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IMPACT OF TARGETING MYC IN METABOLIC REPROGRAMMING AND DIFFERENTIATION OF CANCER

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By

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To my beloved family and friends for always believing in me ♥, and to those who are suffering cancer, especially my aunt Pepa, who passed away from this terrible disease.

A mi querida familia y amigos por creer siempre en mi ♥, y a todos los que sufren de cáncer, especialmente a mi tía Pepa, quien murió por esta terrible enfermedad.

لعائلتي و أصدقائي العزيزين لدعمهم الدائم. و لمرضى السرطان خصوصا لعمتي بيبا التي توفت بسبب هذا المرض.

ABSTRACT

Alterations in several metabolic pathways due to increased energy and biomass demand, as consequence of the uncontrolled proliferation of cancer cells, is known as metabolic reprogramming. Mutations in tumor suppressor genes and oncogenes that initiate cancer development, are responsible directly and indirectly of the changes in major cellular energy production processes, including glycolysis, glutaminolysis, and lipid metabolism.

Neuroblastoma (NB) is a solid tumor that develops extracranially in the sympathetic nervous system, and the most diagnosed cancer during the first year of life. Among several genetic alterations, *MYCN*-amplification occurs in approximately 25% of all cases, associated with poor survival rate. Although the MYCN protein plays a crucial role in NB progression, no inhibitors have been approved for clinical use, as targeting MYCN has proven to be challenging. Thus, other indirect strategies, such as targeting downstream processes controlled by MYCN including metabolism and differentiation, represent alternatives to overcome the drawbacks of directly targeting this oncoprotein.

In clear cell renal cell carcinoma (ccRCC), loss of the *von Hippel-Lindau* (*VHL*) gene provokes constitutive activation of hypoxia inducible factors (HIFs). Due to disruptions in a myriad of metabolic pathways, and in part, as consequence of the continuous stabilization of HIFs, ccRCC is considered a metabolic disease. Moreover, although *MYC* amplification is found only in 5-10 % of the cases, increased MYC signaling has been associated with development of aggressive forms of ccRCC.

In **paper I** we investigated the metabolic changes induced by *MYCN* amplification in NB. By combining proteomics, transcriptome analysis and functional metabolic assays, we demonstrated that MYCN induced changes in several metabolic enzymes, increasing glycolysis and oxidative phosphorylation. We also found that fatty acids were the preferred mitochondrial fuel for energy production in *MYCN*-amplified cells. Moreover, data from tracing experiments with ^{13}C-labeled glucose or glutamine indicated that *MYCN*-amplified NB cells synthetized glutamine *de novo*. Furthermore, targeting fatty acid oxidation resulted in reduction of viability in NB cells with *MYCN*-amplification *in vitro* and in reduction of tumor burden *in vivo*.

Since we found that fatty acid oxidation was relevant for *MYCN*-amplified NB, we further studied the effects of inhibiting *de novo* fatty acids synthesis in **paper II**. Using five different inhibitors targeting two consecutive enzymes in the process, we described that inhibition of the synthesis of fatty acids resulted in striking neuronal differentiation associated with activation of ERK signaling, and reduction of MYCN and MYC levels. Moreover, lipid composition as well as mitochondrial function and morphology of NB cells was altered. In addition, fatty acid synthesis inhibition led to reduced tumor formation and increased differentiation markers in several NB xenograft models. Together, the results in **paper I** and **II** suggested that targeting lipid metabolism could be a potential therapeutic approach for NB patients.

In **paper III** we further analyzed the potential differentiation of NB cells induced by activation of nuclear hormone receptors (NHRs). Our data showed that the simultaneous activation of glucocorticoid receptor (GR), estrogen receptor α (ERα) and retinoic acid receptor α (RARα) potentiated neurite outgrowth, induced changes in the glycolytic and mitochondrial functions, accompanied with lipid droplet accumulation, and reduced proliferation *in vitro* as well as tumor burden *in viv*o. In addition, single cell nuclei analysis revealed a sequential expression of the three NHRs during adrenal gland development. Notably, *in silico* analysis of patient cohorts demonstrated that high expression of these NHRs were correlated with better overall survival. Thus, combination therapy with the concurrent activation of GR, ERα and RARα represents a promising strategy to induce differentiation in NB patients.

Paper IV describes the mechanism behind lipid droplet (LD) accumulation induced by MYC inhibition during hypoxia in clear cell renal cell carcinoma (ccRCC). We found that HIF expression together with MYC inhibition resulted in LD deposition. Our results showed that due to HIF stabilization, glutamine-derived carbons were directed for synthesis of fatty acids, further accumulating in LDs. Importantly, we identified that the *hypoxia inducible lipid droplet associated* (*HILPDA*) gene, was overexpressed upon HIF induction and MYC inhibition, controlling LD formation in ccRCC cells. Hence, our study characterizes the molecular mechanism of LD accumulation in relation to hypoxia and MYC signaling, providing new understanding of metabolic adaption in ccRCC.

Altogether, the data compiled in this thesis describes the important role of the MYC family of proteins in differentiation and metabolism of NB and in the metabolic reprogramming of ccRCC providing new knowledge and potential targets for development of novel therapeutic approaches.

POPULAR SCIENCE SUMMARY OF THE THESIS

Cancer is a disease affecting millions of people worldwide. It can originate almost from any cell in our body that suffers changes in its genes, called mutations, increasing the growth rate. If these tumor cells are contained in the original place where they developed, they are usually relatively easy to remove with chemotherapy, radiotherapy, and/or local surgery. However, these cells might acquire the ability to move to nearby tissues or more distant organs causing metastasis, which is more challenging to cure.

It is usually believed that cancer appears in older adults, but infants, children, and young adults also suffer from this disease. Neuroblastoma (NB) is a tumor of the nervous system affecting children in their first years of life. One of the genes that is often mutated and contributes to NB development is *MYCN*, and it is associated with a worse survival. But why does this gene promote cancer formation? *MYCN* is a member of the *MYC* family of genes that are responsible for many processes in the cells supporting growth and survival. Because it is amplified, there will be many copies of the gene in those cancer cells. The more copies of *MYCN*, the more activation of those processes. For example, MYCN reduces the differentiation of NB cells into neurons and changes the metabolism of cells to provide them with more energy to grow without limit.

Another type of cancer with mutations in genes that change metabolism is clear cell renal cell carcinoma (ccRCC), the most common tumor of the kidney. This cancer mainly develops in adults above 60 years of age. Apart from an increase in MYC activity, these tumors also have increase in the proteins HIF1-α and/or HIF2-α, usually active when our cells lack oxygen. Because the cells detect the lack of oxygen, their metabolism change to quicker produce energy, contributing to their fast growth.

Scientists in the field have tried for years, without much success, to identify a drug that could reduce activation of MYC in tumors. Thus, another way to avoid stimulation of MYC is to analyze how MYC controls different cellular processes with the goal to inhibit them. This thesis studies how MYC changes metabolism and differentiation of cancer cells. In **paper I** we discovered that MYC affects metabolism of sugars (glucose), fats (fatty acids) and the amino acid glutamine. We identified that NB cells prefer fatty acids to obtain energy and we described how stopping this process with a chemical compound reduces tumor growth. **Paper II** was focused on how fatty acid production supports NB survival. By treating the cells with five different chemical compounds, preventing formation of fatty acids, we found that not only NB growth was impaired, but that fatty acid synthesis was associated to differentiation of NB cells to neurons. In **Paper III** we further studied how the differentiation process can be potentiated in NB. To this end, we treated NB cells with common hormones found in the body, estrogen, and cortisol, and with a derivative of vitamin A. These three molecules robustly activated the conversion of cancer cells to neuronal cells. In **Paper IV** we demonstrated how MYC and HIF together regulate lipid accumulation in ccRCC tumors, contributing to cancer progression.

In conclusion, this thesis summarizes our findings describing new potential strategies based on metabolism and differentiation, for treatment of two different types of cancer, one occurring in children and the other in adults.

RESUMEN DE LA TESIS

El cáncer es una enfermedad que afecta a millones de personas en todo el mundo. Se puede originar a partir de casi cualquier célula de nuestro cuerpo que sufra cambios en los genes, llamados mutaciones, incrementando su crecimiento. Si estas células tumorales se mantienen en el sitio original donde se desarrollaron, son relativamente fáciles de eliminar mediante quimioterapia, radioterapia y/o cirugía local. Sin embargo, estas células pueden adquirir la capacidad de migrar a tejidos cercanos o a otros órganos más distantes causando metástasis, siendo en este caso más difícil de tratar.

Normalmente se piensa que el cáncer solo afecta a adultos de avanzada edad, pero los niños y los adultos jóvenes también pueden sufrir esta enfermedad. El neuroblastoma (NB) es un tumor del sistema nervioso que afecta a niños en sus primeros años de vida. Uno de los genes que está normalmente mutado y que contribuye al desarrollo del NB es *MYCN*, asociado con una peor tasa de supervivencia. Pero ¿por qué causa cáncer este gen? *MYCN* es miembro de la familia de genes *MYC*, responsables de muchos procesos en las células, ayudando a su crecimiento y supervivencia. Como esta amplificado, este gen está presente en cuantiosas copias en las células. Cuantas más copias de *MYCN*, más activación de estos procesos celulares. Por ejemplo, MYCN reduce la diferenciación de las células de NB a neuronas, y cambia el metabolismo de las células para ofrecerles más energía y así crecer un número ilimitado de veces.

Otro tipo de cáncer con mutaciones en genes que afectan al metabolismo es el carcinoma de las células renales con células claras (ccRCC), el tipo de cáncer renal más común. Este tumor se desarrolla mayormente en adultos de más de 60 años. Aparte del incremento en la actividad de MYC, estos tumores tienen también elevados los niveles de las proteínas HIF1-α y/o HIF2-α, normalmente activas cuando a nuestras células les falta oxígeno. Como las células detectan que no hay oxígeno, su metabolismo cambia para producir energía de una forma más acelerada que les permita crecer más rápidamente.

Científicos en el campo del cáncer han tratado de encontrar durante años y sin mucho éxito un compuesto que pueda reducir la activación de MYC en tumores. Por este motivo, otra manera de evitar la estimulación de MYC, es analizando como MYC controla los diferentes procesos celulares para tratar de detenerlos. En esta tesis se estudia como MYC cambia el metabolismo y la diferenciación de las células tumorales. En el **artículo I**, descubrimos que MYC afecta al metabolismo de azúcares (glucosa), grasas (ácidos grasos) y del aminoácido glutamina. Identificamos que las células de NB prefieren los ácidos grasos para obtener energía y describimos que, parando este proceso con un compuesto químico, se reduce el crecimiento de los tumores. En el **artículo II**, nos centramos en como la producción de ácidos grasos ayuda a la supervivencia de las células de NB. Tratando estas células con cinco compuestos químicos diferentes que previenen la síntesis de ácidos grasos, descubrimos que reducían el crecimiento del NB, y también que la síntesis de ácidos grasos estaba relacionada con la diferenciación de las células tumorales a células neuronales. En el **artículo III**, estudiamos más a fondo como potenciar el proceso de diferenciación a neuronas en NB. Para ello, tratamos las células tumorales con hormonas que podemos encontrar naturalmente en nuestro cuerpo, como es el

caso de estrógeno, cortisol y un derivado de la vitamina A. Estas tres moléculas activan en gran medida la conversión de células de cáncer a neuronas. En el **artículo IV** demostramos como MYC y HIF regulan juntos la acumulación de lípidos en células de ccRCC, lo cual está asociado con la progresión de este tipo de tumores.

En conclusión, esta tesis resume nuestros descubrimientos en los que describimos nuevas estrategias para tratar dos tipos de cáncer basadas en el metabolismo y la inducción de la diferenciación neuronal, uno afectando a niños y el otro a adultos.

LIST OF SCIENTIFIC PAPERS

I. Ganna Oliynyk*, María Victoria Ruiz-Pérez*, **Lourdes Sainero-Alcolado**, Johanna Dzieran, Hanna Zirath, Héctor Gallart-Ayala, Craig E. Wheelock, Henrik J. Johansson, Roland Nilsson, Janne Lehtiö and Marie Arsenian-Henriksson. *Equal contribution.
MYCN-enhanced oxidative and glycolytic metabolism reveals vulnerabilities for targeting neuroblastoma.
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II. María Victoria Ruiz-Pérez, **Lourdes Sainero-Alcolado**, Ganna Oliynyk, Isabell Matuschek, Nicola Balboni, S.J. Kumari A. Ubhayasekera, Marteinn Thor Snaebjornsson, Kamil Makowski, Kristina Aaltonen, Daniel Bexell, Dolors Serra, Roland Nilsson, Jonas Bergquist, Almut Schulze, and Marie Arsenian-Henriksson.
Inhibition of fatty acid synthesis induces differentiation and reduces tumor burden in childhood neuroblastoma.
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III. **Lourdes Sainero-Alcolado**, Muhammad Mushtaq*, Judit Liaño-Pons*, Aida Rodriguez-Garcia, Ye Yuan, Tong Liu, María Victoria Ruiz-Pérez, Susanne Schlisio, Oscar Bedoya-Reina and Marie Arsenian-Henriksson. *Equal contribution.
Expression and activation of nuclear hormone receptors result in neuronal differentiation and favorable prognosis in neuroblastoma.
Journal of Experimental and Clinical Cancer Research, 2022, 41:226.

IV. **Lourdes Sainero-Alcolado**, Elisa Garde-Lapido, María Victoria Ruiz-Pérez, Marteinn Thor Snaebjörnsson, Irene Stevens, Christine Dyrager, Vicent Pelechano, Almut Schulze, and Marie Arsenian-Henriksson.
MYC inhibition during hypoxia results in accumulation of glutamine-derived lipid droplets in clear cell renal cell carcinoma.
Manuscript, 2022.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Kilian Colas, Susanne Doloczki, Aikaterina Kesidou, **Lourdes Sainero-Alcolado**, Aida Rodriguez-Garcia, Marie Arsenian-Henriksson, and Christine Dyrager.
Photophysical characteristics of polarity-sensitive and lipid droplet-specific phenylbenzothiadiazoles
ChemPhotoChem 2021, 5:632.

II. **Lourdes Sainero-Alcolado***, Judit Liaño-Pons*, María Victoria Ruiz-Pérez, and Marie Arsenian-Henriksson. *Equal contribution.
Targeting mitochondrial metabolism for precision medicine in cancer.
Cell Death & Differentiation, 2022, 29:1304–1317.

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LIST OF ABBREVIATIONS

2-DG	2-Deoxyglucose
ACACA	Acetyl-CoA carboxylase
ACAT1/2	Acyl-CoA cholesterol O-acyltransferases
Acetyl-CoA	Acetyl-Coenzyme A
ACLY	ATP citrate lyase
ACSL	Long chain-Acyl-CoA synthetase
AD	Anno Domini
ADT	Androgen deprivation therapy
AGPAT	1-Acylglycerol-3-phosphate O-acyltransferase
AhR	Aryl hydrocarbon receptor
AIDS	Acquired immunodeficiency syndrome
ALDO	Aldolase
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
ALT	Alternative lengthening of telomeres
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APL	Acute promyelocytic leukemia
AR/*NR3C4*	Androgen receptor
AREs	Androgen response elements
ARF	ADP-ribosylation factor
ARNT	Aryl hydrocarbon nuclear translocator
ASS1	Argininosuccinate synthase 1
ATF4	Activating transcription factor 4
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid
BAK	BCL-2 homologous antagonist killer
BAP1	Breast cancer-associated protein 1
BAX	BCL-2-associated X protein

BC	Before Christ
BCL-2	B-cell lymphoma 2
BCL-X$_L$	B-cell lymphoma-extra-large protein
BET	Bromodomain and extra-terminal domain
bHLH-Zip	Basic helix-loop-helix leucine zipper
BL	Burkitt lymphoma
BMP	Bone morphogenic protein
BPTES	Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide 3
BRCA2	Breast cancer gene 2
BRD4	Bromodomain 4
BSA	Bovine serum albumin
CAD	Carbamoyl-phosphate synthetase 2
CBP	CREB-binding protein
CCNE1	Cyclin E1
ccRCC	Clear cell renal cell carcinoma
CDK1-5	Cyclin-dependent kinase 1-5
CDKN2A	Cyclin-dependent kinase inhibitor 2A
ChRE	Carbohydrate response elements
chRCC	Chromophobe renal cell carcinoma
ChREBP	Carbohydrate-responsive element-binding protein
CKIs	Cyclin-dependent kinase inhibitor
CNS	Central nervous system
CO$_2$	Carbon dioxide
CPT1/2	Carnitine/palmitoyl-transferase 1/2
CS	Citrate synthase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CUX1	Cut-like homeobox 1
DAG	Diacylglyceride
DEX	Dexamethasone
DGATs	Diacylglycerol acyltransferase
DHODH	Dihydroorotate dehydrogenase

DHT	Dihydrotestosterone
DLG2	Discs large MAGUK scaffold protein 2
DNA	Deoxyribonucleic acid
DOX	Doxycycline
E2	17-β-estradiol
EAAs	Essential amino acids
E-Box	Enhancer box
ECAR	Extracellular acidification rate
EMA	European medicines agency
EMT	Epithelial to mesenchymal transition
ENOL	Enolase
EPO	Erythropoietin
ER	Endoplasmic reticulum
EREs	Estrogen response elements
ERK	Extracellular receptor kinase
ERα/*ESR1*	Estrogen receptor α
ERβ/*ESR2*	Estrogen receptor β
ETC	Electron transport chain
EZH2	Enhancer of zeste 2
$FADH_2$	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FATP2	Fatty acid transporter protein 2
FDA	Food and drug administration
FGF1/2	Fibroblast growth factor 1/2
FH	Fumarate hydratase
FIH-1	Factor inhibiting HIF-1
G3P	Glycerol-3 phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLS	Glutaminase
GLUT1	Glucose transporter 1

GnHR	Gonadotrophin-releasing hormone
GPAT	Glycerol-3-phosphate O-acyltransferase
GR/*NR3C1*	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GS	Glutamine synthetase
GSK3β	Glycogen synthase kinase 3 β
GSTO2	Glutathione S-transferase omega 2
HDAC	Histone deacetylase
HIF1/2/3-α	Hypoxia inducible factor 1/2/3-α
HIF1-β	Hypoxia inducible factor 1-β
HILPDA/HIG2	Hypoxia inducible lipid droplet associated
HK2	Hexokinase 2
H-ras	Harvey rat sarcoma virus
HREs	Hormone response elements
HREs	Hypoxia response elements
HSP90	Heat shock protein 90
IDH	Isocitrate dehydrogenase
IDRF	Image-defined risk factors
Ig	Immunoglobulin
INR	Initiator sequences
INRGSS	International neuroblastoma risk group staging system
INSS	International neuroblastoma staging system
iPS	Induced pluripotent stem
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KDa	Kilodalton
KGDHC	α-KG dehydrogenase complex
KLF4	Krüppel-like factor 4
KO	Knockout
K-ras	Kirsten-rat sarcoma virus
LD	Lipid droplet
LDHA	Lactate dehydrogenase

L-DON	6-diazo-5-oxo-L-norleucine
LOH	Loss of heterozygosity
LPCAT2	Lysophosphatidyl-choline acyltransferase 2
LPO	Lipid peroxides
MAPK	Mitogen-activated protein kinase
MAX	MYC associated protein X
MB0-IV	MYC homology boxes 0-IV
MCT1-4	Monocarboxylate transporter 1
MDM2/4	Mouse double minute 2/4 homolog
MEFs	Mouse embryonic fibroblasts
MGA	MAX gene associated protein
MID	MYC interacting domains
miR-23a/b	MicroRNA-23a/b
MIZ-1	MYC-interacting zinc finger protein-1
MLX	MAX like protein X
MNA	MYCN-amplified
MNT	MAX's next tango
MONDOA/MLXIP	MLX interacting protein
MONDOB/MLXIPL	MLX interacting protein like
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MXD1-4	Max dimerization proteins 1–4
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Neuroblastoma
NEFL	Nestin
NES	Neurofilament L
NHRs	Nuclear hormone receptors
NGFR	Nerve growth factor receptor
NLS	Nuclear localization signal

NMNA	Non-MYCN-amplified
NOTCH	Neurogenic locus notch homolog protein
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NPY	Neuropeptide Y
NSCLC	Non-small cell lung cancer
OCR	Oxygen consumption rate
OCT4	Octamer-binding transcription factor 4
ODDD	Oxygen-dependent degradation domain
OXPHOS	Oxidative phosphorylation
P53/TP53	Tumor protein p53
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PAS	Period circadian protein-ARNT-single minded protein
PBRM1	Polybromo 1
PC	Phosphatidylcholine
PCC	Pheochromocytoma
PD-1	Programmed death ligand-1
PDK	Pyruvate dehydrogenase kinase
PDX	Patient-derived xenograph
PE	Phosphatidylethanolamine
PFAS	Phosphoribosyl formylglycinamidine synthase
PGK1	Phosphoglycerate kinase 1
PGL	Paraganglioma
PHDs	Prolyl hydroxylases
PHOX2B	Paired-like Homeobox 2B
PI	Phosphatidyl inositol
PI3K	Phosphoinositide 3-kinase
PKB/AKT	Protein kinase B
PKM2	Pyruvate kinase M2 isoform
PLIN1	Perilipin 1
PLIN2/ADRP	Perilipin 2 / Adipocyte differentiation related protein

PLIN3/TIP47	Perilipin 3 / Tail-interacting protein of 47 KDa
PLIN4/ S3-12	Perilipin 4
PLIN5/MLDP	Perilipin 5 / Myocardial LD protein
PP2A	Protein phosphatase 2A
PPARγ	Peroxisome proliferator-activated receptor γ
PPP	Pentose phosphate pathway
pRCC	Papillary renal cell carcinoma
PRDX	Peroxiredoxins
Pro	Proline
PRPP	5-phosphoribosyl-1-pyrophosphate
PRPS1	PRPP synthase 1
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
PUFAs	Polyunsaturated fatty acids
PUMA	P53 upregulated modulator of apoptosis
RAR	Retinoic acid receptor
RARE	Retinoic acid response elements
RARα/*RARA*	Retinoic acid Receptor α
RB	Retinoblastoma
RNA pol II	RNA polymerase II
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
RXR	Retinoic X receptor
SASP	Senescence-associated secretory phenotype
SCD	Stearoyl-CoA desaturase
SCG2	Secretogranin 2
SDH	Succinate dehydrogenase
SHH	Sonic hedgehog
Ser	Serine
SEs	Sterol esters
SETD2	SET domain containing 2

shRNA	Short hairpin RNA
SLC1A5/ASCT2	Solute carrier 1 A5 / Glutamine transporter
SLC43A1	Solute carrier 43 A1 / EAAs transporter
SM	Sphingomyelin
SOX2	Sex determining region Y-box 2
SQSTM1	Sequestosome 1
SREBPs	Sterol regulatory element binding proteins
STC2	Stanniocalcin 2
TAD	Transcriptional activation domain
TCA	Tricarboxylic acid
TFIIH	Transcription factor IIH complex
TGF-α/β	Transforming growth factor α / β
TGs	Triglycerides
TH	Tyrosine hydroxylase
THF	Tetrahydrofolate
TKI	Tyrosine kinase inhibitor
TNM	Tumors, nodes, and metastases
TPI	Triose phosphate isomerase
TSP-1	Thrombospondin-1
TUBB3	βIII-tubulin
VCAN	Versican
VEGF	Vascular endothelial growth actor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau
v-src/c-src	Viral-src/ cellular-src
WNT	Wingless-related integration site
Wt	Wildtype
α-KG	α-ketoglutarate

1 INTRODUCTION

1.1 HALLMARKS OF CANCER

Hippocrates (460-370 BC), a Greek physician known as the father of medicine, was the first person using the term cancer. To refer to tumors, he used the word *carcinos*, the Greek word for crab. It is believed that the terminology was appointed since tumors show projections interacting with the surrounding tissues and mimicking the shape of a crab. Years later, the Roman physician Celsus (25 BC-50 AD), translated the word *carcinos* to Latin, denoting it as cancer, the Latin word for crab. The term *oncos,* meaning swelling in Greek, was firstly used by another Greek physician, Galen (130-200 AD), and it is still used for naming the specialty treating cancer, oncology. Cancer has been part of human beings through history. The first evidence of cancer dates from ancient Egypt (3000 BC), discovered in bones from mummies, and papyrus mentioning ulcers in the breast, stating that there was no treatment for this disease [1].

