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# **Therapeutic and Prognostic Strategies in Neuroblastoma: Exploring Nuclear Hormone Receptors, MYC Targets, and DIAPH3**

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# Therapeutic and Prognostic Strategies in Neuroblastoma: Exploring Nuclear Hormone Receptors, MYC Targets, and DIAPH3

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Ye Yuan**

The thesis will be defended in public at Peter Reichard, Biomedicum, Karolinska Institutet, Solna, **January 15<sup>th</sup> at 09:30 am.**

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*"Im... Tal... Im Tal, da floss ein kleiner Bach dahin, und im klaren Wasser konnte man die Forellen sehen. Die Fische glitzerten silbern und sprangen lustig umher. Hier, an diesem... leib... lieblichen Ort, wollte der Hirte rasten und sein Mittagsmahl einnehmen."*

--<Heidi> 2015 film 1:11:30/1:50:50



# Popular science summary of the thesis

Neuroblastoma (NB) is a pediatric cancer that originates in the peripheral nervous system. Some low-risk tumors can regress without treatment, but high-risk cases are aggressive and difficult to treat. Much effort has been made to investigate the developmental and cellular origin of this disease and the molecular bases for its behavior, especially in the high-risk type.

In **study I**, we investigated the role of nuclear hormone receptors in *MYCN*-amplified NB. *MYCN* amplification is frequently observed in poor-outcome NB cases. We found that upon combined signaling from the glucocorticoid, estrogen, and retinoic acid receptors, the impact of *MYCN* amplification is reduced, driving cancer cells into a more mature or differentiated state. This is accompanied with metabolic alterations and reduced tumor burden in mice. These results could be translated to clinical use where today retinoic acid is used as single treatment for minimal residual disease.

In **study II**, a comprehensive model for predicting the survival of NB patients was designed. We screened pre-defined target genes of *MYCN* and c-MYC to select those of clinical and biological relevance for NB. We used prediction models in different patient cohorts and showed that a risk score computed for *MYCN* and c-MYC targets could effectively classify patients into different groups with different survival chances. Patients with a high-risk score showed poor clinical outcomes and reduced survival rates. Interestingly, we found that c-MYC and *MYCN* target genes have different expression patterns during development. Together these data highlighted the role of target genes of c-MYC and *MYCN* during development and the NB biology.

In **study III** we examined the role of the *DIAPH3* gene and protein in NB. *DIAPH3* is a member of the formin protein family that regulates the cell skeleton structure, and it has received much attention in various tumors, except in NB. We found that overexpression of *DIAPH3* is associated with poor survival and outcome of NB patients. We also showed that this gene is expressed in tumors with a low maturation grade. Notably we observed that the proliferation of cancer cells was reduced when the gene was silenced, suggesting that *DIAPH3* may be a promising target for future NB treatment.



# Abstract

Neuroblastoma (NB) is a pediatric cancer derived from the cells of neural crest origin that form the sympathoadrenal system. Typically, the tumor cells migrate along the spinal cord and spread to the chest, neck, and/or abdomen. Different clinical behaviors are observed in this disease: some tumors spontaneously regress without treatment, while others are highly aggressive and resistant to current therapies. Approximately 40% of high-risk NB patients have *MYCN* amplification while 10% have *MYC* (*i.e.* encoding c-MYC) overexpression. These patients have undifferentiated tumors with a poor prognosis.

