neuronal differentiation in NB via upregulation of miR-17~92 cluster, which targets NHRs. Specifically, MYCN inhibition results in increased levels of GR and promoted expression of differentiation markers in NB. Inhibition of MYCN was followed by increased GR expression and reduced tumor burden *in vivo*.

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

The detailed understanding of metabolic processes and the targeting of metabolic enzymes to regulate specific bioenergetic functions in cancer cells is critical for successful clinical outcome. Several scientific reports suggest that cancer aggressiveness may be associated with metabolic reprograming and/or metabolic adaptation. Our data identified MYCN as a main regulator of MNA NB metabolism, which enhances energetic parameters in NB. Moreover we demonstrated an importance of mitochondrial biogenesis in MNA NB. Since normal cells relay on OXPHOS energy production, direct targeting of mitochondria may be very toxic. Using selective inhibitors for the main fuel sources in cancer cells as well as MYCN inhibition which result in decreased metabolic activity and decreased cell proliferation may therefore be more specific for cancer cells.

Our data suggests that the balance between the key enzymes involved in FA synthesis has a significant impact on NB cells. Further investigation of the connection between the induced metabolic alterations and neuronal differentiation in NB is needed.

Recently, it was shown that the miR-17~92 cluster is a global regulator of cancer metabolism. In our previous publication, we demonstrated that this cluster is involved in regulation of neuronal differentiation in MNA NB. However, the role of the cluster in NB metabolism remains unclear and needs further studies.

Increased knowledge about the impact of MYCN on metabolic alterations, the detoxification system and mitochondrial processes will provide not only information about the significance of MYCN for tumor development, but also expand the knowledge about cancer cell resistance and survival. Together this will provide new knowledge that can form the basis for novel therapeutic approaches not only for *MYCN*-amplified NB but also for other tumors as MYC is a global oncogene involved in a majority of human cancer.

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