Cancer comprises a group of highly heterogenic diseases caused by the abnormal proliferation of cells and deregulation in programmed cell death. With nearly 10 million deceased in 2021, cancer is a leading cause of deaths worldwide, representing one out of six deaths [2]. Cancer develops due to genetic abnormalities caused by mutations at the somatic or germline levels, as well as a plethora of progressive events inducing genomic instability, some of them also related to environmental factors [3,4].

For long, researchers have put considerable efforts to find the mechanisms involving the multistep process of tumorigenesis. In the last decades, many aspects at cellular, biochemical, and molecular levels implicated in the transformation of normal cells to a malignant phenotype, have been identified. In 2000, Douglas Hanahan and Robert Weinberg, described six capabilities that most, if not all, types of cancer share, known as the hallmarks of cancer [5].

The first one defined is <u>sustained proliferative signaling</u>. Three strategies used in cancer cells explain how they achieve autonomy in growth signals. To not depend on external signs, they are able to synthetize and respond to their own, inducing an autocrine stimulation process that results from a positive feedback loop. Cancer cells also overexpress growth factor receptors to increase their response to signals and show alterations in the downstream effectors for the signaling to be enhanced, promoting cell growth [6,7]. Closely related is the acquired capacity of <u>evading growth suppressors</u>. This antiproliferation signal is mainly regulated by the tumor suppressor retinoblastoma (RB), controlled by the signaling molecule transforming growth factor β (TGF-β). In cancer cells, TGF-β receptors are downregulated or showing inactivating mutations, promoting the inactivation of RB, and thus avoiding the antiproliferation signals [8].

To increase their number, cancer cells not only proliferate but also inhibit the signals inducing programmed cell death, apoptosis. The strategies to <u>resist cell death</u> reside in alterations in several tumor suppressors and oncogenes. The tumor suppressor p53 controls the machinery involved in DNA damage sensing, that triggers the apoptotic cascade. Loss of *TP53* is found

in about 50% of all tumors [9]. In addition, many tumors harbor alterations in the PI3 kinase-AKT/PKB pathway, which normally transmits antiapoptotic signals. Hyperactivation of this pathway is associated to loss of the *phosphatase and tensin homolog* (*PTEN*) tumor suppressor gene, that, when present, weakens phosphoinositide 3-kinase/protein kinase B (PI3K-AKT/PKB) signaling [10].

Cancer cells have <u>enabled replicative immortality</u>, allowing them to propagate unlimited times. Alone, the three hallmarks described above do not result in the massive cell growth observed in tumors. Telomeres are nucleotide sequences in tandem located at the end of the chromosomes that shorten in each cell division cycle. When telomeres are too short, they could compromise genome instability, so cell replication is inhibited, and cells undergo apoptosis or senescence [11,12]. Telomerase is an enzyme responsible to elongate telomeres, which is silent or in very low levels in most somatic cells. However, approximately 90% of the cancer cells have an upregulation on the levels of this enzyme [13], while the remaining 10% show activation of the alternative lengthening of telomeres (ALT) mechanism, maintaining the telomeres in length [14]. This process allows the limitless multiplication of the descendant cells.

Because of the rapid cell proliferation in tumors away from blood vessels, oxygen and nutrients availability will decrease as the tumor grows. However, cancer cells can <u>sustain angiogenesis,</u> which is the process of formation of new blood vessels. Angiogenesis is induced by several initiating signals including vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2) and contained by the presence of thrombospondin-1 (TSP-1). Many tumors have an upregulated expression of these pro-angiogenic markers and inhibit the anti-angiogenic markers promoting an "angiogenic switch" that results in new blood vessels to support tumor growth [15,16].

During cancer development, malignant cells acquire the <u>capacity of invading and forming metastases</u> in distant places. This process follows a multi-step pattern known as the invasion-metastasis cascade [17]. It starts with the invasion of the nearby tissue and their intravasation in blood and lymph vessels, allowing them to travel to further parts of the body. Afterwards, they exit the vessels by extravasation, forming micro-metastasis that grow to form bigger colonies, a step called colonization. Associated to the invasive part of the process is the expression of several transcription factors inducing changes in epithelial cells, termed epithelial to mesenchymal transition (EMT) [18,19].

In 2011 two emerging hallmarks and two enabling characteristics were defined [20]. Genomic alterations in cancer cells are part of the acquisition of the different hallmarks described above. Although they vary between the different types of cancer, these modifications in the genome affecting the DNA repair machinery and destabilizing gene copy number and nucleotide sequence are responsible for a selective advantage contributing to the outgrowth of malignant cells [3,21]. Thus, <u>genome instability and mutations</u> are considered as one enabling characteristic for tumorigenesis. Moreover, infiltration of cells from the immune system induces an inflammatory response that enhances tumorigenesis and its progression. In fact, <u>tumor-</u>

promoting inflammation is associated with the supply of many molecules preparing the tumor microenvironment for a better proliferative scenario for cancer cells [22].

To sustain uncontrolled proliferation, cancer cells reprogram their metabolism. This guarantees not only fuel for cell growth and division, but also to increase the supply of biomolecules to generate building blocks. Although aerobic glycolysis, known as the Warburg effect, was the first metabolic alteration described in cancer cells [23], many metabolic pathways seem to be disrupted, including glutaminolysis, fatty acid synthesis and oxidation as well as other metabolic related processes such as mitochondrial biogenesis [24]. Advances in cancer metabolism in the past years have contributed to the knowledge of how cancer keep up their proliferation state, including reprogramming of energy metabolism as a new hallmark.

The immune system plays an important role in cancer progression. Immune surveillance is the process in which the immune system is always alert, eliminating emerging cancer cells and avoiding neoplasm formation. However, tumors evolved to be able to evade immune system by using different strategies as inhibiting immune checkpoints or disrupting antigen presentation genes [25,26].

Early this year, four new hallmarks were included as essential for tumorigenesis (Figure 1) [27]. During development, progenitor cells are differentiated into their final cell type to assume a specific function within the body. This differentiation state is irreversible, and it is accompanied by inhibition of proliferation. However, cancer cells have the capability to unlock phenotypic plasticity, exiting this status of terminal differentiation by either dedifferentiating to their progenitor state, transdifferentiating to another cell type with a different function, or by blocking the differentiation process in uncomplete differentiated progenitor cells [28].
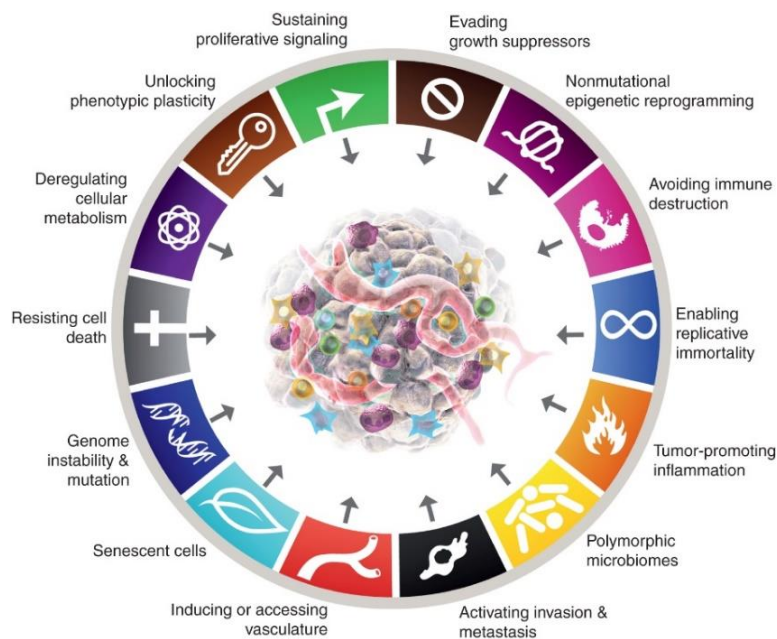
Figure 1: Hallmarks of cancer: new dimensions. The different hallmarks of cancer described during the last two decades are indicated. Reprinted from Hanahan, 2022, Cancer Discovery, with permission from the publisher American Association for Cancer Research.

<u>Non-mutational epigenetic reprogramming</u> induces changes in gene expression and contributes to acquiring the different hallmarks presented above. These epigenetic changes are consequence of the environment, resulting in tumor heterogeneity [29]. Moreover, the different cells that form the tumor microenvironment also present epigenetic alterations in their genome, promoting the development of the malignant capabilities [30].

The microbiome has profound effects in many diseases, including cancer. Different resident microorganisms promote cancer progression and survival and resistance to therapy while others protect from tumorigenesis [31]. The ways in which the microbiome contributes to cancer formation are diverse. For instance, some bacteria release toxins inducing DNA damage or disrupting repair mechanisms [32,33]. Moreover, it has been described the presence of bacteria inside tumors [34]. Thus, <u>polymorphic variation in microbiomes</u> is consider a new hallmark of cancer.

<u>Cellular senescence</u> is the process in which cells undergo proliferative arrest, which is triggered by different stresses in the cell, including nutrient deprivation, deregulation of signaling pathways and DNA damage among others. Although senescence could be a measure to protect from tumor proliferation, senescent cells are shown to contribute to tumor development through the senescence-associated secretory phenotype (SASP), providing signaling molecules to cancer cells to acquire hallmarks mentioned above such us avoiding apoptosis, inducing angiogenesis, evading the immune system and proliferative signaling [35].

After two decades since the first six hallmarks of cancer were described, we have observed a huge advance in cancer research that resulted in the addition of new categories, which help us to better understand the mechanism of tumorigenesis and have opened the way for therapeutic approaches to be exploited in a clinical setting.

1.1.1 Oncogenes and tumor suppressor genes

In cancer, alterations in several genes are considered crucial to trigger tumorigenesis. Depending on their role in cancer development, these genes can be divided in two categories: oncogenes or tumor suppressor genes.

Oncogenes are mutated genes with the potential of initiating cancer. The non-mutated versions of these genes are called proto-oncogenes, participating in normal cell division and growth processes. In 1914, Theodor Boveri postulated the theory suggesting that there must be a factor that when amplified, is responsible for tumorigenesis [36]. It was not until 1969 that George Todaro and Robert Huebner termed this factor "oncogene" [37]. The first oncogene discovered was *v-src* (*viral-src*) by G. Steven Martin in 1970, based on the earlier work from Francis Peyton Rous in 1911 [38]. Rous described that extracts of sarcomas from chickens that were filtered in small pores could induce tumors in healthy chickens. Since these pores were small for cells or bacteria to pass, he concluded that the responsible for cancer was a virus termed Rous sarcoma virus (RSV) [39]. In fact, the factor inducing cancer was not the virus, but a host gene hijacked by the virus. Only few years after, Michael Bishop and Harold Varmus described the presence of a gene with a similar structure to *v-src* in chickens, called *c-src* (*cellular-src*)

[39]. For the discovery of the cellular origin of retroviral oncogenes, they received the Nobel Prize in Physiology or Medicine in 1989.

The discovery of *c-src* increased the interest to find other oncogenes. The MC29 myelocytomatosis virus was also shown to have acquired a gene responsible for malignant tumors in chickens, in this case *v-myc*. Similar as *c-src*, chicken cells also contained the cellular version of *v-myc* (*c-myc*) [40,41]. The *H-ras* and *K-ras* oncogenes were also discovered to be present in the genome of the Harvey and Kirsten rat sarcoma viruses, respectively, and the corresponding genes in cells [42,43].

Differently from the oncogenes found in tumor viruses, another important oncogene, *HIF1A*, encoding for the HIF1-α protein, was first described in 1991 by Gregg L. Semenza while studying the gene encoding erythropoietin (EPO) [44].

On the contrary, tumor suppressors are genes that participate in controlling cell division and proliferation. Mutations in these genes result in uncontrolled proliferation of cells leading to cancer development. The idea of genes that promote cancer formation suggested that in normal cells, other type of genes could regulate abnormal cell division. The experiments of somatic cell hybridization by Henry Harris in 1969 demonstrated that most hybrid cells obtained from fusing tumor with somatic normal cells, could not develop tumors in animals [45]. In 1971, Alfred Knudson described the tumor suppressor gene retinoblastoma (*RB*) and defined the two-hit hypothesis, indicating that for retinoblastoma formation, one mutation in *RB* occurs at germ-line stage followed by a *de novo* mutation on the other allele later on [46,47].

In 1979, a protein with size 53 KDa was described by the independent research groups of Lionel Crawford, David P. Lane, Arnold Levine, and Lloyd Old, in mouse and humans and was named p53. This mysterious protein was discovered bound to the large T antigen of the SV40 virus [48–51]. Due to the findings of oncogenes associated to tumor viruses, it was first believed that *tumor protein 53* (*TP53*) was an oncogene since it was found in high levels in cancer compared to normal cells [52]. However, the first clones of *TP53* were obtained from cancer cells since it was more abundant than in normal cells. The successive experiments proved its oncogenic functions and even interaction with other oncogenes like *H-RAS*. Yet, years after, the non-mutated *TP53* gene was cloned from rat embryo fibroblasts, demonstrating that it is mutated in cancer cells and that wild type (wt) *TP53* did not induce cancer development, thus confirming its role a tumor suppressor gene [53]. Bert Vogelstein demonstrated that *TP53* is frequently loss in several tumor types [54].

In clear cell renal cell carcinoma (ccRCC) abnormalities on the short arm of chromosome 3, not present in normal kidney or other types of kidney cancer were reported [55]. This suggested the potential presence of a tumor suppressor gene that could contribute to ccRCC development. In 1993 Farida Latif *et al.,* found that loss of the *von Hippel-Lindau* (*VHL*) gene, residing on chromosome 3, was associated to sporadic and germ-line mutations in ccRCC, in accordance with the two-hit hypothesis of Knudson [56].

1.1.2 Nuclear hormone receptors in cancer

The nuclear hormone receptors (NHRs) belong to a transcription factor superfamily comprising of 48 genes in humans and 49 in mice. They orchestrate many different processes in the cell related to development, cell proliferation, metabolism, and immune response [57–59]. They are activated by binding to their respective ligand, which include steroid hormones (estrogen and progesterone), lipophilic signals such as thyroid hormones, retinoic acid, vitamin D, fatty acids, and oxysterols [60]. All NHRs share a common structure, with a transactivation region, a ligand-binding domain, a nuclear localization signal, and a domain for binding at hormone response elements (HREs) as monomers, homodimers, or heterodimers [61].

In many types of cancer, the regulation of several NHRs is altered, and their role in tumor development and progression has been established, proposing them as potential therapeutic targets. Due to their mode of action, they are easy to target using small-molecule compounds that mimic their natural ligands [62].

The most known case of targeting NHRs for cancer treatment is the estrogen receptor (ER) in breast cancer. Two ER isoforms have been identified, ERα, encoded by the *ESR1* gene, and ERβ, encoded by the *ESR2* gene. Upon estrogen binding to the receptors, they form homodimers that, in the nucleus, bind to the estrogen response elements (EREs) in the promoter of target genes [63]. ERα is one of the drivers of breast cancer, and different targeted therapies for ER$^+$ breast tumors have been developed, including direct targeting of the ER and drugs disrupting estrogen synthesis (aromatase inhibitors). In the 1970s, tamoxifen, a failed contraceptive drug, was the first hormonal therapy for breast cancer [64]. However, it was later found that it activated ER in other tissues causing uterus and endometrial cancer, as well as thromboembolism [65]. Since then, other ER-targeted drugs have been approved for their use in the clinic to treat breast cancer, with higher affinity and reduced side effects, constituting one of the strategies for ER$^+$ tumors [62].

Glucocorticoids are anti-inflammatory molecules which have also been used in the context of cancer. For example, dexamethasone, the synthetic ligand for glucocorticoid receptor (GR), 25 times more potent than the natural ligand hydrocortisone, is administrated to ameliorate allergic reactions and other side effects of chemotherapy in cancer [66,67]. They exert their function by forming homodimers upon the presence of ligand, and binding to the glucocorticoid response elements (GREs) in the DNA. Although the effect as anti-cancer drug in combination with other strategies has been described for some hematological malignances such as leukemias and multiple myeloma, their effect on solid tumors is less clear, in some cases associated to severe side effects [68].

Retinoids are vitamin A derivatives, with all-*trans* retinoic acid (ATRA) being the predominant natural molecule, present ubiquitously in the body. They are important for many roles during embryonic development as well as in regulating differentiation, apoptosis and cell growth [69–72]. Retinoids exert their functions by binding two different types of NHRs, retinoic acid receptor (RAR) or the retinoic X receptor (RXR), from which three subtypes are described, α, β and γ.

They form heterodimers and bind the retinoic acid response elements (RARE) in the DNA of the promoters of their target genes [73]. In acute promyelocytic leukemia (APL), the use of ATRA in combination with chemotherapy promotes differentiation and increases the long-term survival of the patients to 90% [74]. Moreover, isotretinoin (13-*cis* retinoic acid) is currently used in the clinic as maintenance therapy for high-risk neuroblastoma [75].

Androgens, like testosterone and dihydrotestosterone (DHT), play an important role in the development of the prostate gland. They serve as ligands for the androgen receptor (AR), that upon binding homodimerize and translocate to the nucleus binding to the androgen response elements (AREs) activating the expression of target genes [76]. The gene encoding for AR, *NR3C4*, is the most frequently mutated gene in prostate cancer. The use of anti-androgens in the clinic, such as bicalutamide and flutamide, result in inhibition of the AR preventing the signaling and reducing tumor growth. However, high-risk prostate cancers can progress even in the presence of anti-androgens, becoming castration resistant [77]. Another strategy to target prostate cancer is the androgen deprivation therapy (ADT) through surgical or chemical castration using gonadotrophin-releasing hormone (GnHR) which, together with the anti-androgens, consist in the current treatment for the metastatic prostate cancer [78–80].

1.1.3 Childhood versus adult cancers

Cancer is a rare disease in children, although in developing countries it is the leading cause of death in children over one year of age. Contrary to adult cancer, tumors in children do not appear as result of accumulating mutations during time, lifestyle, or environmental influence. Pediatric cancers have a very low mutational status compared to tumors in adults (Figure 2) [81], and more interestingly, approximately 10 % of childhood tumors do not present any mutations at all, most likely due to epigenetic alterations [82]. In many cases, these genomic aberrations occur prenatally, as part of a developmental disorder.

Because of the differences in origin, the most common childhood tumors are leukemias, central nervous system (CNS) tumors, neuroblastoma, bone tumors and sarcomas [83].

Over the past years, the incidence of childhood cancer has been increasing worldwide. Current treatment options for tumors in children comprise surgery, chemotherapy, and radiation. Although the 5-year survival rate has greatly improved, the severe side effects that these children experience as consequence of the aggressive treatment will affect their development as well as decrease quality of life. These include delay in growth, behavior, and cognitive problems among others, and higher risk to develop another type of tumor as an adult [84]. Thus, finding new targets for these tumors are crucial for developing precision medicine-based therapies that are less harmful and that will improve the life quality of childhood cancer survivors.
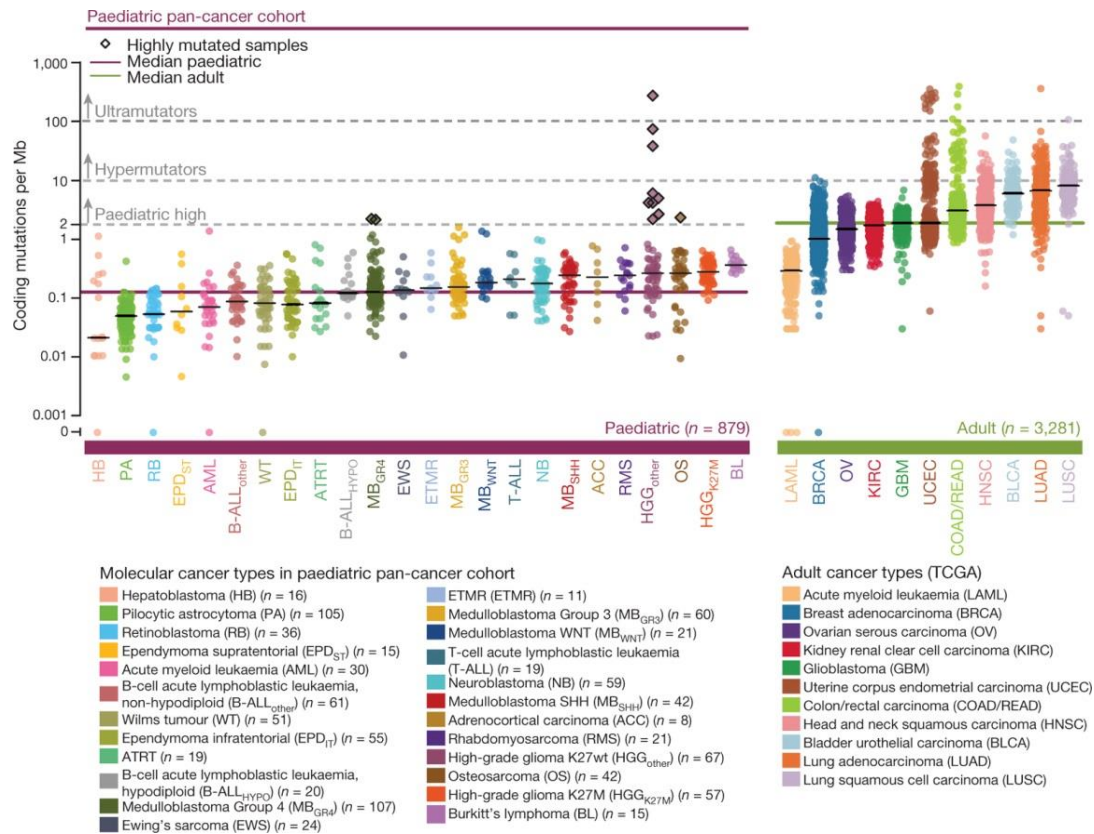
Figure 2: Somatic mutations in childhood and adult cancers. Representation of the sequencing data from several different types of pediatric (879, left) and adult (3281, right) cancers. Reprinted from Göbner *et al*., 2018, Nature, with permission from the publisher Springer Nature (<u>CC BY 4.0</u>).