Our group previously found that the expression and activation of nuclear hormone receptors (NHRs) estrogen receptor alpha (ER $\alpha$ ) by 17- $\beta$ -estradiol (E2), and the glucocorticoid receptor (GR) by dexamethasone (DEX), could trigger differentiation by disrupting the regulation of the miR-17~92 microRNA cluster by *MYCN*. In **paper I**, we sought to investigate whether the simultaneous activation of both ER $\alpha$  and GR has a more beneficial effect compared to the activation of either ER $\alpha$  or GR alone. We examined cell survival, alterations in cell shape as indicated by neurite extension, variations in metabolic pathways, accumulation of lipid droplets, and performed xenograft experiments. Our findings revealed that the simultaneous activation of GR and ER $\alpha$ , compared to their single activation, led to reduced viability and a more robust differentiation. This dual activation also caused changes in glycolysis and oxidative phosphorylation, increased lipid droplet accumulation, and decreased aggressiveness in mouse models. The triple activation with an additional activation of the retinoic acid receptor using all trans-retinoic acid (ATRA), amplified the differentiation phenotype. Bulk-sequencing analysis showed that patients with high levels of NHRs are related to favorable survival and clinical outcome. In summary, our data suggest that combination activation of these NHRs could be a potential differentiation induction treatment.

**Paper II** investigates target genes of c-MYC and *MYCN* to explore if it is possible to obtain a better prognosis prediction using the expression of this group of genes, instead of the expression of *MYC* and/or *MYCN* alone. In addition, we analyzed if there are different prediction power capabilities between c-MYC and *MYCN* target genes, and their different role during sympathoadrenal development. We screened lists of target genes by using comprehensive approaches, including differential expression analysis between clinical risk groups, INSS stages, *MYCN* amplification status, progression status; Univariate Cox regression analysis to select the target genes related to prognosis prediction power, and protein interaction network analysis to select genes that share a meaningful biology function. Following the training and validation of (LASSO) regression prediction models in three different patient cohorts (SEQC, Kocak, and Versteeg), we found that a risk score computed on c-MYC/*MYCN* target genes with prognostic value, could effectively classify patients in groups with different survival probabilities. The high-risk group of patients exhibited unfavorable clinical outcomes and low survival rates. Further, single cell RNA sequencing analysis revealed that c-MYC and *MYCN* targets have different expression patterns during sympathoadrenal development. Notably, genes linked to adverse outcomes were predominantly expressed in sympathoblasts

in comparison to chromaffin cells. In summary, our research provides new insights into the importance of c-MYC/MYCN target genes during sympathoadrenal development and their value in predicting patient outcome.

In **paper III** we studied the function of one member of the formin protein family involved in cytoskeleton modulation: Diaphanous Related Formin 3 (*DIAPH3*). We found that high *DIAPH3* expression in NB tumors are associated with *MYCN* amplification, higher stage, risk, progression and negative clinical outcome. Elevated *DIAPH3* expression was also found in specific cells during mouse sympathoadrenal development and in progenitor cells of the post-natal human adrenal gland. Furthermore, the knockdown of *DIAPH3* resulted in a slight decrease in cell growth and cell cycle arrest. Our study suggests that *DIAPH3* could be a promising target for new therapeutic strategies.

## List of scientific papers

- I. 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.

- II. **Ye Yuan**, Mohammad Alzrigat, Aida Rodriguez-Garcia, Xueyao Wang, Tomas Sjöberg Bexelius, John Inge Johnsen, Marie Arsenian-Henriksson, Judit Liaño-Pons, and Oscar C. Bedoya-Reina#.

**Target genes of c-MYC and MYCN with prognostic power in neuroblastoma exhibit different expressions during sympathoadrenal development.**

*Cancers*, 2023, 15(18), 4599

- III. **Ye Yuan**, Oscar Bedoya-Reina, Marie Arsenian-Henriksson, Aida Rodriguez Garcia and Judit Liaño-Pons.