1.2 METABOLIC REPROGRAMMING IN CANCER

Cancer cells show alterations compared to normal cells in different metabolic pathways due to their increased energy demand resulting from the rapid cell proliferation and to adapt to nutrient deprivation. This process is known as metabolic reprogramming, and was included in 2011 by Douglas Hanahan and Robert Weinberg as one of the hallmarks of cancer (Figure 1) [20]. Among the different alterations, this thesis focuses on deregulation of glucose, glutamine, and fatty acids metabolism.

1.2.1 Aerobic glycolysis and oxidative phosphorylation

Otto Warburg first described in the 1920s that cancer cells show alterations in their metabolism. Normal cells under aerobic conditions obtain energy by metabolizing glucose into pyruvate via glycolysis, and further, to carbon dioxide (CO_2) via oxidative phosphorylation (OXPHOS), producing 36 molecules of adenosine triphosphate (ATP). However, even in the presence of oxygen, he described that cancer cells consumed high amounts of glucose to produce lactate via "aerobic glycolysis", which is less efficient than OXPHOS, producing only two molecules of ATP. This phenomenon is known as the Warburg effect [23]. The difference in efficiency of aerobic respiration is compensated by an upregulation in glucose transporters (GLUT1), allowing an increase in glucose import, an induction of hexokinase 2 (HK2), the rate-limiting enzyme and first step in glycolysis, as well as lactate dehydrogenase A (LDHA), that converts pyruvate into lactate. An increment in glycolytic flux is associated with activation of different

oncogenes, for example *MYC* and *HIF1-α* [85], and deactivation of tumor suppressor genes as *TP53* or *PTEN* [86,87].

The increase in aerobic glycolysis provides proliferating cells with several advantages. If the glycolytic flux is highly enhanced, the amount of ATP produced by aerobic glycolysis could overcome the amount produced by OXPHOS [88], and the intermediaries of the glycolytic pathway can be diverted into other metabolic processes for biosynthetic purposes [89]. These include pentose phosphate pathway (PPP) to produce nicotinamide adenine dinucleotide phosphate (NADPH) and ribose, and one carbon metabolism for synthesis of nucleotides and generation of NADPH [90].

The Warburg effect was based on the idea that all cancer cells show an impairment in their oxidative metabolism translated as an increase in glycolytic flux. But in 1956, Warburg showed that cancer cells consume lower amount of oxygen than normal cells and hypothesized that it could be due to an injury in respiration [91]. Later studies demonstrated that OXPHOS is fully functional in many highly proliferative tumor cells [92,93]. In some, the prevalence of oxidative metabolism over glycolysis is the responsible for ATP production [94]. Moreover, disrupting glycolysis restores OXPHOS activity. For instance, inhibition of LDHA resulted in an increase in mitochondrial respiration, and limiting pyruvate kinase M2 isoform (PKM2) activity, reducing ATP production from glycolysis, did not prevent tumor formation [95,96]. In addition, for cancer cells to sustain their proliferative rate, mitochondrial metabolism is essential [97,98]. Therefore, most tumors produce ATP through OXPHOS despite presenting a high glycolytic profile, indicating that both processes can be enhanced [99]. The exception are tumors with mutations in the tricarboxylic acid (TCA) cycle enzymes such as succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase (IDH), although they adapt their metabolism to obtain TCA intermediates [100,101].

1.2.2 Glutamine metabolism

Apart from glucose, glutamine is another major nutrient for energy production in cancer cells, as well as a carbon source for biomass synthesis. From all amino acids, glutamine is the most abundant in plasma, providing nitrogen that can be used for the synthesis of nucleic acids, other amino acids, as well as glutathione, participating in redox homeostasis [102–104]. Absence of glutamine results in a decrease in the survival of some cancer cells *in vitro*, a phenomenon termed "glutamine addiction", associated to the overexpression of oncogenes as *MYC* [105,106].

Cells with high dependency on glutamine present upregulated expression of the glutamine transporter solute carrier 1 A5 (SLC1A5) [107,108]. Glutamine is converted into glutamate by glutaminase (GLS), an enzyme elevated in many cancer cells [109–111]. Glutamate is further transformed into α-ketoglutarate (α-KG), that is incorporated into the TCA cycle, used as a fuel for oxidative metabolism, process known as glutaminolysis. Via this pathway, glutamine provides TCA cycle intermediates that are used for anaplerotic purposes [112]. Cells with a defective mitochondrial oxidative function, as well as cells in hypoxia, or those in which HIF

is otherwise activated, depend on reductive carboxylation of glutamine to produce citrate, a source of acetyl-CoA necessary for lipid synthesis [113].

In some tumors, expression of the glutamine synthetase (GS) enzyme that catalyzes the addition of ammonia to glutamate to produce glutamine, is upregulated. Moreover, non-small cell lung cancer (NSCLC) cells and *MYCN*-amplified neuroblastoma cells *in vitro*, and some glioblastoma tumors *in vivo* can perform *de novo* glutamine synthesis from glucose-derived carbons. In all of them MYC levels are elevated [114–116].

1.2.3 Fatty acid metabolism

1.2.3.1 *Fatty acid synthesis*

In most normal tissues, lipids are obtained exogenously from the diet, as *de novo* fatty acid synthesis is restricted to liver, adipose tissue, and mammary glands during lactation. However, in cancer cells lipid biosynthesis is reactivated independently of the availability of exogenous fatty acids. In the mid-1950s, it was shown that different types of tumors were able to use acetate- and glucose-labeled carbons to synthesize lipids [117]. Years later, another study described that almost all fatty acids are synthetized *de novo* in cancer cells [118]. The increased in the synthesis of fatty acids could occur as result of the high energy demand in tumor cells or as a response to deprivation of exogenous lipids.

The two key enzymes that participate in *de novo* fatty acids synthesis are acetyl-CoA carboxylase (ACACA) and fatty acids synthase (FASN). In the first step, ACACA synthetizes malonyl-CoA from acetyl-CoA. Malonyl-CoA is further converted into long-chain fatty acids by FASN. The activation of *de novo* fatty acid synthesis in cancer cells is caused, in part, by the overexpression of these two lipogenic enzymes [119]. Expression of ACACA and FASN is regulated by different transcription factors from the basic helix-loop-helix leucine zipper (bHLH-Zip) family, the sterol regulatory element binding proteins (SREBPs) [120]. Moreover, other transcription factors inducing *de novo* fatty acids synthesis are MONDOA (also termed MLXIP, MAX like protein X interacting protein), and carbohydrate-responsive element-binding protein (ChREBP, also known as MLXIPL) [121,122]. MYC regulates the expression of MONDOA, promoting glutaminolysis and lipid synthesis. Moreover, inhibition of MONDOA results in apoptosis, while exogenous oleic acid prevented this process, indicating the importance of fatty acid synthesis for cancer cells survival [121].

1.2.3.2 *Fatty acid β-oxidation*

Fatty acid oxidation (FAO) is another energy source, producing two times more ATP than carbohydrates per gram of dry mass. Thus, when nutrients are abundant in the cells, fatty acids are the preferred storage biomolecule. β-oxidation occurs in mitochondria via shortening fatty acids in two carbons per cycle, generating NADH (nicotinamide adenine dinucleotide), FADH2 (flavin adenine dinucleotide) and acetyl-CoA, until the last cycle in which they produce two Acetyl-CoA molecules from four carbon fatty acids. The rate limiting step of β-oxidation is catalyzed by the carnitine/palmitoyl-transferase 1 (CPT1) enzyme, which resides

in the outer mitochondrial membrane. CPT1, together with CPT2, mediate the transportation of long-chain fatty acids to mitochondria for their oxidation [123].

It has been described that a subtype of diffuse large B-cell lymphomas expresses high levels of enzymes involved in FAO and prefer fatty acids as mitochondrial fuel, especially palmitate, even when they have other nutrients available [124]. Moreover, an isoform of CPT1, CPT1C, usually expressed in brain, has been found to be upregulated in cancer cells, promoting FAO as a source for ATP production, enhancing tumor growth and resistance to metabolic stress [125].

1.3   METABOLISM AS A TARGET FOR PRECISION MEDICINE IN CANCER

Cancer cells use different ways to adapt their metabolism according to their energy needs to sustain their survival. As described above, metabolism of glucose, glutamine, and fatty acids play an important role in tumorigenesis. The increased knowledge on how metabolic pathways are regulated, their main enzymes, and how they affect cancer proliferation, have provided novel targets for cancer treatment. However, the translation of metabolic inhibitors to clinical use has proven challenging, as we have recently summarized [126]. Yet, drugs targeting metabolism are prescribed with successful results and several other pro-drugs are currently enrolled in clinical trials.

Several inhibitors targeting the main enzymes in glycolysis have been identified. 2-Deoxy-glucose (2-DG) is an inhibitor of HK2, the first and rate limiting enzyme of glycolysis. Although it induces apoptosis and inhibits cell growth *in vitro* in several tumor types [127,128], its use was limited due to high toxicity [129]. Another strategy for targeting glycolysis is the inhibition of LDHA, since its depletion caused a reduction in tumor burden in mouse models and xenograft experiments of different cancers [95,130,131]. Several inhibitors of LDHA have been described, with the most promising being GNE-140. However, none of them entered clinical trials due to toxicity [132].

Alterations in the enzymes participating in the TCA cycle are also frequently shown in tumors, inducing the aberrant accumulation of their metabolic products. These metabolites in turn induce signaling activation and epigenetic changes promoting cancer progression and are thus termed oncometabolites. Many drugs have been identified to successfully inhibit enzymes that when mutated acquire gain of function (Figure 3), like isocitrate dehydrogenase (IDH) and the multienzyme α-KG dehydrogenase complex (KGDHC). Two drugs targeting IDH, AG-221 and AG-881, are currently in clinical trials for acute myelogenous leukemia [133,134], while CPI-163, targeting KGDHC, is in the first phases of clinical trials for the treatment of hematological malignances as well as small cell lung cancer (SCLC) [135,136].

Targeting the electron transport chain (ETC) has for long been exploited for cancer treatment (Figure 3). Two well-known drugs, tamoxifen and metformin, target complex I of the ETC. While tamoxifen is used for breast cancer [137], metformin is currently one of the most prescribed drugs worldwide, for treatment of type II diabetes, and is in clinical trials as a potential option for colon, breast, and prostate cancer [138,139]. For targeting complex III, the malaria drug, atovaquone has been enrolled in clinical trials as a combination strategy with common

chemotherapeutics for NSCLC [140] and acute myeloid leukemia (AML) [141]. Doxorubicin, frequently used for the treatment of several hematological and solid tumors, is an inhibitor of complex IV [142].

Glutamine addiction has been observed in several types of cancer. Targeting GLS disrupts antioxidant glutathione production, elevates reactive oxygen species (ROS) levels and induces apoptosis in cancer cells [143,144]. 6-diazo-5-oxo-L-norleucine (L-DON) was the first glutamine analog described for inhibiting GLS. However, it also targets amino acids transporters and transglutaminases, showing lack of specificity, as well as inducing severe side effects [145]. Other compounds have shown to exclusively target GLS. For instance, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide 3 (BPTES) has effectivity in targeting glutaminolysis, but has a low solubility in aqueous solvents and low bioavailability [146]. CB-839 appears as a more potent GLS inhibitor, currently in several clinical trials for both hematological and solid tumors [147] (Figure 4).
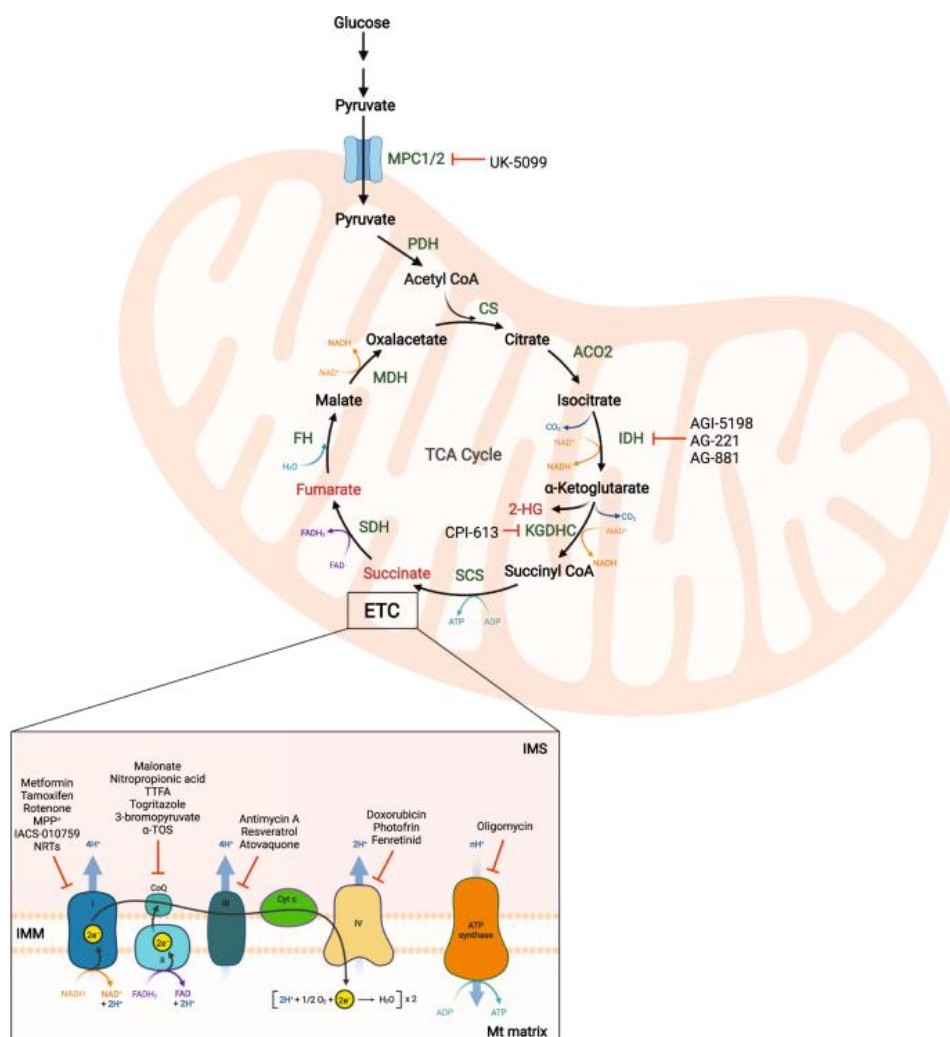
Figure 3: Inhibitors of the TCA cycle and the ETC. IDH and KGDHC enzymes from the TCA harbor gain of function mutations, and several inhibitors have been identified to target their activity. The ETC has also been extensively studied in cancer with many inhibitors available to target all the complexes. Reprinted from Sainero-Alcolado *et al*., 2022, Cell Death & Differentiation, with permission from the publisher Springer Nature (<u>CC BY 4.0</u>).

In the past years, targeting fatty acid synthesis has become an attractive approach for anticancer treatment, as different FASN (cerulenin, C75 and orlistat) [148], and ACACA inhibitors (TOFA and soraphen A) demonstrated to reduce tumor growth and survival in different tumor models [149,150]. Orlistat is an intestinal lipase inhibitor drug approved by the European Medicines Agency (EMA) for treatment of obesity that was discovered to also be able to inhibit FASN [151]. Furthermore, TVB-2640, a FASN inhibitor, is currently in clinical trials for breast and colon cancer [152,153].

The most common strategy to inhibit FAO is by targeting the rate limiting enzyme of the process, CPT1. Different studies have demonstrated that its inhibition reduces survival of several different cancer cells and could potentially be used as a new therapeutic strategy [125,154,155]. Although etomoxir is commonly used for the inhibition of CPT1 in tumor cells, its application is limited to experimental settings due to high toxicity and off-target effects. Other drugs targeting FAO have been described, including teglicar (ST1326) and perhexiline, showing a higher selectivity for CPT1 [156,157] (Figure 4).
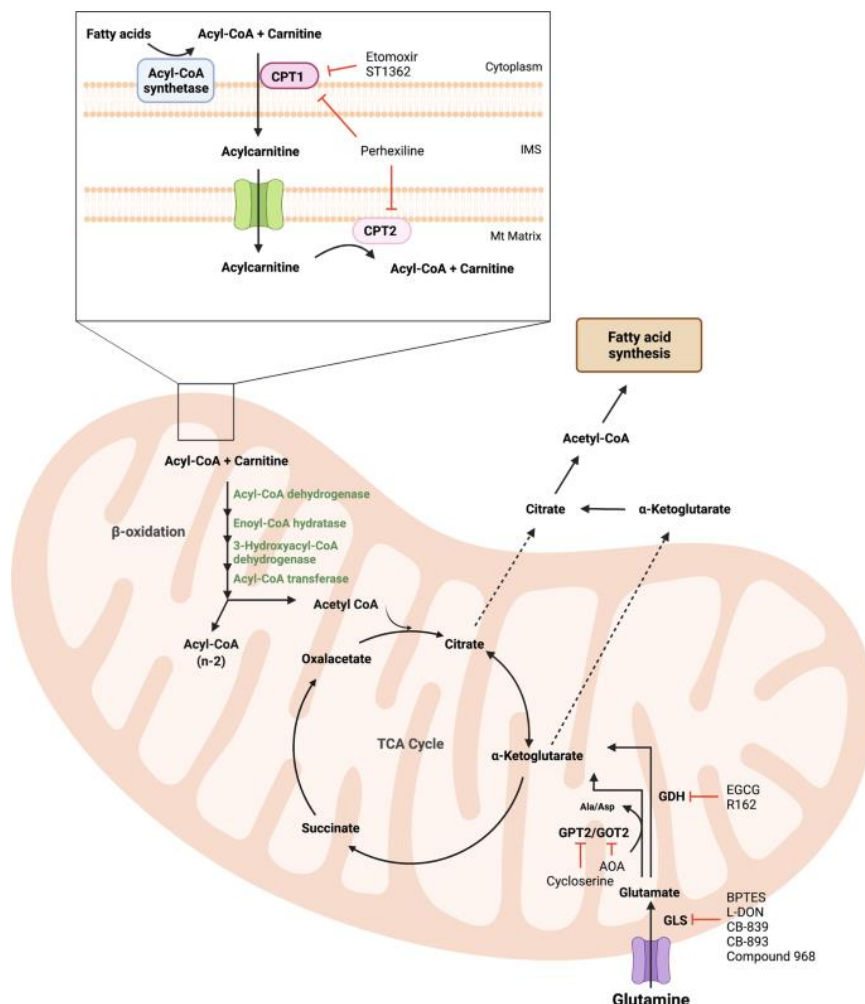
Figure 4: Inhibitors of glutamine metabolism and FAO. Different inhibitors for the rate limiting enzyme of FAO and several enzymes participating in the metabolism of glutamine have been described. Reprinted from Sainero-Alcolado *et al*., 2022, Cell Death & Differentiation, with permission from the publisher Springer Nature (CC BY 4.0).

Recent advances in the field of cancer metabolism have shown that targeting key enzymes regulating the most vulnerable metabolic pathways in different tumor types could serve as a potential therapeutic strategy. Yet, many inhibitors available show poor specificity and high toxicity, thus more studies are needed to reach this goal. Importantly, combination treatment of metabolic inhibitors together with conventional cytochemical remedies could result in a better approach to potentiate the anti-cancer efficacy.
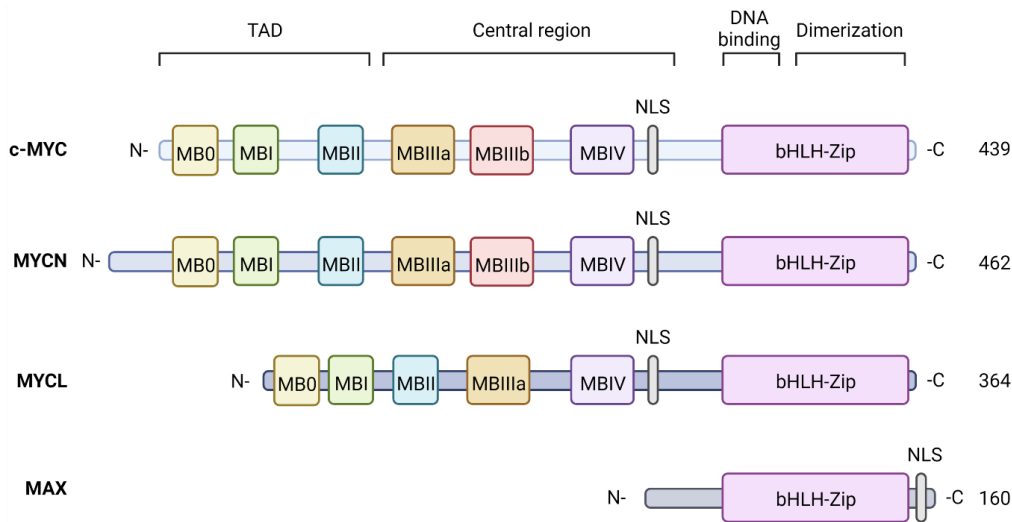
1.4 MYC FAMILY

1.4.1 The MYC family protein network

The *MYC* (*v-myc* avian myelocytomatosis viral oncogene homolog) family of proto-oncogenes comprises three members: *c-MYC*, *MYCN*, and *MYCL*. As described above, *v-myc* was initially discovered as an oncogene in the retrovirus MC29, causing myelocytomatosis, a type of leukemia in chickens. The cellular version of this gene, found in all vertebrate genomes, was named *c-MYC* (from now on referred as *MYC*). Years later, the other two paralogs, *MYCN* and *MYCL* were identified amplified in human neuroblastoma and SCLC respectively [158–160].

The three family members encode for the c-MYC, MYCN, and MYCL proteins, sharing a similar structure but with different expression patterns. The levels of MYCN increase in the early embryo, and is important for the development of the heart, lungs, central nervous system, limbs, kidneys, and the inner ear. After the start of organogenesis, MYCN expression is downregulated, and MYC is required to support growth and proliferation in multiple organs and tissues [161]. However, MYC expression is downregulated upon terminal differentiation. In adults, MYCN is only present in the intestinal stem cells to control homeostasis of the tissue, while MYC is expressed in highly proliferative and regenerative tissues such as epidermis and intestines, and in B lymphocytes [162,163]. The MYCL expression pattern during development is similar to MYCN, and especially found in the forebrain, the hindbrain and the kidneys, while in adults it is expressed during normal development of dendritic cells [164,165]. Yet, MYCL is dispensable for embryonic development [164–166].

MYC proteins are transcriptions factors that contain a bHLH-Zip region at the carboxy-terminus, important for dimerization and DNA binding at specific enhancer box sequences (E-boxes) located in the promoter or enhancer regions of their target genes [167]. Moreover, they incorporate a nuclear localization sequence (NLS) located at the central region, close to the carboxy terminus [168]. At the amino terminus, these proteins contain the transcriptional activation domain (TAD). The MYC structure is also composed by several highly conversed regions termed MYC homology boxes (MB0, MBI, II, III a and b, and IV), located at the TAD and in the central region, and important for protein stability and transcriptional activation and repression (Figure 5). Although the three proteins share a similar structure, MYCL lacks MBIIIb box [169,170].

Figure 5: Schematic representation of the structure of the MYC family of proteins and their dimerization partner MAX. All of them share a common structure with a transactivation domain, central region, and bHLH-Zip domain for DNA binding and dimerization with MAX. Created with Biorender.com.

MYC controls the transcription of 15% of all human genes. While most transcription factors bind at specific DNA sequences stimulating transcription of their target genes, MYC also enhances the production of transcripts of active genes. This role of increasing the transcriptional output of genes have a huge impact in many cellular processes, including proliferation, and thus, explains the vast oncogenic role of MYC in cancer [171,172].

The expression of MYC is highly regulated at several levels. MYC transcription is controlled by different mitogenic factors activating signaling pathways that induce proliferation including rat sarcoma (RAS), mitogen-activated protein kinase (MAPK), Janus kinase-signal transducer and activator of transcription (JAK/STAT) and wingless-related integration site (WNT)/β-catenin [173–176]. Moreover, MYC protein stability is regulated by post-transcriptional phosphorylation in two residues located at the MBI: serine 62 (Ser62) and threonine 58 (Thr58). Phosphorylation at Ser62 by the extracellular receptor kinase (ERK), cyclin-dependent protein kinase 1 (CDK1), CDK2, and CDK5 enhances MYC stability. Ser62 is necessary for MYC phosphorylation at Thr58 by glycogen synthase kinase 3 β (GSK3β), resulting in MYC protein degradation via the proteasome [177,178].

The MYC protein is intrinsically disordered. Upon binding to MYC-associated protein X (MAX), the carboxy terminus adopts an ordered structure, forming a heterodimer that allows binding to E-boxes on the promoters of target genes [179]. The complex then recruits histone acetyltransferases that will maintain an open chromatin configuration, allowing target gene transcription [180]. The structure of MAX also contains a bHLH-Zip domain but lacks the TAD resulting in a smaller protein (Figure 5) [181,182]. While MYC half-life is approximately 20 minutes, MAX is stable for 24 hours, generating a non-limiting pool. MAX can form homodimers that are unable to induce gene transcription, limiting MYC binding, and thus inhibiting the expression of target genes [183]. However, MYC can also function without MAX, as shown in the rat pheochromocytoma (PCC) cell line PC12. These cells are homozygous for

a mutation in *Max*, resulting in a non-functional MAX protein. However, even in the absence of its partner protein, overexpression of *Myc* in these cells prevented the differentiation phenotype induced by *Ras* and promoted apoptosis [184]. Moreover, a rat fibroblast cell line, in which *Myc* was knocked out (KO), substituted by a mutant version of *Myc* without bHLH domain that was not able to bind to *Max*, still stimulated proliferation [185]. Mutations in *MAX* have also been described to drive tumorigenesis, for instance, associated with sporadic and hereditary PCC and paraganglioma (PGL) [186]. In addition, the p.His28Arg mutation in MAX results in increased vascularity and aggressiveness in endometrial cancer [187].

MYC and MAX belong to a large network comprising several bHLH-Zip transcription factors, with MAX and MAX like protein X (MLX) in the center of the interactions (Figure 6). Apart from MYC and itself, MAX can bind MAX dimerization proteins 1-4 (MXD1-4), MAX's next tango (MNT), and MAX gene associated protein (MGA). The interaction between MXDs and MAX act as repressors of some of the genes activated by MYC, as their overexpression decreases MYC/RAS induced co-transformation [188–190]. Moreover, increased levels of MXD1 and 2 reduces proliferation and increases G0/G1 cell arrest [191–193]. Due to the antagonist role of MXD1-4 and MGA with MYC, and their common loss or inactivation in cancer cells, they could act as potential tumor suppressors.

MNT is known to be the major MYC antagonist. For instance, mouse embryonic fibroblasts (MEFs) with lack of *Mnt* (*Mnt-/-*) mimic cells with MYC overexpression. These cells show increased proliferation and growth, more sensitivity to apoptosis and have higher levels of genes controlled by MYC such as cyclin E1 (*Ccne1*) and cyclin dependent kinase 4 (*Cdk4*). Moreover, in these MEFs, MYC expression is reduced, most likely to compensate the lack of MNT [194–196]. However, depending on the context, MNT can either act as a tumor suppressor or an oncogene. While alterations in *Mnt* in mouse mammary epithelium and T cells leads to mammary adenocarcinomas and T cell lymphomas, loss of *Mnt* can also prevent MYC induced tumorigenesis as result of increased apoptosis [197–199]. In humans, loss of heterozygosity (LOH) at the *MNT* locus occurs in approximately 20% of the cases in sarcomas, hepatocellular and lung carcinomas, and uterine carcinosarcomas [200].

Depending on its interaction with MAX, MGA can act both as a repressor or an activator. Apart from the bHLH-Zip domain, it also contains a T-loop DNA binding domain, and it is the least studied member of the family. However, it is known that MGA suppresses MYC transcriptional activation [201]. Inactivating mutations in MGA are found in lung adenocarcinomas and in high risk chronic lymphocytic leukemia, suggesting its potential as tumor suppressor [202,203].
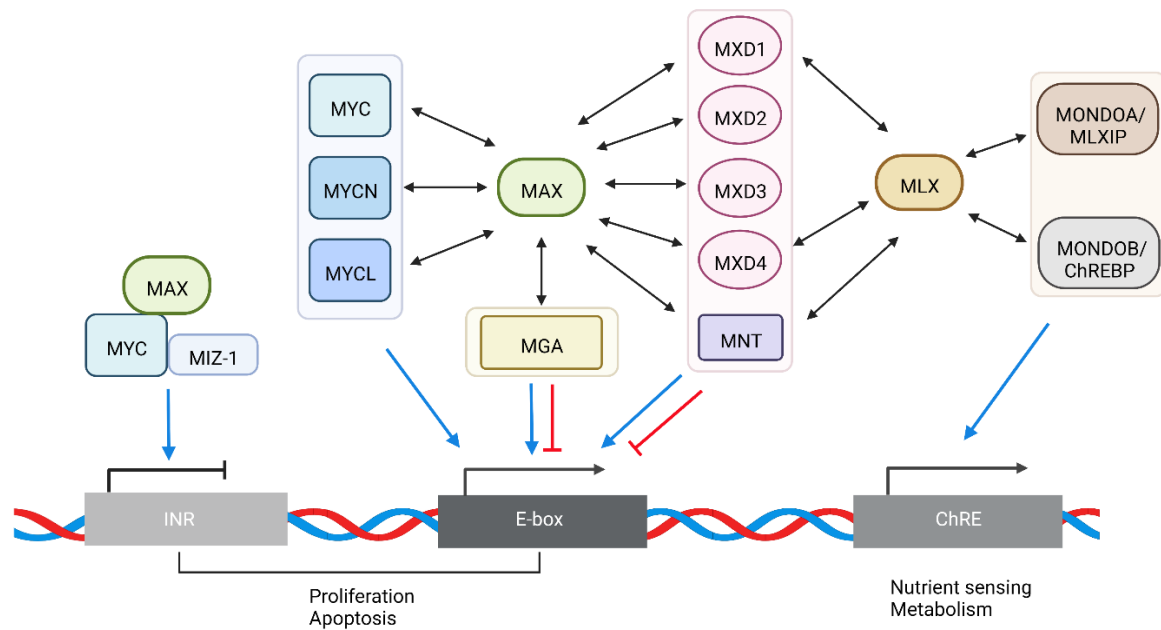
Figure 6: Proximal MYC interacting network. The MYC family of proteins form dimers with MAX, binding to the E-boxes and activating the transcription of genes related to proliferation and apoptosis. Moreover, MYC, bound to MAX, can interact with MIZ-1 repressing the activation of target genes. MXD1-4, MNT and MGA dimerize with MAX, while MXD1, MXD4 and MNT can also bind to MLX activating or repressing gene transcription. MONDOA and ChREBP form dimers with MLX and bind to ChRE sequences activating the expression of genes related to metabolism. Created with Biorender.com.

The proteins MLX, MONDOA (MLXIP), and ChREBP (MONDOB/MLXIPL) play an important role in the control of metabolism. Activated by the presence of intracellular glucose, they regulate expression of genes involved in glucose and lipid metabolism by binding to carbohydrate response elements (ChRE) [204]. Moreover, MXD1, MXD4 and MNT can also bind to MLX, keeping their transcriptional repression function [205,206]. In acute lymphoblastic leukemia (ALL), MONDOA is overexpressed promoting differentiation and apoptosis [207].

In addition, the expression of MYC target genes can be repressed via its interaction to MYC-interacting-zinc finger (MIZ-1), forming a ternary repressive complex binding to the transcriptional initiator sequences (INR). MIZ-1 contains two MYC interacting domains (MID), while it does not interact with MAX. Moreover, even though MIZ-1 can bind to all members of the MYC family proteins, the interaction is stronger with MYC [208].

1.4.2 Cell processes governed by MYC are deregulated in cancer

MYC proteins play an important role in regulation of several normal cellular functions including metabolism, apoptosis, proliferation, cell growth and inhibition of differentiation. Thus, MYC alterations in cancer would induce profound changes in the behavior of cells.

The MYC protein is deregulated in approximately 70 % of all human cancers. These alterations range from amplification and overexpression to chromosomal translocations. Indeed, *MYC* is the most amplified oncogene in human cancers [209,210]. The first discovery of *MYC*-amplification

was in 1982 in HL60 leukemia cells [211]. *MYCN* and *MYCL* were also identified due to their amplification in neuroblastoma and small cell lung cancer, in 1983 and 1985, respectively [159,160]. Importantly, translocation of *MYC* under the promoter of immunoglobulin (Ig) heavy chain enhancer is a hallmark in Burkitt lymphoma (BL), as first shown in 1982, shortly after MYC discovery [212,213]. Despite being deregulated transcriptionally, MYC protein abundance is also impacted in several ways. For instance, factors affecting its stability due to alterations in proteins regulating proteasomal degradation or point mutations, although the latter are rare, occurring in BL, ALL and acquired immunodeficiency syndrome (AIDS)-associated lymphomas [214–218]. Moreover, mutations in signaling pathways that control the transcription of MYC, as PI3K/AKT, MAPK, and WNT, can also lead to an increase in its transcription [176,219,220].

The cell cycle is, in part, highly controlled by MYC. It has been demonstrated that overexpression of MYC in quiescent cells leads to cell cycle entry. Cell cycle progression and cell proliferation are induced by MYC via regulating the expression of the genes participating in these processes. The cell cycle is mediated by cyclins and CDKs. MYC induces the expression of CDK4 and cyclin D2 which in turn phosphorylate and inactivate RB during G1 phase. Moreover, MYC upregulates the levels of cyclin E1 and E3, forming a complex with CDK2, critical for G1/S transition, and of cyclin B required for CDK1 to enter mitosis [221–223]. In addition, MYC controls the levels of cyclin-dependent kinase inhibitors (CKIs) p15, p21 and p27, responsible for blocking cell cycle progression [208,224]. Alteration of MYC in tumors impairs the expression of CKIs and induces the expression of cyclins and CDKs, stimulating uncontrollably the cell cycle [225].

Apart from driving cell cycle progression, MYC also promotes apoptosis to limit cell growth in inappropriate environments [226]. The tumor suppressor p53 induces apoptosis and inhibits cell proliferation in response to cellular stress, including hypoxia, DNA damage, expose to chemicals or radiation. The protein mouse double minute 2 homolog (MDM2) controls the levels of p53 by inhibiting its transcriptional activity and inducing its degradation. MYC stabilizes p53 protein by inducing the expression of ADP-ribosylation factor (ARF), which in turn will inhibit MDM2. Stabilization of p53 translates in an increase on the levels of the pro-apoptotic proteins BCL-2-associated X protein (BAX), p53 upregulated modulator of apoptosis (PUMA) and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) [227]. Moreover, MYC inhibits the expression of the anti-apoptotic markers B-cell lymphoma 2 (BCL-2) and B-cell lymphoma-extra-large protein (BCL-X$_L$), but induces expression of BCL-2-associated X protein (BAX) and BCL-2 homologous antagonist killer (BAK), necessary for apoptosis induction in the mitochondria [228–231]. However, in cancer cells with deregulated MYC expression the apoptotic response is low, indicating that mutations in other effectors of the death program might be also altered in cancer. For instance, overexpression of BCL-2 or loss of p53 [232].

Since this thesis focuses on the effects of MYC in metabolism and differentiation of cancer cells, the role of MYC in these two processes will be explained in more detail.

1.4.2.1   MYC induction of metabolic rewiring

MYC has a major role in the metabolic adaption of tumors as it increases glycolysis and lactate production and export, and in addition has effects on glutaminolysis, lipid metabolism and mitochondrial mass and function [233] (Figure 7).

The levels of GLUT1, HK2 and pyruvate dehydrogenase kinase (PDK), the enzyme that promotes lactate production by blocking the use of pyruvate by the TCA cycle, are upregulated by MYC [234,235]. Furthermore, the expression of LDHA and the lactate transporter, monocarboxylate transporter 1 (MCT1), are also controlled by MYC, resulting in an increase in lactate production from pyruvate [235,236]. In addition, MYC indirectly regulates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and triose phosphate isomerase (TPI) [235].

Glutamine addiction is mainly driven by the MYC oncoprotein. It has been shown that deprivation of glutamine in MYC-overexpressing cells leads to apoptosis [237]. MYC promotes glutaminolysis by increasing the levels of the glutamine transporter SLC1A5 (also known as ASCT2) [238], and elevating GLS levels via the inhibition of miR-23a and miR-23b [239].

Essential amino acids (EAAs) are necessary signaling molecules used for the synthesis of proteins. In cancer, MYC deregulation is associated with an increased in the levels of the receptors for several of these EAAs, including the solute carriers SLC7A5 and SLC43A1. Elevated EAA levels activate a positive feedback loop, and in turn induce *MYC* mRNA translation [240].
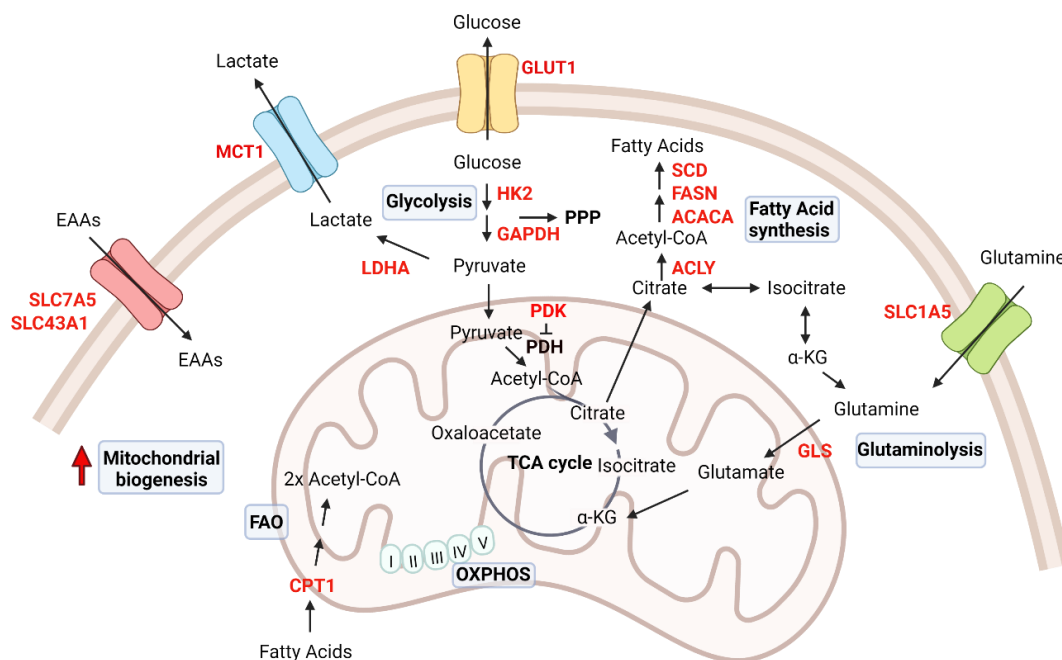
Figure 7: Metabolic reprogramming induced by MYC. Several metabolic pathways are altered in cancer due to MYC deregulation. MYC induces glycolysis, fatty acid synthesis, glutaminolysis, fatty acid oxidation and mitochondrial biogenesis. In red, the enzymes upregulated by MYC. Created with Biorender.com.

Both glucose and glutamine can be used for acetyl-CoA production in the TCA cycle. Acetyl-CoA is the precursor for lipid biosynthesis by ACACA and FASN. MYC activates lipid synthesis by upregulating the expression of these two lipogenic enzymes, as well as by inducing the production of citrate and elevating the levels of ATP citrate lyase (ACLY) and stearoyl-CoA desaturase (SCD) [241–243]. Moreover, MYC requires MONDOA to induce the expression of SCD and FASN, promoting the synthesis of fatty acids [121]. In addition, MYC also induces mitochondrial biogenesis and β-oxidation. MYCN inhibition in neuroblastoma cells results in a reduction of enzymes participating in FAO, which in turn leads to accumulation of cytoplasmic lipid droplets [244].

As MYC regulates the expression of several enzymes important for mitochondrial activity, enhanced MYC expression is associated with increased mitochondrial mass and function as well as OXPHOS [245].

1.4.2.2 Differentiation controlled by MYC

Since MYC induces cell cycle entry and progression, it was assumed that it inhibits terminal differentiation. In fact, the role of MYC on differentiation was one of its first functions described [246,247]. MYC levels are drastically downregulated upon cell differentiation. However, the mechanism by which MYC prevents this process is not associated with its role in proliferation [248,249]. Instead, it depends on the repression of several transcription factors involved in the differentiation of different lineages, such as c-JUN for neuronal and GATA1 for erythroid differentiation [184,250].

Many studies have highlighted the role of the MXD protein family in differentiation. For example, they are expressed in differentiated cells, although the different members appear to be tissue specific [251]. Moreover, they are upregulated in differentiation experiments *in vitro*, and their ectopic expression results in growth arrest and alters MYC transformation function, inhibiting tumor growth. This indicates that MXD proteins, with their ability to dimerize with MAX, repress the transcription of MYC target genes [252]. One study demonstrated that MYC/MAX complexes are replaced by MXD/MAX during HL60 differentiation [253]. During this switch, MXD proteins interact with the repressor SIN3 and class I histone deacetylases (HDAC), reducing the accessibility for other activators [254].

In addition, MYC is one of the four factors described by Yamanaka to reprogram human differentiated cells into induced pluripotent stem (iPS) cells, together with octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2) and Krüppel-like factor 4 (KLF4) [255]. However, while MYC is not crucial for the process, it increases the efficiency of reprogramming [256,257]. In fact, due to the tumorigenic potential of MYC, 20% of the mice generated from iPS cells developed tumors [127]. These findings indicate that the role of MYC in maintaining stemness is linked with inhibition of differentiation and tumorigenesis.

1.4.3 Targeting MYC in cancer

The deregulated expression of MYC in many different tumors is associated with poor prognosis and increased resistance to chemotherapy. Additionally, MYC regulates the expression of a plethora of genes with function in different cellular processes contributing to cancer cell survival, proliferation and invasiveness [258]. These two aspects make MYC a promising target for cancer therapy.

MYC downregulation leads to growth arrest, enhanced apoptosis, differentiation, and tumor regression in many human cancer model systems [259]. Yet, why is there no MYC inhibitor approved for the treatment of cancer patients? Since the past 20 years, considerable efforts have been made to develop a specific MYC inhibitor for clinical purpose without much success, and MYC has been defined as an undruggable protein for many reasons. First, MYC lacks a binding pocket, usually used as target in drug discovery. Second, due to its vast effects in basic cellular processes, MYC inhibition may cause toxicity and serious side effects on normal cells and tissues, that could cause the candidate compound to fail in clinical trials. Third, MYC is a nuclear transcription factor with an intrinsically disordered structure until its dimerization with its partner protein. Despite of these drawbacks, several direct and indirect strategies have been developed for targeting MYC, including disruption of MYC stabilization and dimerization with MAX, inhibition of MYC transcription and alterations in processes induced by MYC [260]. Some of these strategies are described below.

Direct strategies for MYC targeting

A stable conformation in MYC structure is achieved upon dimerization with its partner MAX. Moreover, for its transcriptional activity, MYC requires the interaction with MAX to bind the E-boxes in the DNA. Different small molecules acting as MYC-MAX dimerization inhibitors have been identified and characterized. The first generation of these inhibitors, 10058-F4 and 10074-G5, were described to disrupt the MYC-MAX complex, leading to cell death and induction of differentiation *in vitro* [261]. However, they showed low specificity and cell penetration, with an IC$_{50}$ of 42 μM for 10058-F4 and 20 μM for 10074-G5, limiting their potential use in the clinic [260,262].