**DIAPH3 is expressed during sympathoadrenal development and correlated with poor prognosis in neuroblastoma.**

*Manuscript*, 2023

## PUBLICATIONS NOT INCLUDED IN THIS THESIS

- I. Muhammad Mushtaq\*#, Judit Liaño-Pons\*, **Ye Yuan**, Jiansheng Wang, María Victoria Ruiz-Pérez, Yi Chen, Elena Kashuba, Felix Haglund de Flon, Bertha Brodin, and Marie Arsenian-Henriksson#. **EZH2 inhibition sensitizes retinoic acid-driven senescence in Synovial sarcoma. EZH2 inhibition sensitizes retinoic acid-driven senescence in Synovial sarcoma.**

*Manuscript in Revision*, 2023

- II. Judit Liaño-Pons#, Fenja L. Fahrig, **Ye Yuan**, Lea Schort, Maria Esteve Garcia, Oscar C. Bedoya-Reina, and Marie Arsenian-Henriksson#. **Targeting Peroxiredoxin 6 as a Differentiation Inducing Strategy for Neuroblastoma.**

*Submitted manuscript*, 2023

- III. Lourdes Sainero-Alcolado, Giuseppe Santopolo, **Ye Yuan**, Judit Liaño-Pons#, and Marie Arsenian-Henriksson#. **Defining Neuroblastoma: from origin to precision medicine.**

*Submitted review*, 2023

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## List of abbreviations

4-HPR	N-(4-hydroxyphenyl) Retinamide
ADR	Adrenergic
ALK	Anaplastic Lymphoma Kinase
ALKi	ALK inhibitors
ALL	Acute Lymphoblastic Leukemia
APCs	Antigen-Presenting Cells
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ASCT	Stem Cell Transplantation
ATM	ataxia telangiectasia mutated
ATP	Adenosine Triphosphate
ATR	ATM and Rad3-related kinase
ATRA	All-trans Retinoic Acid
AUC	Area Under Curve
bHLH-Zip	Basic helix-loop-helix leucine zipper
BRD4	Bromodomain Containing Protein 4
CAM1	Calmodulin 1
CCA	Cyclohexane Carboxylic Acid
CDKs	Cyclin-dependent kinases
ChIP	Chromatin Immunoprecipitation
ChIP-seq	ChIP-sequencing
CHK	Checkpoint kinase
CKIs	Cyclin-dependent kinase inhibitor
CMA	Chaperone-Mediated Autophagy
COG	Children's Oncology Group
COJEC	Cisplatin, Vincristine, Carboplatin, Etoposide, and Cyclophosphamide
CRC	Colorectal Cancer
CR	Complete Remission
CTL	Cytotoxic T Lymphocyte
CTD	C-Terminal Domain

DAD	Diaphanous Autoregulatory Domain
DEX	Dexamethasone
DID	Diaphanous Inhibitory Domain
GNB-N	Ganglioneuroblastoma, nodular
DLG2	Discs Large MAGUK Scaffold Protein 2
E2	Estradiol
E-Box	Enhancer box
ECAR	Extracellular Acidification Rate
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen-Receptor
ER $\beta$ 2	ER Beta Splice Variant
ERBB3	Receptor Tyrosine-Protein Kinase erbB-3
FACS	Fluorescence-Activated Cell Sorting
FasL	Fas Ligand
FDR	False Discovery Rate
FH	Formin Homology
G1	Gap1
G2	Gap2
G4	G-Quadruplex
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBD	GTPase-binding domain
GD2	Ganglioside 2
GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GR	Glucocorticoid Receptor
GTP	Guanosine Triphosphate
HDC	High-Dose Chemotherapy
HDAC	Histone Deacetylase

HER2	Human Epidermal Growth Factor Receptor 2
HIF	Hypoxia Inducible Factor
hMSCs	Human Mesenchymal Stem Cells
HRE	Hormone Response Elements
IDRF	Image-Defined Risk Factors
INRG	The International NB Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LASSO	Least Absolute Shrinkage and Selection Operator
LIM	Lin-11, Isl-1, and Mec-3
LOH	Loss of Heterozygosity
LUAD	Lung Adenocarcinoma
MAPK	Mitogen-Activated Protein Kinase
MAX	MYC Associated Factor X
MES	Mesenchymal
MMP	Matrix Metalloproteinase
MTAs	Microtubule Target Agents
MXD	MAX dimerization protein
MYC	Myelocytomatosis
NEFL	Neurofilament Light Chain
NES	Neuroepithelial Stem Cell Protein, Nestin
NB	Neuroblastoma
NGFR	Growth Factor Receptor
NHRs	Nuclear Hormone Receptors
NK	Natural Killer
NR3C1	Nuclear Receptor subfamily 3 group C member 1
NT	Neural Tube
NTAD	N-Terminal Transactivation Domain

OCR	Oxygen Consumption Rate
ODC1	Ornithine Decarboxylase 1
OLS	Ordinary Least Squares
OS	Overall Survival
PBSC	Peripheral Blood Stem Cell
PEST	proline (P), glutamate (E), serine (S), and threonine (T)
PD	Progressive Disease
PDAC	Pancreatic Ductal Adenocarcinoma
PDX	Patient Derived Xenografts
PHOX2B	Paired-Like Homeobox 2B
PI	Phosphatidyl Inositol
PI3K/AKT	Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)
PLK1	Polo-Like Kinase 1
PNTs	Peripheral Neuroblastic Tumors
PTEN	Phosphatase and Tensin Homolog
PTMA	A-Prothymosin
PTPRD	Protein Tyrosine Phosphatase Receptor Type D
P53/TP53	Tumor Protein P53
RA	Retinoic Acid
RB	Retinoblastoma
RAR $\alpha$ /RARA	Retinoic Acid Receptor A
ROC	Receiver Operating Characteristic
ROCK	Rho-Associated Kinase
ROS	Reactive Oxygen Species
SAHA	Suberoylanilide Hydroxamic Acid
SASP	Senescence-Associated Secretory Phenotype
SCG2	Secretogranin 2
SCPs	Schwann Cell Precursors
SIF	Small intensely fluorescent
scRNA-seq	single-cell RNA sequencing
SIOPEN	Society Of Pediatric Oncology Europe Neuroblastoma Group

SOX2	SRY-box 2
SNS	Sympathetic Nervous System
TERT	Telomerase Reverse Transcriptase
TH	Tyrosine Hydroxylase
TNFR1	Tumor Necrosis Factor Receptor 1
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TrkA	Tropomyosin receptor kinase A
TrkB	Tropomyosin receptor kinase B
t-SNE	t-distributed stochastic neighbor embedding
TSG	Tumor Suppressor Gene
TSP-1	Thrombospondin 1
TUBB3	Tubulin Beta 3 class III
TSS	Transcription Start Site
XRT	Radiation Therapy

# **1 Literature Review**

## **1.1 Cancer**

- 1.1.1 Hallmarks of cancer
- 1.1.2 Oncogenes and tumor suppressor genes
- 1.1.3 Cell cycle
- 1.1.4 Cell death
- 1.1.5 Cytoskeleton modulation
- 1.1.6 MYC proteins

## **1.2 Pediatric cancers and their features**

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## **1.4 Neuroblastoma**

- 1.4.1 Incidence and epidemiological features of Neuroblastoma
- 1.4.2 Origin and development of Neuroblastoma
- 1.4.3 Classification and risk stratification of Neuroblastoma
- 1.4.4 Genetic alterations of Neuroblastoma
- 1.4.5 Treatment of Neuroblastoma
- 1.4.6 Neural differentiation in Neuroblastoma
- 1.4.7 Metabolism in Neuroblastoma
- 1.4.8 Hypoxia in the initiation and progression of Neuroblastoma
- 1.4.9 Cytoskeletal modulation in Neuroblastoma

## **1.5 Methods for statistical analysis**



## 1.1 Cancer

### 1.1.1 Hallmarks of cancer

The hallmarks of cancer are a variety of characteristics that cancer cells acquire during the intricate process of tumor development. The original version of this framework included six biological properties [1], but it has now been expanded to include fourteen traits [2,3]

The first six characteristics of cancer consist of:

**(1) Sustaining proliferative signaling:** Cancer cells are continuously growing and dividing. They achieve this by manipulating growth signals, either through self-production or by altering their environment to receive these signals, leading to uncontrolled proliferation and growth [4,5].