To improve the potency of 10074-G5, a variant compound 3jc48-3 was developed, showing higher stability and potency than the original 10074-G5 molecule [263]. The small molecule KJ-Pyr-9, a MYC inhibitor found in a pyridine library, has a potent MYC-MAX inhibitory effect, with a K$_D$ of 6.5 nM, and demonstrated a reduction in the growth of *MYC*-amplified cancer cells *in vivo* [264]. Moreover, the small molecule MYCMI-6 disrupts MYC-MAX interaction resulting in suppressed growth *in vitro* and reduced tumor proliferation and induced apoptosis in an *in vivo* model for neuroblastoma [265]. A recently described inhibitor is MYCi975 with good tolerability and potent effect *in vivo*, increasing infiltration of immune cells [266].

One of the most potentially promising mechanisms for MYC targeting to date, is the use of the small peptide Omomyc, which comprises 90 amino acids corresponding to the bHLH-Zip domain of MYC, with substitutions in four amino acids [267]. Omomyc preferentially

heterodimerize with MAX and not MYC, or forms homodimers, acting as a dominant negative of MYC, inhibiting its binding to DNA by blocking E-boxes with inactive dimers [268,269]. Studies in different mouse models have demonstrated the efficacy of Omomyc independently of the type of cancer and with minimal to no toxicity, suggesting its potential applicability as anti-MYC drug in patients [270–273]. Omomyc is currently in clinical trials for the treatment of several solid tumors, with positive results from phase I indicating good tolerability, stabilizing the disease and with few side effects. This makes Omomyc to be the first MYC-targeted drug to successfully complete phase I and it is expected to start phase II shortly [274].

Indirect strategies for MYC targeting

As direct MYC targeting is challenging, different indirect approaches have been developed, for instance, blocking MYC transcription by disrupting the interaction of transcriptional and epigenetic regulators. An example is the use of bromodomain and extra-terminal domain (BET) inhibitors. These BET inhibitors replace bromodomains, as BRD4, in binding acetylated lysines in histones, promoting the transcription of several genes of the cell cycle, including *MYC*. One of these inhibitors, JQ1 produces cell cycle arrest and senescence, and reduces tumor growth in acute myeloid leukemia, multiple myeloma, and neuroblastoma both *in vitro* and *in vivo* [275–277]. JQ1 was the first-in-class compound showing the potential use of BET inhibitors for cancer treatment. However, it failed in clinical trials due to poor pharmacokinetics and cytotoxicity [278]. Despite the lack of success on its own, combination strategies of JQ1 with immune therapy or other epigenetic directed approaches, have demonstrated a more potent synergistic effect in several cancer mouse models [279,280]. Another inhibitor, I-BET762 decreases MYC levels resulting in reduction of tumor burden in prostate cancer, pancreatic ductal adenocarcinoma cells and in a patient-derived tumor model of prostate cancer [281,282]. Similarly, OTX-015 downregulates MYC expression and its transcriptional program, showing antitumor effect *in vitro* and *in vivo* in different types of cancer including neuroblastoma, hematological malignances, breast, and prostate cancer [283–286]. Even though OTX-015 entered clinical trials for the treatment of hematological and solid tumors, it was discontinued due to toxicity and adverse side effects [287,288]. Several other BET inhibitors are currently in phase I-II clinical trials for different types of cancer [289].

Another strategy is targeting CDK7, the catalytic subunit of the transcription factor IIH complex (TFIIH), that phosphorylates serine residues of RNA pol II, facilitating transcription initiation and elongation. Inhibition of CDK7 by THZ1 reduces MYC expression, showing anti-tumor effects in neuroblastoma [290].

In addition, targeting MYC protein stability has also been studied. The serine/threonine protein phosphatase 2A (PP2A) regulates MYC protein stability by dephosphorylating Ser62. Activators of this phosphatase have shown promising results in different types of cancer alone or in combination with other strategies [291,292]. Similarly, targeting proteins involved in the ubiquitination and deubiquitination of MYC for its degradation can be used as a strategy to target protein stability [293–295].

1.5 HYPOXIA

1.5.1 Hypoxia effectors and signaling pathway

Rapid growth and aberrant vascularization in solid tumors result in a reduction of oxygen availability. This condition of low oxygen is called hypoxia. To adapt to the hypoxic environment, cancer cells activate the expression of several signaling pathways, where the most important are the expression of the hypoxia inducible factors (HIFs). These consist of an oxygen sensitive α subunit (HIF1-α, HIF2-α and HIF3-α) and a constitutive expressed HIF1-β subunit [296].

The HIF1-β protein is also known as aryl hydrocarbon nuclear translocator (ARNT). This subunit was discovered before HIF1-α, and it was described to bind to aryl hydrocarbon receptor (AhR), necessary for its transportation to the nucleus [297]. All HIF members share a bHLH-PAS motif, crucial for the dimerization between the α subunits with HIF1-β for binding DNA at the hypoxia response elements (HREs). Moreover, two transactivation domains (N-TAD and C-TAD), important for their transcriptional activity, are found in the structure of HIF1 and 2-α, which share 48% similarity in their amino acid sequence. While the N-TAD is important for maintaining HIF stability, the C-TAD interacts with the co-activators. In addition, HIF1-α and HIF-2α contain two nuclear localization signals while only one is present in HIF3-α protein. Importantly, all three α subunits contain an oxygen-dependent degradation domain (ODDD), for mediating oxygen sensing (Figure 8) [297–299].

The HIF3-α protein exits in ten distinct variants that in difference to HIF1-α and HIF2-α, lack the TAD, but instead have a Zip domain, but are also regulated by oxygen. The different variants may have different functions if not opposite in some cases, although their regulation and biological roles are still poorly understood [300–302].
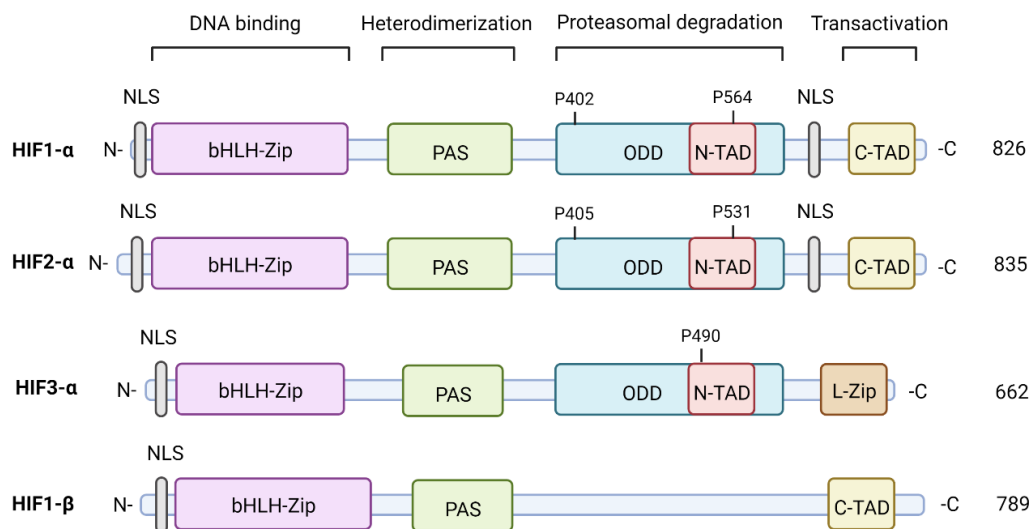
Figure 8: Schematic representation of the structure of the HIF proteins. All of them contain a bHLH-Zip domain for DNA binding, a PAS domain for heterodimerization with HIF1-β. All members except HIF1-β contain a ODDD where hydroxylation for proteasomal degradation occurs. Created with Biorender.com.

In normoxia, or normal oxygen concentration, HIF1-α is hydroxylated on proline residues P402 and P564, HIF2-α on P405 and P531, and HIF3-α on P490, located at the ODDD by prolyl hydroxylases (PHDs), triggering the recognition by the VHL protein [303,304]. VHL recruits elongin B, elongin C, and Cullin-2 to form an E3 ubiquitin ligase complex that will ubiquitinate HIF1/2-α, targeting it for proteasomal degradation [303]. Upon hypoxia, the PHDs are not able to hydroxylate HIF1/2-α subunits, since they are highly dependent on oxygen, as well as 2-oxoglutarate, ascorbate, and Fe (II) to catalyze the reaction [305]. Therefore, HIF1/2-α will dimerize with HIF1-β forming heterodimers that will translocate to the nucleus and bind to DNA at the HREs, activating transcription of the target genes [306]. As mentioned before, *VHL* is a tumor suppressor lost in approximately 90% of ccRCC tumors. Due to *VHL* loss the stabilization of HIF1-α and HIF2-α leads to a pseudohypoxic state associated with poor prognosis [307] (Figure 9).

Another way of regulating HIF is by controlling the transcriptional activation of its target genes, regulated by the co-activator CREB-binding protein (CBP)/p300. Hydroxylation in asparagine 803 (Asn-803) by asparaginyl hydroxylase (also known as factor inhibiting HIF-1, FIH-1) blocks the interaction with the co-activator [308,309].

Apart from the action of hydroxylases, HIFs are also regulated by oncogene activation in cancer, deregulating different signaling pathways. For instance, activation of PI3K/AKT/mammalian target of rapamycin (mTOR) and RAS pathways increases translation of HIF1-α [310]. Moreover, loss of p53 in several tumor types also upregulates HIF1-α expression [311].
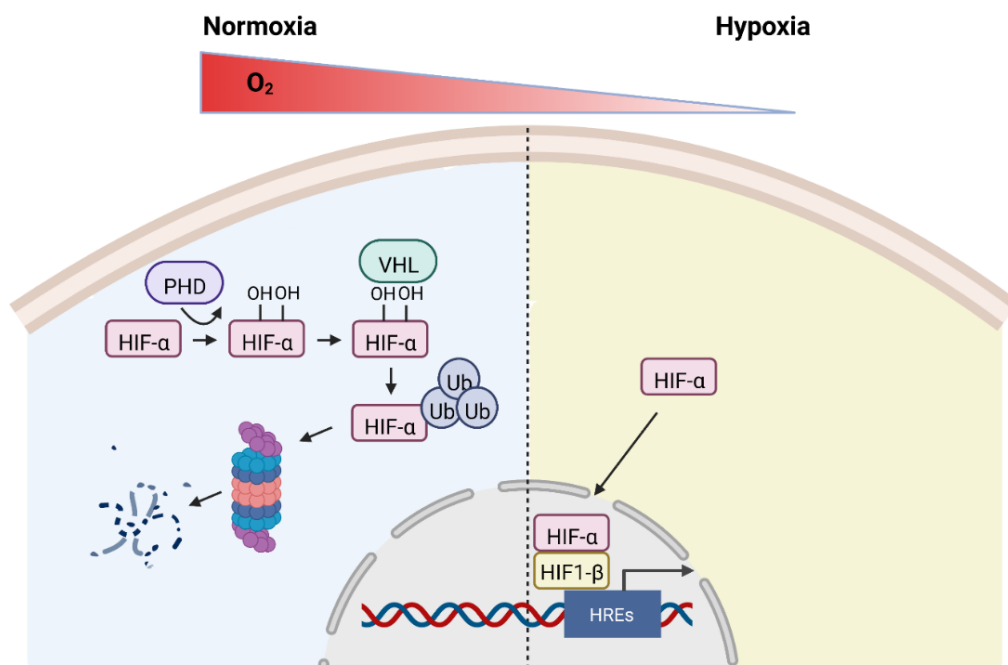
Figure 9: Normoxia vs hypoxia signaling pathways. In presence of oxygen PHDs hydroxylate HIF-α in two proline residues that are recognized by VHL, targeting them for proteasomal degradation. In hypoxia, when oxygen concentration is low, HIF-α can bind to HIF1-β in the nucleus activating the transcription of the hypoxia response genes. Created with Biorender.com.

1.5.2 Cell processes controlled by HIFs

Hypoxia inducible factors participate in different processes, including metabolism, angiogenesis, erythropoiesis, apoptosis, and proliferation. While in some cases HIF1-α and HIF2-α regulate the expression of the same targets, as for example vascular endothelial growth factor (VEGF) and GLUT1, resulting in overlapping functions, the differences in their TAD imply that they have exclusive targets. HIF1-α is known as a regulator of metabolic reprogramming by upregulating glycolysis, and HIF2-α is involved in tumor growth and cell cycle progression, mediating the transcription of cyclin D1, OCT4, and transforming growth factor α (TGF-α) [312,313]. Moreover, erythropoiesis and absorption and metabolism of iron are highly dependent on HIF2-α [314,315]. However, in absence of one of them, HIF1-α and HIF2-α can substitute the functions of each other in specific contexts [316,317].

As metabolic reprogramming is more relevant to the work discussed in this thesis, the role of HIF in controlling this process will be detailed in the following section.

1.5.2.1 HIFs induction of metabolic rewiring

Together with MYC, HIF1-α mediates the metabolic reprogramming that cancer cells need to satisfy their energy requirements. MYC levels are repressed by HIF1-α expression while HIF2-α enhances MYC activity [318,319]. But as many cancer cells show increased MYC levels, the inhibitory role of HIF1-α is repressed and both oncogenes collaborate to increase glycolysis under hypoxia [320].

Due to the lack of oxygen during hypoxia, cancer cells are unable to depend on OXPHOS to obtain ATP. Since the ratio of ATP/AMP (adenosine monophosphate) decreases, it induces the allosteric regulation of the glycolytic enzyme phosphofructokinase (PFK), activating glycolysis [321]. Following the allosteric control, upregulation of HIF1-α activates the expression of different glycolytic proteins: GLUT1 and GLUT3 glucose transporters, as well as several glycolytic enzymes such as HK1 and HK2, PFK, aldolase (ALDO), phosphoglycerate kinase-1 (PGK1), enolase (ENOL), LDHA, and the lactate transporter monocarboxylate transporter 4 (MCT4), elevating the glycolytic flux [322–324]. In addition, HIF1-α also activates PDK, avoiding the entrance of pyruvate into the mitochondrion and reducing the TCA cycle flux [325]. Mitochondrial autophagy is also activated by HIF1-α, and together with a reduction in cytochrome c oxidase (complex IV of electron transport chain), the formation of ROS is prevented, protecting the cells from oxidative damage [326,327].

A different case is observed for HIF2-α. Although it can upregulate the levels of GLUT1, it does not elevate other glycolytic enzymes [328]. In conclusion, the impact of HIF1-α in metabolic reprogramming results in switching ATP production from OXPHOS to glycolysis.

Moreover, HIF1-α stabilization induces the reductive carboxylation of glutamine, contributing to the synthesis of fatty acids, in part to compensate the lack of citrate due to decreased activity of the TCA cycle [329,330]. In addition, glutaminolysis is enhanced by HIF1-α as a result of increased levels of GLS [331].

Uptake of fatty acids and other lipids mediated by peroxisome proliferator-activated receptor γ (PPARγ) is upregulated during hypoxia [332]. Enhanced fatty acid synthesis via the upregulation of FASN [333], and reduction of FAO due to CPT1 inhibition by HIF1-α, contribute to the accumulation of fatty acids in lipid droplets (LDs) [334]. In fact, hypoxia promotes the expression of several LD associated proteins, including perilipin 2 (PLIN2) and hypoxia inducible lipid droplet associated (HILPDA/HIG2), the last one inhibiting adipose triglyceride lipase (ATGL) and impairing lipolysis [335,336].

1.5.3 Targeting HIFs in cancer

Like MYC, inhibition of HIFs has proven to be challenging. However, different strategies have been described including inhibitors of the expression, protein stabilization and dimerization, inhibition of their transcriptional activity, and targeting upstream or downstream pathways.

Some drugs inhibiting signaling pathways regulated by HIF include the anti-VEGF monoclonal antibody bevacizumab, the tyrosine kinase inhibitors (TKI) for the VEGF receptor sorafenib and sunitinib are approved by the Food and Drug Administration (FDA) and are currently used for cancer treatment, especially for ccRCC [337–339]. However, patients develop resistance to VEGF inhibitors by compensating with upregulation of other angiogenic factors [340]. Inhibitors of mTOR also reduce HIF expression, representing a potential therapeutic strategy [341,342]. To overcome the resistance to the treatment, several combinatory strategies have been adapted, such as simultaneous treatment with TKI and mTOR inhibitors, or TKI and the immune checkpoint inhibitors programmed death ligand-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [343,344].

Several inhibitors have been described to disrupt the dimerization with HIF1-β. In the case of HIF2-α, PT2399 and PT2385 were the first ones identified, but even though they successfully dissociate the HIF2-α/HIF1-β complex, tumors acquire resistance [345,346]. A second-generation inhibitor, MK-6482, shows an improved potency and specificity, currently in a phase III clinical trial, indicating its potential as a HIF2-α inhibitor [347,348]. Acriflavine inhibits the dimerization of both HIF1-α or HIF2-α to HIF1-β, being a potent candidate to those cancers with overexpression of both proteins [349].

Other approaches include disrupting the interaction with the transcriptional co-activators p300/CBP and induction of HIF1/2-α proteasomal degradation. Chetomin, a fungal metabolite, inhibits the interaction of both HIF1-α and HIF2-α with p300/CBP, resulting in anti-tumoral effects [350]. Yet, due to its toxicity, this small molecule never reached clinical trials [351]. By binding to heat shock protein 90 (HSP90), HIF1-α blocks its proteasomal degradation. Thus, HSP90 inhibitors have been described to reduce HIF1-α stability. Geldanamycin and SCH66336 are two examples of such. However, since other proteins also bind to HSP90 to avoid their proteasomal degradation, they are not very specific for targeting HIF1-α [352,353].

1.6 LIPID DROPLETS

1.6.1 Structure of lipid droplets

Lipid droplets (LDs) are unique organelles formed by a phospholipid monolayer, with a central core rich in neutral lipids, especially sterol esters (SEs) and triglycerides (TGs) (Figure 10). While in white adipocytes LDs are mainly composed by TGs, in other non-adipose cells different ratios of TGs:SEs can be found. Moreover, depending on the cell type, other lipids in small proportions like retinyl esters, free cholesterol and ether lipids accumulate in LDs [354–356].
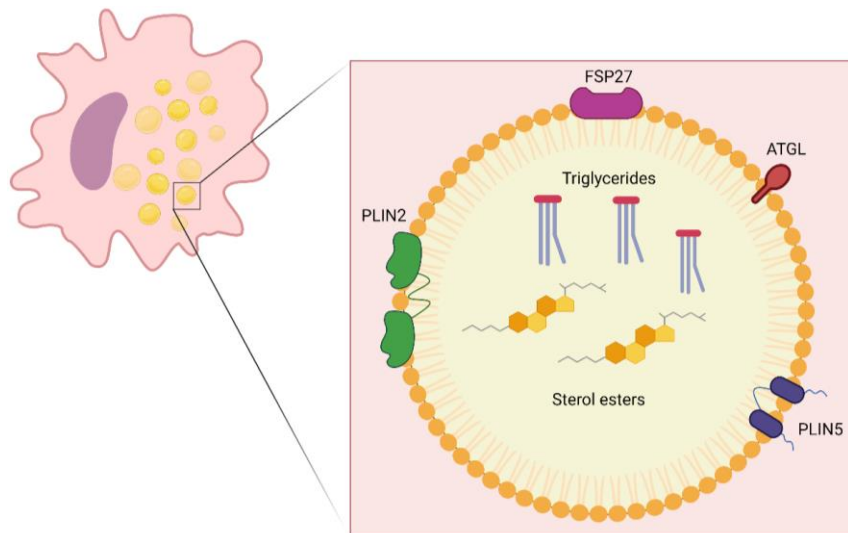
Figure 10: Structure of a lipid droplet. Triglycerides and sterol esters accumulate in the core of LDs. Several different proteins important for LDs to perform their function are located in the phospholipid monolayer. Created with Biorender.com.

The membrane of the LDs is predominantly composed by phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM) [357,358]. Structural and functional proteins are embedded or localized at the phospholipid monolayer, playing an important role in lipid homeostasis and function of LDs. Using proteomics, approximately 200 different LD associated proteins have been identified [359,360]. The most common are the PAT proteins consisting of Perilipin (PLIN1), Adipocyte differentiation related protein (ADRP/ADFP/PLIN2), and Tail interacting protein 47 KDa (TIP47/PLIN3) [361–363]. Other members of these family include S3-12 (PLIN4) and myocardial LD protein (MLDP/OXPAT/LSDP5/PLIN5) [364,365]. The different PLIN proteins are the most studied and are used as markers for detecting the presence of LDs.

Lipid droplets are formed upon the increase in intracellular free fatty acids, to avoid lipotoxicity. Although the biogenesis of LDs is still poorly understood, they are believed to derive from the endoplasmic reticulum (ER) [366,367]. The process of neutral lipid synthesis is controlled by enzymes and proteins within LDs in multiple steps (Figure 11). First, free fatty acids need to be activated by binding acyl-CoA, a step performed by long chain acyl-CoA synthetase (ACSL) [368]. Glycerol-3 phosphate (G3P) will be converted to phosphatidic acid (PA) via glycerol-3-phosphate O-acyltransferase (GPAT) and 1-acylglycerol-3-phosphate O-

acyltransferase (AGPAT). In the last two steps, PA will form diacylglycerol (DAG) via phosphatidic acid phosphatase (PAP), to finally generate TG by diacylglycerol acyltransferase (DGATs) enzymes.

The synthesis of sterol esters is performed by the enzymes acyl-CoA cholesterol O-acyltransferases (ACAT1 and ACAT2), that together with DGAT are located in the ER [369,370]. As the amount of neutral lipids increases due to high abundance of fatty acids, they begin to accumulate between the bilayer of the ER. When enough neutral lipids are accumulated, a nascent LD is released to the cytoplasm. These organelles are mainly formed *de novo*, but fission of preexisting LDs can also derive in new ones [371].
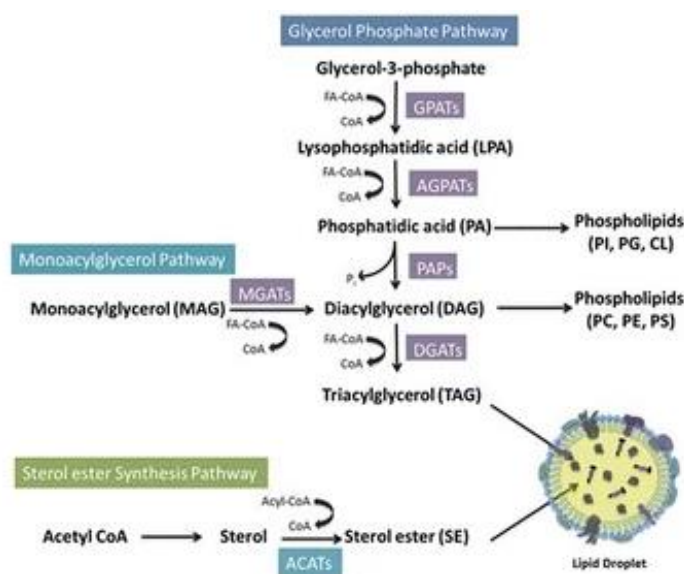
Figure 11. Triglyceride and sterol ester synthesis pathways during the formation of LDs. The intermediate lipid species and the enzymes participating in both processes are indicated. Adapted from Onal *et al*., 2017, Lipids in Health and Disease, with permission from the publisher Springer Nature (CC BY 4.0).