**(2) Evading growth suppressors:** This is achieved by deactivating tumor suppressor proteins; two well-known examples are retinoblastoma-associated (RB) and p53 proteins. RB reacts to extracellular growth-inhibitory signals, and its absence could lead to continuous growth and division of cells [6]. P53 is an intracellular sensor to detect damage and stress. It can repair damage by stopping the cell cycle, however it can also cause apoptosis in extreme or irreversible circumstances [7].

**(3) Resisting cell death:** Apoptosis is a programmed cell death that involves blebbing formation, cytoskeleton breakdown, and fragmentation of the nucleus. Tumor cells develop strategies to evade apoptosis, such as losing p53 function or altering the balance of pro- and antiapoptotic factors [7,8].

**(4) Enabling replicative immortality:** Telomerase is an enzyme that extends telomeres by adding repeat segments to the ends. In normal cells, telomeres get shorter during each division, which could lead to senescence or crisis. However, in cancer cells, telomerase activity maintains telomere length, allowing continuous division and avoiding these growth barriers [9].

**(5) Inducing angiogenesis:** Tumors require nutrients and oxygen, to achieve this, an "angiogenic switch" would be triggered, leading to the formation of new blood vessels. This process is modulated by key regulators include vascular endothelial growth factor A (VEGF-A) that promotes angiogenesis, and thrombospondin 1 (TSP-1) that inhibits it [10].

**(6) Activating invasion and metastasis:** As carcinomas progress, they often lose important adhesion molecules like E-cadherin. The process of invasion and metastasis cascade, includes several steps: local invasion, intravasation into vessels, transit through lymphatic and

hematogenous systems, extravasation into distant tissues, and the growth of micro metastases into macroscopic tumors. Transcription factors such as Snail, Slug, Twist, and Zeb1/2 are vital important in regulating the epithelial-mesenchymal transition (EMT) [11].

In the updated 2011 review, Hanahan and Weinberg added two emerging hallmarks [2]:

**(7) Reprogramming Energy Metabolism:** Otto Warburg demonstrated that cancer cells frequently rely on fermentation for energy production, even in the presence of oxygen. Although this aerobic glycolysis process is less effective than mitochondrial oxidative phosphorylation. It provides intermediate metabolites that are important for producing nucleotides, amino acids, and lipids, which are essential for rapidly dividing cancer cells [12-14].

**(8) Evading Immune Destruction:** Studies in immunodeficient mice have shown that deficiencies in various immune cells such as CD8<sup>+</sup> T cells and natural killer (NK) cells could increase tumor formation. It highlight the importance of innate and adaptive immunity in surveillance of cancer [15]. Clinical epidemiology also showed patients with higher infiltration level of Cytotoxic T lymphocytes (CTLs) and NK cells have better prognoses [16].

In their 2011 review, Hanahan and Weinberg updated two enabling characteristics [2]:

**(9) Genome instability:** Cancer cells often exhibit increased rates of mutation, that could be induced by mutagenic agents or a malfunction of genomic maintenance machinery or the surveillance systems. A key player involved in this process is the *p53* gene, which is also referred to as the "guardian of the genome" [17].

**(10) Tumor-Promoting Inflammation:** Inflammation provides bioactive molecules to the tumor microenvironment. It releases factors or enzymes that support cell proliferation, invasion and metastasis, prevent cell death, and promote the formation of blood vessels that facilitate cancer development [18-20].

In 2022, new dimensions of hallmarks and characteristic were proposed by Hanahan [3], which include:

**(11) Unlocking phenotypic plasticity:** This hallmark include three parts: Dedifferentiation is that the cell reverts to a progenitor-like state and allow the cell proliferate again [21]; Trans-differentiation in which cells initially on a differentiation path and then change to the different developmental trajectory [22]. Blocked differentiation involves progenitor cells that have not

fully differentiated and may undergo regulations that block their progression into completely differentiated [23].

**(12) Non-mutational epigenetic reprogramming:** This process is independent from DNA instability and mutations. It can regulate cell growth and differentiation, and its flexible and reversible characteristics make it vulnerable to external and environmental factors [24].

**(13) Polymorphic microbiomes:** The microbiome has a crucial importance in the development, progression, and treatment resistance in cancers. Studies indicate that the host microbiome could promote inflammation and influences the effectiveness of immune checkpoint inhibitor treatments [25].

**(14) Senescent cells:** Senescence is an irreversible state of cell cycle arrest that serves as a mechanism to maintain tissue homeostasis. It involves cell morphology changes, metabolism reprogramming, and the presentation of a senescence-associated secretory phenotype (SASP) [26]. Traditionally, senescence was viewed as a defense against cancer, however, recent studies also suggests that in some contexts senescent cells can actually promote tumor progression by generating a cytokine shield that protects non-aging tumor cells against the immune system [27].

### 1.1.2 Oncogenes and tumor suppressor genes

An oncogene is a gene whose product has the potential to initiate cancer. In particular, the aberrant activation of an oncogene can trigger unregulated cellular proliferation, which is an important characteristic of cancerous transformation [28]. Proto-oncogenes can transform to active oncogenes through mechanisms such as, point mutation, gene amplification, chromosomal translocation, insertion, deletions, or truncations [29]. Oncogenes include, for example, the *RAS* family which consist of *HRAS*, *KRAS*, and *NRAS*. These are important for cell signaling and growth [30]. Another example is *MYC* whose overexpression can lead to uncontrolled cell growth and division. Its amplification can be found in many types of cancer, for example, breast, lung, and osteosarcoma [31,32]. The *human epidermal growth factor receptor 2 (HER2/neu*, also known as *ERBB2*) encodes epidermal growth factor receptor (EGFR) 2 that involved in cell growth and division. *HER2/neu* amplification is most commonly associated with breast cancer, but can also occur in other types of cancer [33].

A tumor suppressor gene (TSG), also referred to as an anti-oncogene, is the gene whose product controls and influences the behavior of a cell during the crucial activities of cell division and replication, and tumorigenesis [34]. For example, p53 is a tumor suppressor gene that is

frequently found in up to 50% of all cancers and is thus called “the guardian of the genome” [35,36]. The main function of p53 protein includes halting cell growth, repairing DNA, and inducing cell death (apoptosis) [35]. Mutant p53 proteins exhibit properties that help cells to grow and evade programmed cell death [37].

### 1.1.3 Cell cycle

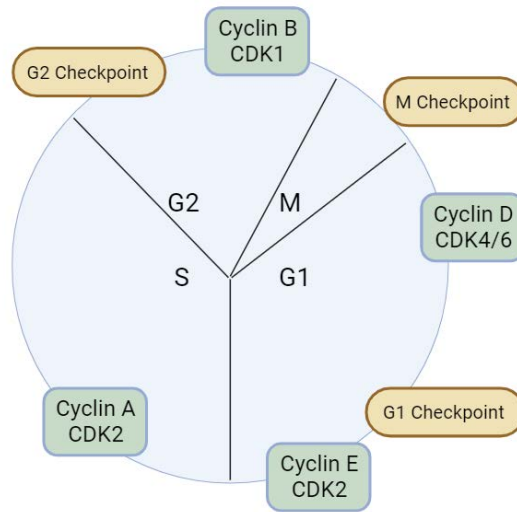
The cell cycle is a strictly controlled process comprising of interphase, mitosis, and cytokinesis phases, which lead to the growth and division of a cell [38]. The interphase consists of Gap1 (G1), Synthesis (S), Gap2 (G2) phase, and mitosis (M) which can be divided into four phases: Prophase, Metaphase, Anaphase, and Telophase [39].