1.6.2 Role in cancer

Lipid droplets are dynamic organelles that are in continuous biogenesis and breakdown according to nutrient needs. Although changes in energy metabolism are responsible for LD accumulation, several studies demonstrated that their formation is also induced in situations of cellular stress as imbalances in redox homeostasis, oxygen deprivation, inflammation, autophagy, cell death, and mitochondrial dysfunction [372–375].

For a long time, LDs have been considered as inert fat storage organelles [376]. However, they serve as lipid homeostasis regulators, controlling nutrient and oxidative stress and promoting survival of cancer cells [377–379]. Their accumulation occurs in many types of cancer, including ovarian, colon and renal cell carcinoma [380–382].

1.6.2.1 Energy storage and lipid trafficking

As discussed earlier, synthesis of fatty acids is enhanced in cancer cells. Lipids can be obtained from the extracellular milieu and from symbiosis with the tumor microenvironment, including

lipid droplets in adipocytes [381,383]. However, if the extracellular lipid content is exhausted, cells might acquire lipids from internal storage in LDs, autophagy of organelles, or phospholipid lipolysis [384,385]. Lipid droplets are in the center of all these processes regulating the traffic of lipids from extracellular or internal sources and their use for specific cellular needs.

Two proteins are responsible for nutrient and energy sensing: AMP-activated protein kinase (AMPK) and mTOR. In cancer cells, due to the constitutive activation of proliferative signaling pathways by oncogenes, AMPK and mTOR are dysregulated, contributing in large part to metabolic reprogramming. AMPK detects low energy and glucose levels inhibiting anabolic pathways such as synthesis of fatty acids and other lipids, and activate FAO, mitochondrial biogenesis, and OXPHOS [386,387]. mTOR is negatively regulated by AMPK and promotes cell growth and survival. In nutrient rich environments, cancer cells will accumulate the excess of fatty acids in form of TGs in LDs. But during starvation, AMPK activates mitochondrial oxidation, LDs will be directed towards mitochondria and fatty acids from these organelles will be used for energy production [388]. The progressive supply of fatty acids from LDs protects cancer cells from nutrient deprivation increasing their survival for prolonged times. In fact, FAO enzymes, including the rate limiting CPT1, are elevated in this situation and its targeting, together with downregulation of ATGL, prevents the survival effect of LDs in cancer cells [389,390].

Accumulation of LDs during hypoxia has been observed in different tumor types [336,391]. Under oxygen deprivation, LD formation contributes to ER and redox homeostasis, while during reoxygenation LDs provide mitochondria with fatty acids, promoting in both cases cell proliferation and survival [336,374].

1.6.2.2 Avoiding lipid peroxidation

The increased amount of free polyunsaturated fatty acids (PUFAs), and in a less extend DAG, cholesterol, and ceramides, leads to lipid peroxidation and thus, imbalances in lipid metabolism. The process of lipids accumulating in non-adipose tissues is known as lipotoxicity and has damaging consequences for the cells such as mitochondrial dysfunction, defective signaling pathways, DNA and protein damage and ROS production [392,393].

PUFAs, mostly present at the cell membrane, can be oxidized by ROS, forming iron-dependent lipid peroxides (LPO), which in high amounts induce a unique form of programmed cell death denominated ferroptosis [394]. For instance, induction of ferroptosis by inhibiting antioxidant pathways results in cell death in different types of cancer [395–397]. Lipid droplets protect cancer cells from ferroptosis by controlling the trafficking of PUFAs and emerging as antioxidant organelles [390]. In addition, PUFAs contained in LDs and released via ATGL enzyme may accumulate in the phospholipid cell membrane sensitizing cancer cells to ferroptosis [396].

1.6.2.3 Chemoresistance

Alterations in lipid metabolism, including the presence of LDs, have been associated with resistance to conventional chemotherapeutics in cancer cells [398,399]. For example, elevated

expression of a LD associated enzyme, lysophosphatidyl-choline acyltransferase 2 (LPCAT2), correlated with an increase in LD content and resulted in reduction of chemotherapy-induced ER stress in a model of colon cancer [400]. In addition, *de novo* accumulation of LDs induced by chemotherapeutic drugs has also been reported [401]. Doxorubicin and 5-fluorouracil, induced the accumulation of LDs and cell membrane lipid fluidity, which could trigger invasion and metastasis in liver and colon cancer [401].

Collectively, considering the crucial role of LDs in regulating lipid trafficking, storage, and release, contributing to fatty acids supply, accumulating toxic lipids, and promoting cell survival, they are emerging as promising targets for cancer treatment.

1.7 NEUROBLASTOMA

Neuroblastoma (NB) is a rare type of tumor of the sympathetic nervous system affecting children and young adults. It is the most common solid tumor that develops extracranially, and the most deadly cancer in childhood [402]. Neuroblastoma accounts for approximately 7% of all childhood cancers, with 11 to 13 cases per million [403].

The cell of origin is still unknown, although it is believed to arise from neural crest-derived precursors from the sympathoadrenal lineage.

Stages, classification, and origin

Neuroblastoma is a heterogeneous disease with a variable clinical presentation, from tumors that regress spontaneously without any clinical intervention to highly disseminated disease with poor patient survival and resistance to therapy. The International Neuroblastoma Staging System (INSS) which uses a post-surgical classification of tumors was developed in 1988 [404]. This system distinguishes several stages: 1, 2 (A and B), 3, 4, and 4S. While in children diagnosed with stage 1 NB the tumor can be completely removed and cancer cells do not reach lymph nodes near the tumor area, in stage 4 the primary tumor has spread to distant lymph nodes and formed metastasis in bones, bone marrow, and other organs. Stage 4S is a special case of NB affecting infants younger than one year where the tumor has spread to the skin, liver, and/or bone marrow (with less than 10% of the cells in the bone marrow showing cancer characteristics). Interestingly, very frequently, tumors from 4S group regress spontaneously without any therapy [405].

However, due to the limitations in the INSS system another classification strategy was suggested in 2009, the International Neuroblastoma Risk Group Staging System (INRGSS) [406]. Contrary to INSS, this method used pre-surgical imaging tests by observing the presence or absence of image-defined risk factors (IDRF). This method classifies the tumors in L1, L2, M, or MS. Tumors from L1 stage are contained in the area they started and no IDRF are found. In L2, the tumor has also not spread but IDRF are identified. Tumors that metastasize comprise group M, and MS, like group 4S in INSS system, are tumors that spread to skin, liver and/or bone marrow (with 10% of the cells in the bone marrow showing tumor characteristics) that develop in children under 18 months. Including other prognostic factors such as age, histologic

classification, grade of differentiation, MYCN and neurotrophic receptors status, chromosomal aberrations (11q and 1p deletion), and cell ploidy, INRGSS defines the risk of NB in very low, low, intermediate, or high risk. This strategy provides the best classification of the tumors to determine the therapeutic approach.

Neuroblastoma is a developmental disorder. Understanding its origin is essential to study the tumorigenic process. The neural crest is a transient structure appearing after the neural tube closure during embryogenesis. Progenitor cells from the neural crest will differentiate into several cell types including peripheral sensory and enteric neurons, Schwann cells, melanocytes, adrenal medulla, and cells from the craniofacial skeleton [407]. Neuroblastoma develops in the sympathetic nervous system, mainly in the adrenal medulla and paraspinal ganglia, but lesions can also be found in neck, chest, and pelvis [402]. Many different signaling pathways are involved in neural development such as sonic hedgehog (SHH), WNT, neurogenic locus notch homolog protein (NOTCH), bone morphogenic protein (BMP), tyrosine kinase receptors, and TGF-β [408]. Activation of MYCN via the WNT pathway is essential for the proliferation and migration of neural crest progenitors, while its expression is downregulated for inducing terminal differentiation of sympathetic neurons [409]. Deregulated MYCN expression and other alterations are involved in NB development, as described below.

1.7.1 Genetic alterations

Although most NB cases occur due to spontaneous mutations, some patients have a hereditary predisposition for the disease. Familial neuroblastoma accounts for the 1-2% of the cases, presenting an autosomal dominant inheritance. *Paired-like Homeobox 2B* (*PHOX2B*) and *Anaplastic Lymphoma Kinase* (*ALK*) have been identified as NB predisposition genes [402]. The *PHOX2B* gene is important for the normal development of the autonomic nervous system, and missense or frameshift mutations in this gene accounts for 6-10% of familial NB cases [410]. Activating mutations in the tyrosine kinase domain of ALK are responsible for 50% of familial NB cases, and in 10-12% of sporadic NB [411]. Other germline mutations associated with predisposition to NB have been identified, including *TP53*, *breast cancer gene 2* (*BRCA2*) and *HRAS*, but their clinical relevance needs to be further studied [412].

Sporadic mutations leading to NB development include gene amplifications, polymorphisms, and chromosomal alterations. Amplification of *MYCN* is found in approximately 20% of all NB cases and in 40% of high-risk NB, and is a marker for poor prognosis [413]. During sympathoadrenal development, the excess of progenitor cells that did not undergo differentiation is eliminated by apoptosis [414]. The presence of MYCN during the maturation process blocks apoptosis and induces proliferation, leading to NB formation [415]. *In vivo* studies demonstrated that MYCN overexpression in sympathoadrenal precursors results in NB progression [415].

The gain of the distal portion of 17q is the most common chromosomal alteration found in NB and a marker for poor prognosis. Moreover, it is correlated with *MYCN* amplification and 1p36 deletion [416]. LOH at 11q is also associated with poor outcome, occurring in approximately 33%

of neuroblastomas, but inversely correlated with *MYCN* amplification, representing a marker for aggressive subtypes with a non-*MYCN*-amplified status [417].

1.7.2 Neuroblastoma and metabolism

In the last decade there has been an emerging interest on studying cancer metabolism, although only recently the rewiring of energy pathways occurring in NB started to be described. Since many metabolic enzymes are directly regulated by *MYCN*, its amplification in NB has a profound impact in metabolic reprogramming.

Elevated levels of the glucose metabolism enzymes, HK2 and GLUT1, inducing a shift towards glycolysis, are associated with *MYCN* amplification in NB [418]. Moreover, the expression of LDHA is increased in NB. Although it was firstly described to be dependent on MYCN [419], a later study demonstrated that LDHA expression is not correlated to *MYCN* amplification, but necessary for NB growth and tumorigenicity [420].

An increased in amino acids synthesis and upregulation of their transporters is also correlated with amplification of *MYCN*. For instance, two solute carriers SLC7A5 and SLC43A1, controlling the intracellular levels of several EAAs such as isoleucine, leucine, phenylalanine, and valine, are transcriptionally activated by MYCN. If these transporters are depleted, *MYCN* mRNA translation is reduced. This data indicates a positive feedback loop that sustains NB cells growth by amplifying their expression [240]. In addition, by increasing the mRNA expression of activating transcription factor 4 (ATF4), MYCN upregulates the expression of enzymes participating on the synthesis of non-essential amino acids, and increases the levels of their transporters [421,422]. Moreover, enhanced glutamine metabolism is essential for NB cells proliferation and survival, since glutamine deprivation induces apoptosis [237]. Together with ATF4, MYCN induces the expression of the glutamine transporter SLC1A5 (ASCT2). Glutaminolysis is also stimulated by MYCN by direct activation of GLS transcription [423,424]. Furthermore, as we describe in paper I, *MYCN*-amplified NB cells synthetize glutamine *de novo* [116].

Nucleotide pools in NB cells are also regulated by MYCN by promoting purine and pyrimidine biosynthesis, as well as one-carbon metabolism [422,425,426]. In coordination with ATF4, MYCN increases the levels of enzymes producing glycine and the one carbon unit 10-formyl-tetrahydrofolate (THF) [422]. Moreover, 5-phosphoribosyl-1-pyrophosphate (PRPP) synthase 1 (PRPS1), phosphoribosyl formylglycinamidine synthase (PFAS), carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and carbamoyl-phosphate synthetase 2 (CAD), and dihydroorotate dehydrogenase (DHODH), responsible for the synthesis of purines and pyrimidines are also upregulated by MYCN in NB [426].

One study published this year shows that MYCN regulates the transport of fatty acids in NB cells via directly targeting *SLC27A2*, encoding for fatty acid transporter protein 2 (FATP2) [427]. Moreover, by activating the expression of MONDOA, MYCN increases the levels of the transcription factors SREBPs, promoting fatty acids and cholesterol synthesis [121]. Further discussed in paper I-III, we identified that FAO is the preferred source for energy production

in *MYCN*-amplified NB cells, and that inhibition of both fatty acid synthesis and FAO reduces proliferation *in vitro* and tumor burden *in vivo* [116,428]. We also described that activation of several NHRs in NB cells induces the accumulation of LDs [429,430].

A mechanism independent of MYCN has been described to alter energy metabolism in NB. The transcription factor Cut-like homeobox 1 (CUX1) upregulates the levels of glycolytic enzymes, promoting glycolysis, growth and metastasis in NB cells [431].

Despite the vast role of MYCN in regulating the metabolic reprogramming in NB, there are many aspects that need to be further studied to develop metabolism-based therapies. For instance, the metabolic interplay between NB cells, the tumor microenvironment, and immune cells. In addition, many of the metabolic research in NB is mainly focused on high-risk cases with *MYCN*-amplification. Expanding the knowledge on the less aggressive groups could provide valuable insights on the role of metabolism in the different stages of NB.

1.7.3 MYCN control of differentiation in neuroblastoma

Expression of MYCN is crucial for normal embryonic development. Several studies in mice demonstrated that deletion of *MYCN* results in prenatal lethality [427,432]. However, its downregulation is needed for differentiation of the sympathoadrenal precursors. Amplification of *MYCN* correlates with an undifferentiated phenotype in NB, which is associated with bad prognosis [433]. Yet even though MYCN levels decrease for differentiation to occur, its expression is necessary to activate the differentiation program in NB cells [434].

The tropomyosin receptor kinase (TRK) family is essential for the correct development of the central and peripheral nervous systems. The three members of this family are *TRKA*, *TRKB* and *TRKC*, with different expression patterns. While *TRKC* and *TRKA* are crucial for early and late development of sympathetic neurons, respectively, *TRKB* is only expressed at early stages of sensory neurons development [435]. Enhanced expression of *TRKB* is associated with an aggressive phenotype in NB, and it is correlated with *MYCN*-amplification status. In addition, MYCN suppresses *TRKA* expression, considered as a marker for good prognosis [432].

Moreover, MYCN inhibits differentiation via the upregulation of the miRNA17~92 cluster, resulting in downregulation of several NHRs. As discussed in paper III, inhibition of MYCN or expression and subsequent activation of the NHRs, induces neurite outgrowth, expression of differentiation markers, and reduces tumor burden in NB cells [429,430,436,437].

Different therapeutic strategies have been developed to induce differentiation in NB cells. In the clinic, 13-*cis* retinoic acid (RA) is currently part of the maintenance treatment regimen for high-risk NB patients. Treatment with RA reduces tumor growth and promotes the expression of differentiation markers in NB cells [75,438]. Inhibition of MYCN with the small molecule 10058-F4 or with the use of short hairpin RNA (shRNA) against MYCN, also promotes the expression of neuronal differentiation markers in different NB cell lines [244,439]. In addition, and as shown in paper II, the use of fatty acid synthesis inhibitors results in neurite outgrowth and differentiation of NB cells [428].

1.7.4 Current treatments

For the treatment of NB, both clinical and biological risk factors, as described in the classification systems are considered. Briefly, for very low-risk and low-risk NB patients who are less than one year old, the procedure is observation without surgery unless the tumor grows. For patients older than one year and who present a localized tumor, surgery is performed, and in some cases, the procedure is accompanied by chemotherapy. For patients with intermediate risk, the approach includes tumor excision when possible and chemotherapy. In patients older than 12-18 months with tumors that are unresectable radiotherapy is used [440]. For tumors classified as high-risk NB, the treatment includes a combination of surgery, followed by high-dose chemotherapy, autologous stem cell transplantation, radiotherapy, immunotherapy, and treatment with 13 *cis*-RA. Despite this regimen, still 50% of high-risk patients cannot be cured [441]. Thus, new therapeutic approaches and agents are needed to improve the overall survival of the patients.

1.8 CLEAR CELL RENAL CELL CARCINOMA

Clear cell renal cell carcinoma (ccRCC) is the most common and aggressive type of kidney cancer, accounting for 75% of all cases. It is one of the sixth most common cancers diagnosed in men and the nineth in women [442]. These tumors provide the example of metabolic reprogramming in cancer, since many altered genes are related to energy pathways such as glucose or glutamine metabolism, and thus, labeled as a metabolic disease [443,444].

1.8.1 Stages, classification, and origin

The classification system for ccRCC comprises four stages (I to IV) complemented with the TNM system (Tumors, Nodes and Metastases) [445]. This classification provides detailed information about how large the tumor is, its growth into nearby areas and lymphatic nodes, and if it has spread to distant parts of the body affecting other organs. Tumors can be T 0-4, referring from tumors that are invisible, less than 7 cm, bigger than 7 cm, or spreading around or beyond the kidney and nearby tissues. Moreover, cancer cells can invade lymph nodes in different grades from N0, no cancers cells observed in any of the nodes, N1 only one node contains cancer cells, and N2 cancer cells are present in more than one node. Finally, M0 or M1 indicates the absence or presence of metastases in distant parts from the original tumor site. The different stages also indicate the grade of the disease, from stage I where the tumor is contained in the kidney and is less than 7 cm, to stage IV where the tumor has spread to nearby tissues and the lymph nodes, or to further tissues within the body.

In addition, ccRCC tumors can be graded depending on the cell morphology to predict how fast they will grow and the likelihood of spreading. The former grading system was the Fuhrman system, ranging from grade 1 with cells with small and round nuclei, resembling normal cells and absence of nucleoli, to grade 4 with bizarre and giant nuclei, multilobulated and chromatin clumped [446]. However, due to discordances and limitations in the classification, the International Society of Urology Pathology (ISUP) proposed a novel grading system, with

grade 1-3 based on the nucleoli prominence and grade 4 with multinucleated giant cells with signs of rhabdoid and/or sarcomatoid differentiation [447].

Apart from ccRCC, the other two main types of kidney cancer comprise papillary RCC (pRCC) with 15%, and chromophobe RCC (chRCC) 5% of the cases, respectively [448]. Although the mutations characterizing each of the RCC types are well known, mainly affecting enzymes of energy metabolism, identifying the different cell of origin from which each type arises is important to understand the tumorigenic process. Due to the high cellular heterogeneity within the kidney nephron and the differences observed between the different RCC tumors, it is believed that they originate from different parts [449]. Recent studies demonstrated that ccRCC and pRCC share one gene signature, characteristic from the proximal tubular cells while chRCC has similarities with the gene program from the collecting duct-residing intercalated cells [450,451]. In addition, the expression of these characteristic gene program is lost in aggressive forms of ccRCC indicating the undifferentiated nature of the tumor, while HIF-driven gene expression is observed at more advanced stages of the disease [450].

1.8.2 Genetic alterations

The most common genetic mutation in ccRCC is the loss of the *VHL* tumor suppressor gene in approximately 90% of all cases [450]. This event results in the constitutive stabilization of HIF1/2-α activating a vast number of target genes contributing to cancer progression. While 60% of these tumors express both HIF1-α and HIF2-α, the remaining 30% only express HIF2-α. In fact, HIF2-α has been linked to ccRCC progression, being the oncogenic driver for the disease [318,452].

However, although loss of *VHL* alone is a main factor for ccRCC development, it is not sufficient to drive tumorigenesis [453]. Different efforts have been made to develop animal models that resemble the disease by deleting *VHL* combined with the loss of other tumor suppressors or activation of oncogenes [454,455].

Other genetic alterations found in ccRCC include amplification and deletion of several regions including 3p and 14q chromosome loss and 5q gain [456,457], harboring potential tumor suppressor and oncogenes, respectively. For instance, 3p chromosome contains the genes *Polybromo 1* (*PBRM1*), *BRCA-associated protein 1* (*BAP1*) and *SET Domain Containing 2* (*SETD2*). Depletion or silencing of these genes *in vitro* results in increased proliferation, migration, and DNA replication stress [458,459]. Moreover, gain or overexpression of *Sequestosome 1* (*SQSTM1*), *Enhancer Of Zeste 2* (*EZH2*), *Stanniocalcin 2* (*STC2*), and *Versican* (*VCAN*), located in chromosome 5q are associated with ccRCC progression [460–462]. Additionally, other focal amplifications include *Murine Doble Minute 4* (*MDM4*), regulator of p53 and *JAK2*, while focal deleted regions affecting the tumor suppressor genes *Cyclin-dependent Kinase Inhibitor 2A* (*CDKN2A*) and *PTEN* are also common in this cancer type [457]. Genomic amplification of *MYC* at 8q24 occurs in only 5-10% of the patients and overexpressed in 20% of these tumors, yet it plays an important role in the metabolic reprogramming of ccRCC [463].

1.8.3 Metabolism of ccRCC

The constitutive stabilization of HIF1-α has a high impact in the metabolic reprogramming of ccRCC, favoring aerobic glycolysis and decreasing OXPHOS. Upregulation of glycolytic enzymes as well as LDHA and PDK, prevent the entry of pyruvate to the TCA cycle, shifting the flux towards lactate production [323,325]. This increase in the glycolytic flux provides intermediates that supply several biosynthetic pathways. One example is the PPP, generating ribose 5-phosphate and NADPH necessary for the generation of lipids and nucleotides, while NADPH is also used to reduce glutathione to regulate the levels of ROS [90]. Moreover, ccRCC cells display a lower mitochondria content associated with a reduction in respiration [464,465].

A hallmark of ccRCC cells is the accumulation of LDs and glycogen in their cytoplasm [466]. In fact, SCD and FASN enzymes responsible for cholesterol and fatty acid synthesis, respectively, are increased in ccRCC, associated with tumor growth, aggressiveness, and poor patient survival [467,468]. Inhibition of CPT1 in addition to the reduced mitochondrial activity results in a decrease on FAO, further contributing to the accumulation of LDs [334].

Glutamine consumption is elevated in ccRCC compared to normal kidney. This is in part due to the reprogramming in glutamine metabolism induced during hypoxia to generate fatty acids via reductive carboxylation [330]. In addition, these cancer cells use glutamine for glutathione synthesis maintaining the antioxidant balance protecting against ROS [444].

Moreover, the levels of argininosuccinate synthase-1 (ASS1), enzyme responsible for arginine synthesis are downregulated in ccRCC, and deprivation of arginine reduces tumor growth [469].

1.8.4 Current treatments

Patients with ccRCC present a worse prognosis compared to other types of kidney cancer, with 5-year overall survival of 70% for patients with localized disease and which is reduced to 14% for those with metastasis [470]. Treatment of ccRCC includes surgery, radiotherapy, and chemotherapy, but these strategies are very ineffective in advanced or metastatic stages. The different chemotherapeutic drugs used include anti-VEGF/VEGF-R TKIs (sunitinib, sorafenib and bevacizumab) and inhibitors of mTOR signaling pathway (everolimus and temsirolimus). However, the single treatment resulted in a modest efficiency with acquisition of resistance due to activation of other compensatory vascular signaling pathways, as well as off-target effect that negatively impacted the life of the patients [471].

Another alternative is the use of immune checkpoint inhibitors like nivolumab (anti-PD-1) or ipilimumab (anti-CTLA-4). Yet, it resulted in better survival than the TKIs for only few patients that could not receive the immune checkpoint inhibitors. Thus, combination strategies have been proposed as the best approach for targeting ccRCC. These include simultaneous treatment with VEGFR TKI and anti-PD1 monoclonal antibodies for those patients that are symptomatic and that require a prompt disease control, while combination of anti-PD-1 and anti-CTLA-4 demonstrated a more sustained disease control with time [471,472].

In conclusion, the low rate of overall survival and event free survival in patients with metastatic ccRCC requires new therapeutic approaches to face the disease. Hence, new targets and combination strategies are needed to improve the response to treatment in patients.

2 RESEARCH AIMS

The overall aims of this thesis were to (i) study the metabolic changes after targeting MYC(N) in neuroblastoma and clear cell renal cell carcinoma, and to (ii) investigate the interplay between MYCN inhibition, lipid metabolism and differentiation in neuroblastoma, to identify new targets for treating these cancer types.

The specific aims for the different papers constituting this thesis are to:

  I. Explore how MYCN regulates metabolic reprogramming of neuroblastoma cells.

  II. Study how *de novo* fatty acid synthesis influences neural differentiation in neuroblastoma.

  III. Analyze the effect of the combined expression and activation of three MYCN regulated nuclear hormone receptors, GR, ERα and RARα, on the induction of differentiation in neuroblastoma cells.

  IV. Delineate the molecular mechanism behind lipid droplet accumulation upon MYC inhibition and the interplay with hypoxia signaling in clear cell renal cell carcinoma.

3 RESULTS AND DISCUSSION

3.1 PAPER I. MYCN-ENHANCED OXIDATIVE AND GLYCOLYTIC METABOLISM REVEALS VULNERABILITIES FOR TARGETING NEUROBLASTOMA

Many commonly deregulated cellular processes during tumorigenesis are controlled by the MYC family. For instance, MYC regulates several metabolic pathways such as glycolysis, oxidative phosphorylation, glutaminolysis, and mitochondrial biogenesis. In NB, *MYCN*-amplification is found in nearly 20% of all cases and in 40% of high-risk tumors, associated with poor clinical outcome. However, how MYCN orchestrates metabolism in *MYCN*-amplified (MNA) NB tumors is still not well understood. One previous publication from our research group demonstrated that MYCN inhibition in NB cells induced accumulation of lipid droplets, suggesting its important role in metabolic reprogramming in this cancer type [244].

In **paper I**, our goal was to analyze the metabolic alterations induced by MYCN in NB. To this end, we performed proteomics after MYCN inhibition with a short hairpin RNA against MYCN (sh*MYCN*) induced by doxycycline (DOX) treatment, in the MNA cell line SK-N-BE(2) (BE(2) in short). Comparing our results with the mRNA expression data of 649 patients obtained from the NB Kocak dataset, we concluded that our *in vitro* model correlates with patient data and is thus an adequate system to study the effect of MYCN in NB metabolism. Analysis of the significantly expressed proteins with differences between BE(2)-sh*MYCN* ON and BE(2) sh*MYCN* OFF cells revealed changes in several metabolic pathways. We found that MYCN increased the expression of several enzymes involved in glycolysis (HK2, aldolase C, ALDOC, LDHA), in the antioxidant systems (glutathione S-transferase omega 2, GSTO2; peroxiredoxins, PRDX) and mitochondrial enzymes (citrate synthase, CS and IDH2), associated with poor patient survival. To analyze the impact of MYCN inhibition in glycolysis and OXPHOS, we performed functional metabolic analysis in different MNA and non-*MYCN*-amplified (NMNA) cell lines. Glycolysis was evaluated by measuring the extracellular acidification rate (ECAR), showing a decrease in all glycolytic parameters upon MYCN inhibition, and reduced values in NMNA NB cells. Oxygen consumption rate (OCR) was measured to assess OXPHOS activity. Likewise, BE(2)-sh*MYCN* OFF and NMNA NB cells displayed lower mitochondrial respiration activity compared to the MNA cells. Moreover, upon induction of metabolic stress, BE(2) sh*MYCN* ON cells showed an increase in both glycolysis and OXPHOS, while BE(2)-sh*MYCN* OFF and NMNA NB cells exhibited an elevation in the glycolytic response but not respiration parameters.

Since OXPHOS was enhanced in MNA cells, we wondered which of the main fuels, glucose, glutamine, or fatty acids, mitochondria used in these cells. We observed that MNA NB cells are more dependent on FAO and are completely independent of glutamine compared to BE(2)-sh*MYCN* OFF and NMNA cells. Several studies have shown that cancer cells with increased expression of MYC are addicted to glutamine [112]. The fact that MNA cells were independent of glutamine for mitochondrial respiration was therefore unexpected. Thus, we proceeded to analyze the impact on viability of reducing glucose or glutamine levels in the media. Deprivation of glucose drastically decreased MNA cells viability to 10%, whereas glutamine

starvation only diminished viability to 70% compared to complete media. We further studied the metabolic phenotype by performing isotope tracing experiments with U-^{13}C$_6$-Glucose and U-^{13}C$_5$-glutamine in BE(2)-sh*MYCN* ON cells as well as after five days of MYCN downregulation. Our results demonstrated that most of the intracellular glutamine was unlabeled, indicating that it was synthesized *de novo* from glucose via α-ketoglutarate in BE(2)-sh*MYCN* ON cells, whereas BE(2)-sh*MYCN* OFF cells obtained glutamine from the media.

A thorough analysis of the expression data from BE(2)-sh*MYCN* ON versus OFF cells revealed that MYCN upregulated many of the enzymes participating in FAO. Furthermore, functional metabolic assessment in the presence of BSA-palmitate, increased the oxygen consumption of BE(2)-sh*MYCN* ON cells compared to BE(2)-sh*MYCN* OFF and NMNA cells, suggesting the importance of MYCN for FAO capacity. Analysis of publicly available NB patient datasets showed that expression of *CPT1C*, the rate limiting enzyme of FAO, was associated with worse prognosis. Inhibition of CPT1C with two different compounds, etomoxir or teglicar, reduced the viability of MNA cells, the ability to form colonies, and resulted in accumulation of LDs. Notably, medium with delipidized serum, containing 2/3 less lipids than normal serum, also decreased MNA cells viability.

Finally, to analyze the impact of inhibiting FAO *in vivo*, we performed xenograft experiments with two MNA cell lines, SK-N-BE(2) and IMR-32, and one NMNA cell line, SK-N-AS, and animals were treated with etomoxir daily until experimental endpoint. Tumors derived from MNA cells showed a significant reduction in tumor growth after CPT1C inhibition, while no differences were observed in tumors from the SK-N-AS cells. Moreover, the former tumors presented less expression of the proliferation marker Ki67 and the hypoxia marker HIF1-α.
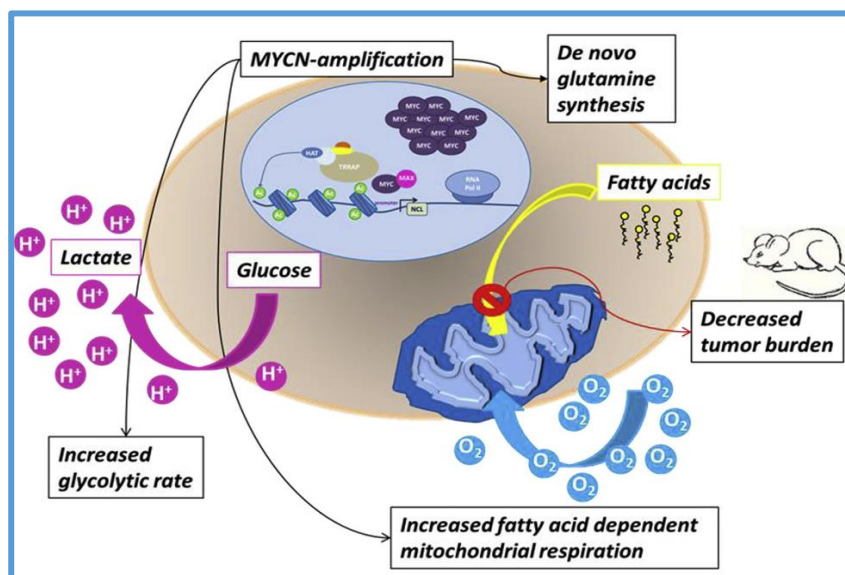
Figure 12: Graphical abstract of paper I. *MYCN* amplification increases glycolysis, fatty acid dependent OXPHOS and induces *de novo* synthesis of glutamine in NB cells. Moreover, targeting FAO decreases tumor burden *in vivo*. Reprinted from Oliynyk *et al.,* 2019, iScience, with permission from the publisher Elsevier (CC NC-ND 4.0).

To adapt to a new environment where nutrients and oxygen may be scarce and to maintain the highly proliferation and biosynthetic rate, cancer cells need to reprogram their metabolism. In part, these metabolic alterations occur as consequence of the disruption of the expression of oncogenes and tumor suppressor genes. The MYC family of proteins is involved in controlling many different energy pathways. Yet, how MYCN regulates metabolism in MNA NB tumors is still not well described.

In **paper I**, our aim was to provide a profound understanding on how MYCN impacts energy metabolism in MNA NB. With a combination of proteomics and gene expression data, we found that MYCN upregulated enzymes from glycolysis, OXPHOS, ETC and antioxidant pathways, as well as proteins involved in mitochondrial respiration, translation, biogenesis, and dynamic processes. Importantly, we demonstrated that MNA NB cells have enhanced glycolysis and OXPHOS, suggesting a highly energetic metabolic phenotype. We identified that MNA NB cells synthetize glutamine *de novo* from glucose, demonstrating the metabolic adaptation capacity of these cells. Moreover, our results showed that MNA NB cells were more dependent on FAO for mitochondrial respiration compared to NMNA cells. We further showed that inhibition of this process, by targeting CPT1C, reduced cell viability *in vitro* and tumor burden in a MNA xenograft model, while not affecting NMNA cells and tumors (Figure 12).

Together, our findings indicated that targeting fatty acid oxidation could be a potential therapeutic approach for MNA NB tumors.

3.2 PAPER II. INHIBITION OF FATTY ACID SYNTHESIS INDUCES DIFFERENTIATION AND REDUCES TUMOR BURDEN IN CHILDHOOD NEUROBLASTOMA

Poorly differentiated NB tumors are associated with a more aggressive phenotype and poor outcome. It has been shown, that MYCN inhibits differentiation of NB cells and that treatment with RA results in MYCN downregulation [473,474]. Induction of differentiation using RA is currently used as maintenance therapy for residual disease in patients with high-risk NB [75,475].

In our study from 2013, we described that MYCN inhibition induced neuronal differentiation and lipid droplet accumulation in NB, suggesting a possible interplay between these processes [244]. Moreover, in **paper I** we identified that MNA NB cells used fatty acids as source for energy production, indicating the importance of lipid metabolism in this type of cancer.

In **paper II**, we investigated the role of fatty acid synthesis in NB biology. This is a cytosolic process controlled by two enzymes, ACACA and FASN. In this study, we used five different inhibitors, TOFA and soraphen A, targeting ACACA, and cerulenin, orlistat and UB006, inhibiting FASN, in a panel of MNA and NMNA NB cell lines. Treatment with fatty acid synthesis inhibitors reduced proliferation and induced cell death, accompanied by decreased MYCN or MYC levels. Unexpectedly, inhibition of fatty acid synthesis resulted in induction of differentiation in both MNA and NMNA cells, as shown by neurite outgrowth and expression of neuronal differentiation markers (βIII-tubulin, TUBB3; neuropeptide Y, NPY; secretogranin 2, SCG2), and in reduction of the progenitor marker SOX2. Importantly, MYCN downregulation and neuronal differentiation was also observed in human patient-derived xenograft (PDX)-derived cell cultures. Targeting FASN using siRNA led to a more robust neurite outgrowth and expression of differentiation markers, demonstrating that the induction of differentiation was indeed due to fatty acid synthesis inhibition.

To analyze if reduced lipid content in the culture medium also could lead to neuronal differentiation, we cultured NB cells in medium containing delipidized serum and observed a robust induction of differentiation upon lipid deprivation. Rescue experiments, by adding exogenous BSA-oleate, prevented neurite outgrowth. Reduction of glucose or glutamine levels did not provoke neuronal differentiation, indicating that this process was fatty acid dependent.

Next, the impact of MYCN inhibition on fatty acid synthesis was assessed with isotope tracing experiments with U-^{13}C$_6$-Glucose and U-^{13}C$_5$-glutamine in the MNA cell line SK-N-BE(2). Inhibition of MYCN increased the contribution of glutamine to fatty acid synthesis via reductive carboxylation, while reducing the amount of pyruvate used for anaplerosis processes, and the amount of several fatty acid species including palmitic, palmitoleic, stearic and oleic acids.

Inhibiting fatty acid synthesis altered lipid composition, reducing the levels of both saturated and unsaturated fatty acids. Moreover, it also resulted in changes in mitochondrial count, structure, and electron density. Functional metabolic assays revealed that inhibitors of fatty acid synthesis reduced OXPHOS parameters while slightly increasing glycolysis.

To understand the molecular mechanism behind neuronal differentiation after fatty acid synthesis inhibition, we used a phospho-kinase array kit in lysates from SK-N-BE(2) cells treated with TOFA or cerulenin. An increase in ERK phosphorylation, and thus activation, was observed. Treatment with the ERK inhibitor SCH772984 in combination with TOFA or cerulenin prevented induction of differentiation, demonstrating the importance of this signaling pathway in promoting the phenotype.

To evaluate the potential effect of fatty acid synthesis inhibition in tumor proliferation *in vivo*, we performed xenograft experiments. First, tumors derived from the MNA cell lines IMR-32 were treated with TOFA, Orlistat, or UB006, showing in all cases a reduction in tumor volume and weight. Staining of the tumors showed a decrease in expression of the proliferation marker Ki67 and MYCN and an upregulation of differentiation related proteins. Since UB006 evidenced the strongest effect on NB tumor progression, we studied its impact in two additional xenograft models using two NB cell lines, the MNA, SK-N-BE(2) and the NMNA SK-N-AS. As found in IMR-32, tumor volume and weight were decreased by the treatment in both cases independent of *MYCN* status.

Furthermore, using three different NB patient databases, we found that high expression of *ACACA* and *FASN* was correlated with poor survival. Comparing the levels of both genes in the different NB stages, *FASN* was more expressed in stages 3 and 4 and to a lesser extent in 4S, while *ACACA* did not show significant differences between any of the groups.
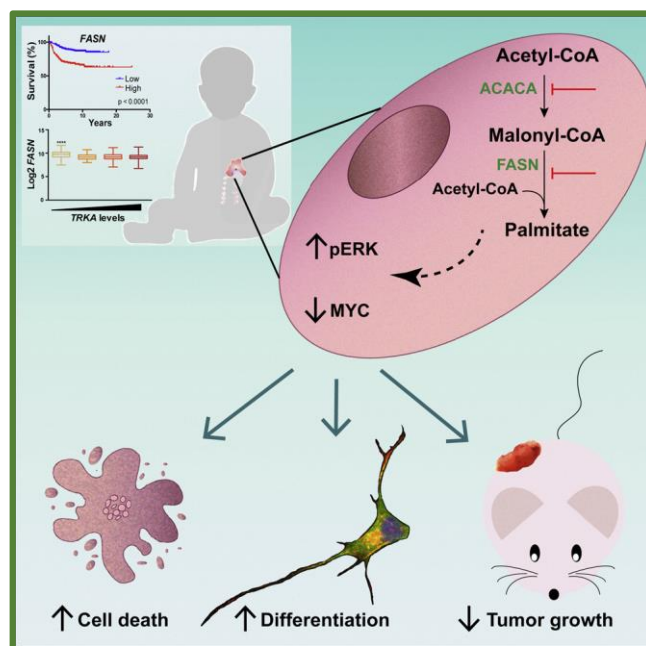
Figure 13: Graphical abstract of paper II. High levels of fatty acid synthesis enzymes are associated with poor prognosis in NB. Targeting the synthesis of fatty acids induces cell death and differentiation *in vitro*, and reduces tumor burden *in vivo*, associated with increased ERK signaling and MYC downregulation. Reprinted from Ruiz-Pérez *et al*., iScience, 2021, with permission of the publisher Elsevier (CC BY 4.0).

Activation of *de novo* fatty acid synthesis is restricted to liver, adipose tissue, and the lactating mammary gland. However, many tumor types display an upregulation in this pathway due to

the metabolic alterations occurring during tumor development to adapt to the microenvironment.

In NB differentiation, MYCN levels are initially elevated while decreasing in the later stages. Here, we described that inhibition of fatty acid synthesis reduced proliferation and MYCN or MYC levels, suggesting a possible regulation of the process. Moreover, differentiation was shown to be dependent on lipid metabolism since deprivation of glucose or glutamine did not increase the expression of differentiation markers or induce neurite outgrowth and supplementing the medium with exogenous oleate prevented the phenotype. Functional metabolic assays revealed a decrease in mitochondrial respiration, and we found changes in mitochondrial structure upon fatty acid synthesis inhibition, compensated by an increase in glycolysis. This is in accordance with **paper I,** showing that MYCN inhibition was associated with damage to cristae and a changed electron density in mitochondria, as well as reduction of OXPHOS capacity. Differentiation was prevented by inhibition of ERK signaling, which has been previously associated with neural differentiation via activation of TRKA. Our analysis of patient datasets showed that patients with high levels of *FASN* had lower *TRKA* expression, further validating this observation. Furthermore, high expression of either FASN or ACACA was associated with poor survival of NB patients, and our *in vivo* experiments demonstrated that inhibition of fatty acid synthesis reduced tumor growth (Figure 13).

To conclude, in **paper II** we showed that inhibition of fatty acid synthesis could be used as a strategy to induce NB differentiation and reduce tumor cell proliferation.

3.3 PAPER III. EXPRESSION AND ACTIVATION OF NUCLEAR HORMONE RECEPTORS RESULT IN NEURONAL DIFFERENTIATION AND FAVORABLE PROGNOSIS IN NEUROBLASTOMA

In **paper II**, we referred to induction of differentiation as a strategy to target NB. Although RA is the standard approach for differentiation therapy, survival of high-risk patients is only improved when it is combined with other strategies. We have previously reported that MYCN, via upregulation of the *miR-17~92* cluster, represses the expression of several NHRs, including the glucocorticoid receptor (GR) and estrogen receptor α (ERα) [429,437]. Thus, inhibition of MYCN induces their expression and the subsequent activation leads to induction of neuronal differentiation. However, during our experimental condition GR or ERα activation resulted in partial differentiation as all cells did not mature.

In **paper III,** our objective was to study the effect after concurrent expression and activation of GR, ERα, and retinoic acid receptor α (RARα). We first further explored the impact of GR activation on differentiation. For that, we used the stable MNA cell line BE(2) transduced with GR (BE(2)-GR) or the empty vector (EV, BE(2)-EV) as control. Importantly, RARα levels were readily detectable, and overexpression was thus not necessary. Activation of GR with its synthetic ligand dexamethasone (DEX) induced neurite outgrowth and expression of differentiation markers similarly to what was observed by the positive control all-*trans* retinoic acid (ATRA), the ligand of RARα. Moreover, the combination of DEX+ATRA slightly increased the differentiation phenotype. We extended our analysis to two NMNA cell lines, SH-SY5Y and SK-N-AS, also overexpressing GR. Likewise, combination of DEX+ATRA resulted in induction of neuronal differentiation in SH-SY5Y cells, and in glial differentiation in SK-N-AS cells. To analyze the effect of GR expression we compared the ability of the cells to produce tumors in female nude mice, without any treatment. Tumor volume was significantly reduced in BE(2)-GR cells, with decreased expression of the Ki67 proliferation marker compared to BE(2)-EV tumors.

To assess differentiation after the combined activation of three NHRs, we generated BE(2)-GR+EV and BE(2)-GR+ERα cells. Treatment with 17-β-estradiol (E2, the ligand for ERα), DEX and ATRA resulted in a profound reduction of viability and colony formation capacity in BE(2)-GR+ERα cells. Additionally, the triple combination robustly induced neurite outgrowth in BE(2)-GR+ERα cells as well as expression of several differentiation markers including *TRKA*, *Neurofilament L* (*NEFL*), *discs large MAGUK scaffold protein 2 DLG2* and reduced the levels of *MYCN* and progenitor cells markers such as *nestin* (*NES*) and *SOX2*. Moreover, a potent differentiation phenotype was also observed in two additional MNA NB cell lines IMR-32 and KCNn69, upon concurrent activation of GR, ERα and RARα.

In **paper I** we defined the impact of MYCN inhibition in metabolic rewiring of NB cells, while in **paper II**, we analyzed the interplay between lipid metabolism and induction of differentiation. Moreover, in an earlier study, we showed that ERα activation in MNA NB cells impaired both the glycolytic and oxidative functions [429]. Hence, we performed functional metabolic assays to evaluate the results of combining the activation of two or three NHRs on

NB metabolism. Both glycolysis and oxygen consumption were higher in BE(2)-GR+EV cells than in those co-expressing both receptors. Single treatment with E2 or DEX increased the glycolytic parameters, an effect further potentiated by their combination, while simultaneous activation of GR and ERα decreased OXPHOS. Triple activation with E2+DEX+ATRA enhanced glycolysis but did not impact respiration. Since MNA NB cells depend on lipid metabolism, as shown in **paper I**, we studied the presence of LDs after treatment with the ligands. Accumulation of LDs was observed upon single, double, as well as triple activation of the three receptors.

To compare the tumor growth potential of BE(2)-GR+EV and BE(2)-GR+ERα cells *in vivo*, we performed xenograft experiments in female nude mice, and no treatment was administered. Simultaneous overexpression of GR and ERα cells robustly reduced tumor volume and weight. Staining of the tumors derived from BE(2)-GR+ERα cells demonstrated a low expression of the Ki67 proliferation marker while different neuronal differentiation markers were upregulated. Interestingly, tumors generated from BE(2)-GR+EV were more vascularized as shown by an increase on the endothelial cell marker endomucin.

In NB patients, survival analysis using two patient cohorts demonstrated that high expression of both GR and ERα correlated with a better prognosis and with elevated levels of the differentiation markers *tyrosine hydroxylase* (*TH*), *nerve growth factor receptor* (*NGFR*) and *SCG2*. We next divided the patients according to high or low GR+ERα expression and *MYCN* status. Interestingly, no MNA patients were identified with high levels of both receptors, validating that MYCN regulates their expression. As expected, NMNA patients with high GR+ERα showed the best overall survival rate. Gene set enrichment analysis revealed that cytoskeleton, differentiation, and metabolism related processes were presented with significant differences between high and low GR+ERα patients. Furthermore, patients with elevated expression of GR+ERα+RARα had a better prognosis compared to patients with overexpression of two receptors.

Importantly, we also interrogated the expression of *GR* (*NR3C1*), *ERα* (*ESR1*), and *RARα* (*RARA*) during chromaffin cell development using single-cell nuclei analysis of embryonic and post-natal human adrenal glands. Expression of *ESR1* was identified in the progenitor cell population, while *NR3C1* was upregulated in both the chromaffin and progenitor cluster, while *RARA* was not significantly expressed in any of the cell populations. In addition, we performed a pseudotime reconstruction as a proxy to study the differentiation process of chromaffin cells in post-natal human adrenal glands. The data clarified that *ESR1*, *NR3C1* and *RARA* were sequentially expressed during the development from progenitor to differentiated chromaffin cells.

Treatment of high-risk NB consists of surgery, chemotherapy, and autologous hematopoietic stem cell transplantation. To avoid the relapse of residual cells, 13-*cis* RA (isotretinoin) is administered to induce differentiation as maintenance therapy. However, the benefit of RA alone is limited in high-risk patients when not combined with other treatment strategies. Thus,

many lines of research are focused on identifying new differentiation approaches for NB treatment.
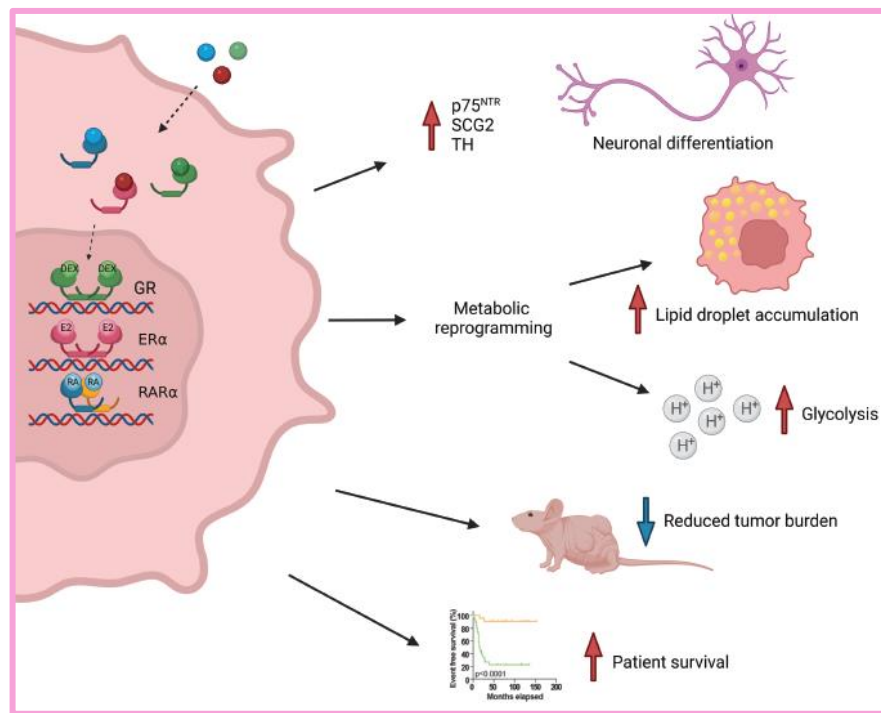
Figure 14: Graphical abstract of paper III. Simultaneous activation of GR, ERα and RARα results in induction of neuronal differentiation, metabolic reprogramming manifested by increased LD accumulation and glycolysis, increased patient survival and reduced tumor growth. Reprinted from Sainero-Alcolado *et al*., 2022, Journal of Experimental and Clinical Cancer Research, with permission from the publisher Springer Nature (CC BY 4.0).

In **paper III**, we investigated how the simultaneous activation of three NHRs could potentiate differentiation in NB. We described that triple treatment with E2+DEX+ATRA induced a robust neurite outgrowth and elevated the expression of several differentiation markers. These results were also validated *in vivo*, and we identified that ERα expression reduced angiogenesis in the tumors. In **paper II** we found the link between metabolism and differentiation, and in **paper III** we discovered that E2 and DEX shifted metabolism towards glycolysis and reduced OXPHOS capacity, while ATRA increased both processes, inducing a more energetic phenotype. Since the three ligands demonstrated stimulation of differentiation, we concluded that glycolysis might play a more important role in this phenotype. In an earlier study we showed that stimulation of ERα with a combination of E2 and NGF resulted in LD accumulation due to alterations in fatty acid usage [429]. Here, we also demonstrated LD formation after treatment with E2, DEX, ATRA, or their combination. Our metabolic assays indicated a strong induction of glycolysis with either of the ligands, which could be to compensate the reduction in mitochondria function. We previously showed that alterations in mitochondrial activity affecting β-oxidation, lead to cytosolic LD accumulation [244]. Our *in vitro* data was validated *in silico* by analyzing NB patient expression data which showed an increase in overall survival and expression of differentiation markers in patients with high levels of all three receptors, GR, ERα, and RARα. Single-cell analysis revealed that *GR* contributes to early

and late stages of adrenal gland development, while *ERα* is essential in the first stages of the sympathetic nervous system maturation and *RARA* was expressed at later stage of the differentiation phase (Figure 14).

Collectively, our data suggest that simultaneous activation of GR, ERα and RARα in NB cells induces neuronal differentiation accompanied by metabolic alterations, and reduced tumor burden, providing a new potential strategy for NB differentiation therapy.

3.4 PAPER IV: MYC INHIBITION DURING HYPOXIA RESULTS IN ACCUMULATION OF GLUTAMINE-DERIVED LIPID DROPLETS IN CLEAR CELL RENAL CELL CARCINOMA

In **paper I** and **II**, our results showed the crucial role of MYCN in rewiring metabolism in NB, especially lipid processes. In our earlier work, we demonstrated that inhibiting MYCN using different strategies, including the MYC-MAX dimerization inhibitor 10058-F4 (F4), the bromodomain inhibitor JQ1, and short hairpin RNA (shRNA) directly targeting MYCN, all led to lipid accumulation, concluding that MYCN downregulation was driving this phenotype [244].

To investigate whether LD formation upon MYC inhibition was also observed in other types of cancer, we performed a screening in nearly 60 different cancer cell lines using three MYC inhibitors, F4, another MYC-MAX dimerization inhibitor 10074-G5 (G5), and JQ1. The results showed that the LD accumulation pattern varied between cell lines from the same cancer type and, in some cases, also between the three inhibitors. Interestingly, a striking difference was observed in the ccRCC cell line RCC4 *VHL-*, with loss of *VHL*, and the same cell line in which *VHL* has been reintroduced (RCC4 *VHL+*). While the RCC4 *VHL-* cell line accumulated LDs after MYC inhibition, this phenotype was not perceived in RCC4 *VHL+* cells. To analyze whether this effect was restricted to the RCC4 cell line, we studied lipid formation in two additional ccRCC cell lines, 786-O and A498, also with loss and reintroduction of the *VHL* gene. A similar pattern of lipid accumulation occurred only upon MYC inhibition in the *VHL-* cells.

To validate that accumulation of LDs was a direct result of MYC inhibition and not an off-target effect of any of the compounds, we employed two additional strategies to reduce its activity. First, we transiently transduced RCC4 cells using sh*MYC* constructs, and second, we transduced 786-O cells with a vector containing the Omomyc sequence. Omomyc is a dominant negative peptide derived from four amino acids substitutions in the bHLH-Zip of MYC. Similar to the MYC inhibitors tested, both sh*MYC* and Omomyc also induced lipid accumulation in *VHL-* cells.

In normoxia, VHL regulates stability of HIFs by binding the hydroxylation sites in two proline residues and targeting them for proteasomal degradation. When oxygen decreases, HIFs are not hydroxylated, thus being permanently stable. Since the only difference between the three cell pairs was *VHL* status, we interrogated if increasing HIF expression in the *VHL+* cells also could lead to lipid deposits. By culturing the cells in presence of $CoCl_2$ causing pseudohypoxia or in a hypoxia chamber at 1% O_2, we demonstrated that *VHL+* cells with elevated HIF levels indeed presented with lipid deposits. Furthermore, inhibition of HIF transcriptional activators using chetomin, prevented LD formation in *VHL-* cells.

In cancer cells, fatty acids could originate from glucose, glutamine or from media uptake. Thus, we proceeded to investigate the origin of the lipids contained in LDs. Cells were cultured in medium containing delipidized serum, low glucose or low glutamine and the presence of LDs was analyzed. Reduction of glutamine drastically reduced LD formation in *VHL-* cells in

normoxia, and in both *VHL+* and *VHL-* cells in hypoxia. Moreover, functional metabolic assays showed that even though *VHL-* had a low mitochondrial respiration rate, they were more dependent on glutamine for oxidation, while glucose was the main fuel in *VHL+* cells. Since glutamine seemed to play an important role in the metabolism of *VHL-* cells, we inhibited GLS, the enzyme responsible for converting glutamine to glutamate, a metabolite that enters the TCA cycle in the mitochondria. Inhibition of GLS using BPTES or L-DON prevented lipid storage in *VHL-* cells. In addition, BPTES remarkably reduced the viability of 786-O *VHL-* Omomyc cells upon DOX induction and reduced LD formation in these cells. Glutamate derived from glutamine can be further metabolized via reductive carboxylation to acetyl-CoA, a precursor for fatty acid synthesis. Inhibition of FASN and ACACA using UB006 and TOFA, respectively, in combination with MYC inhibitors, also prevented LD accumulation.

Since LDs were only observed in *VHL-* cells, we next studied the differences in lipid composition compared to *VHL+* cells upon MYC inhibition with JQ1. Lipidomic analysis revealed that both cells had elevated levels of phosphatidic aid (PA). However, while *VHL-* cells were rich in triglycerides (TG), one of the main components of LDs, *VHL+* were more enriched in phosphoinositol (PI) species, indicating a different use of PA between them. To investigate if glutamine could be derived from the synthesis of fatty acids, we performed isotope tracing experiments with U-$^{13}C_6$-glucose and U-$^{13}C_5$-glutamine in RCC4 *VHL+* and *VHL-* cells treated 72 h with JQ1. In *VHL+* cells treated with JQ1, palmitate was generated from glucose while in *VHL-* cells, glutamine was the preferred carbon source.
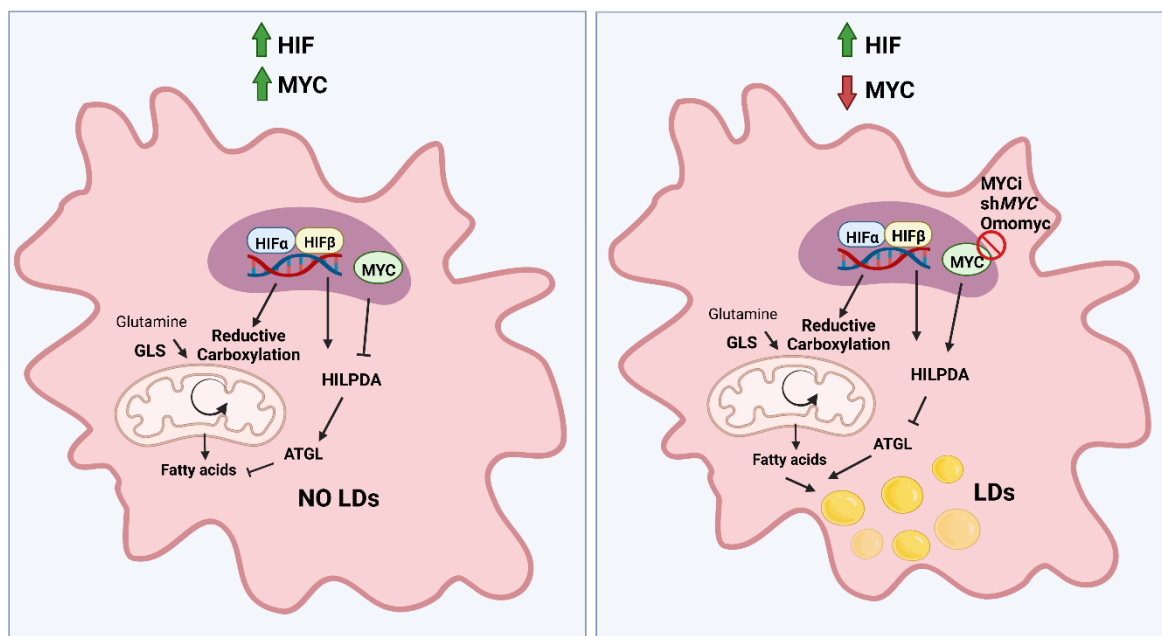
Figure 15: Graphical abstract of paper IV. HIF stabilization directs glutamine for the synthesis of fatty acids. HIF also induces the expression of HILPDA, while MYC may repress its expression. When MYC is active, inhibition of HILPDA results in activation of ATGL promoting lipolysis, and thus, LDs will not accumulate (left panel). Inhibition or downregulation of MYC increases HILPDA expression, inhibiting ATGL and stimulating fatty acid accumulation in LDs (right panel). Created with Biorender.com (from Sainero-Alcolado *et al.,* 2022, manuscript).

To understand the interplay between HIF stabilization, MYC inhibition and LD accumulation, we performed RNAseq. Many lipid metabolism related processes were significantly altered after MYC downregulation, including lipid particle organization. The gene hypoxia inducible lipid droplet associated (*HILPDA*) gene was one of the most significantly upregulated in *VHL-* upon JQ1 treatment. While it is known to be regulated by HIF, using the ENCODE database we observed that MYC also binds to its promoter. Moreover, *HILPDA* was upregulated in *VHL-* cells upon MYC inhibition while reduced after BPTES treatment, validating the correlation with the conditions in which we observed LD accumulation.

Metabolic reprogramming is a hallmark of ccRCC. Deposits of glycogen and lipids as well as genetic deregulations in hypoxia signaling and TCA cycle components are usually observed in these tumors [476]. However, LDs are also present in other types of cancers as previously described by other groups which also is supported by our screening. Nevertheless, formation of LDs proved to be cell line dependent. In the past years, the role of LDs in tumorigenesis has gained more interest, since many studies have suggested that they might be a crucial organelle supporting cancer cell survival [384]. In ccRCC, the constitutive activation of HIF has been described to induce LD accumulation [477]. Moreover, we have previously shown that MYCN inhibition in NB is associated to lipid deposits [244]. In **paper IV,** our aim was to explore the interplay between hypoxia and MYC inhibition in LD formation. Our data indicated that combined MYC downregulation and stabilization of HIFs are necessary for this process. Moreover, we showed that HIF activation derives carbons from glutamine for the synthesis of fatty acids, further converting to PA. In *VHL-* cells, PA was used for the formation of TGs while *VHL+* cells were producing PI related lipid species, explaining why LDs are only observed in *VHL-* cells. Finally, we identified that *HILPDA*, whose expression is known to be regulated by HIF, was upregulated after MYC inhibition in *VHL-* cells, and that MYC binds to its promoter, most likely repressing its expression.

Altogether, we demonstrated that HIF stabilization reprograms glutamine metabolism for synthesis of fatty acids. Upregulation of HILPDA due to HIF induction and MYC inhibition leads to fatty acid accumulation in the form of TGs in LDs (Figure 15). Further studies will reveal whether this new mechanism could be used as a basis for developing new approaches to target ccRCC.

4 CONCLUSIONS

The expression of MYC is deregulated in approximately 70% of all human cancers. As a pleiotropic transcription factor, MYC governs many different aspects of the cells. This thesis is focused on two of the various processes controlled by MYC: metabolism and differentiation.

Targeting MYC has been proven to be challenging due to the lack of an enzymatic pocket and its intrinsically disordered structure. Thus, the undruggable nature of the MYC protein has given rise to other targeting approaches. This includes inhibition of MYC interaction with partner proteins, its transcription, induction of post-translational modifications affecting MYC degradation, and targeting the downstream processes regulated by MYC [478].

In **paper I**, we found that *MYCN* amplification in NB is associated with a high metabolic profile with enhanced glycolysis and OXPHOS. In addition, MNA NB cells can synthetize glutamine *de novo*, and are dependent on fatty acids for mitochondrial respiration. Since fatty acids are crucial for energy production, inhibition of the rate limiting enzyme participating in fatty acid oxidation, CPT1C, results in inhibition of MNA cell proliferation *in vitro* and tumor burden *in vivo*.

Paper II is a continuation of **paper I**. As fatty acid metabolism is a vulnerability in MNA NB, we determined the effects of inhibiting fatty acid synthesis in these cells. Inhibition of FASN and ACACA with five different small molecules reduces NB proliferation and induces neuronal differentiation. The impact on differentiation of fatty acid synthesis inhibitors is mediated via ERK signaling activation. Moreover, *in vivo* experiments showed that targeting these two enzymes diminishes NB proliferation. *In silico* analysis further validated that high expression of *FASN* and *ACACA* are correlated with worse survival in NB patients.

In **paper III,** we focused on studying the differentiation potential of NB cells upon activation of three different NHRs. Combination of E2, DEX and ATRA robustly enhances neuronal differentiation, shown as increased neurite outgrowth and expression of differentiation markers, and reduced tumor burden. Moreover, activation of GR, ERα and RARα is also associated with enhanced glycolysis and lipid droplet accumulation. Moreover, we identified that patients expressing high levels of these receptors either alone or in combination are correlated with a better prognosis. Finally, we showed that *ERα*, *GR* and *RARα* are sequentially expressed during chromaffin cell differentiation of the developing adrenal gland.

Paper IV evaluated the impact of MYC targeting in the accumulation of LDs, and the interplay with hypoxia signaling in ccRCC. Using three MYC inhibitors, shRNA against MYC, and Omomyc expression, we showed that LD formation in *VHL-* cells is dependent on combined MYC inhibition and HIF stabilization. Metabolic assays identified that these lipids are generated from glutamine and form TGs in *VHL-* cells, while they are derived from PI synthesis in *VHL+* cells, explaining the differences in LD accumulation. Moreover, we discovered that MYC inhibition and HIF stabilization increase HILPDA levels, promoting glutamine-derived lipid deposition in the *VHL-* cells.

Results from **paper I**-**IV** established that targeting lipid metabolism represents a promising strategy for the treatment of both childhood NB as well as adult ccRCC.

In conclusion, we demonstrated the role of MYC in the metabolic reprogramming and differentiation of cancer, providing novel potential targets that could pave the way for the development of new approaches for these types of tumors.

5 POINTS OF PERSPECTIVE

In the four papers comprising this thesis new metabolic targets related to MYC deregulation, important for the development of NB and ccRCC, have been identified.

A decade ago, metabolic reprogramming was recognized as a crucial hallmark for cancer development and progression. Since then, many different studies have focused on targeting metabolic enzymes to combat tumor formation. Several of these strategies have made it to clinical practice, for instance the use of anti-folates for acute lymphocytic leukemia [479]. However, while many of the prodrugs proved to be excellent candidates *in vitro* they failed in the clinical stages. In part, this is due to the high heterogeneity of tumors between individuals. Thus, precision medicine has gained more interest to provide each patient with optimal treatment.

Here, we described that MNA NBs are highly dependent on fatty acids. Also, we identified that targeting lipid metabolism could serve as a potential therapeutic strategy for affected children. In addition, we defined how MYC impacts lipid accumulation in ccRCC cells during hypoxia. Understanding how cancer cells regulate lipid metabolism is crucial for identifying new targets to develop approaches for cancer treatment.

Remedies directed to different aspects of tumor progression could provide a more effective approach. For instance, we showed that fatty acid synthesis inhibition is closely related to induction of neuronal differentiation, associated with a better prognosis in high-risk NB. Thus, combination strategies targeting lipid metabolism with conventional maintenance treatment with RA, could further potentiate the differentiation phenotype and survival. Furthermore, a combination of estradiol, cortisol and RA activating their respective receptors, could potentially be exploited in the clinic as a robust approach for NB cells differentiation. Cortisol and RA are already used in children with cancer. When it comes to estradiol, the hormone which turned out to be the most efficient of the three ligands, further studies using low concentrations in combination with the other two will be needed to avoid any severe side effects on the growing child. Lipid droplet accumulation is a well-known characteristic of ccRCC and other types of tumors, associated with cancer development, survival, and resistance to conventional chemotherapy. Investigating the mechanism leading to their formation as well as their role in cancer will provide essential knowledge for development of new combination strategies to improve patient survival.

It is important to mention that most of the chemical compounds used in our studies have not passed clinical trials due to high toxicity. However, our research could serve as a proof-of-principle on which targets would need better inhibitors with higher specificity, better tolerance, and fewer side effects.

In this thesis, I have participated in the identification of three metabolic targets for NB, namely CPT1C, FASN, and ACACA, and a new approach for potentiating differentiation by combined activation of GR, ERα and RARα. Importantly, I have spearheaded the identification of the

molecular mechanism involving LD accumulation during hypoxia after MYC inhibition in ccRCC. Together, this work provides novel insights on the *MYC* oncogene in tumor metabolism that should be explored in further studies for future clinical applicability.

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