**Cell cycle check points** are important to cell cycle control because they detect errors that occur in the process of DNA replication, chromosome segregation and trigger a cell cycle arrest to repair the damages. For example, the G1/S checkpoint (also known as the restriction point) assesses the presence of growth factors and DNA damage [40]. The G2/M checkpoint ensures that the chromosome is appropriately duplicated and that there is no DNA damage [41].

**Cyclins** together with their partner cyclin-dependent kinases (CDKs) are crucial regulators of the cell cycle. The first discovery was cyclin A and B, which associate with CDK1 (also known as CDC2) [42,43]. Together, they regulate mitosis by phosphorylating substrates including cytoskeleton proteins, histone H1, and possibly components of the mitotic spindle [44,45]. The degradation of cyclins A and B is necessary for cells to exit mitosis [46]. Cyclin E is induced during G1 and interacts with CDK2, thus facilitates cells to move from G1 to S phase. Cyclin E/ CDK2 complexes keep Rb in a hyperphosphorylated state (inactive) and activate a positive feedback loop for the accumulation of E2F [47], which is a transcriptional factor that regulate genes in S phase [48]. This is shown in **Figure 1**.

**Cell cycle arrest** is referred to the event when a cell’s normal progression is stopped due to DNA damage or malnutrition. For example, a cell can pause the cell cycle to allow for DNA repair when it detects DNA damage before proceeding with DNA replication and cell division. Checkpoint kinases coordinate this reaction, including the ataxia telangiectasia mutated (ATM) kinase, ATM and Rad3-related kinase (ATR), and checkpoint kinase (CHK)1/2 [49-51]. Moreover, tumor suppressor proteins like p53, p21, and p16 can block activity of CDK and lead to stop of the cell cycle [51,52]. Inhibiting growth factor signaling may also arrest the cells to stop in a distinct cell cycle phase [53]. For instance, inhibition of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway through antagonist of PI3K, phosphatase and

tensin homolog (PTEN) or PI3K inhibitor could leads to G2 phase cell cycle arrest [54]. Additionally, microtubule-depolymerizing agents can induce G1/G2 arrest before cells enter mitosis [55].



**Figure 1. Cyclins, CDKs and check points in the cell cycle.** This Figure shows the cyclins with their paired CDKs across different cell cycle phases. Different checkpoints are also illustrated. Created with Biorender.com

#### 1.1.4 Cell death

When a biological cell stops functioning, it is referred to have undergone cell death. Traditional major types of cell death are programmed cell death and necrosis [56].

**Apoptosis** is a programmed form of cell death characterized by cell shrinkage, chromatin condensation (pyknosis), cell membranes blebbing, and formation of apoptotic bodies [57]. The process of apoptosis is regulated by a complex of caspases proteins which could be divided into two categories: initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6, and -7) [58]. The initiation of apoptosis includes extrinsic, intrinsic and the perforin/granzyme pathway. In the extrinsic (death receptor) pathways death receptors like Fas, Tumor necrosis factor receptor 1 (TNFR1), and TNF-related apoptosis-inducing ligand (TRAIL) receptors, are activated upon binding to their respective ligands (*e.g.*, Fas Ligand (FasL), TNF-A, and TRAIL) [59]. The intrinsic (mitochondrial) pathway could be triggered by

DNA damage, oxidative stress, and other factors that could produce cellular stress. The intrinsic pathway is regulated by the B-cell lymphoma 2 (BCL-2) family proteins, which includes both pro-apoptotic and anti-apoptotic members. The pro-apoptotic members [*e.g.*, BCL-2 associated X (BAX) and B-cell antagonist killer (BAK)], promote the release of cytochrome c from the mitochondria. While the anti-apoptotic members [*e.g.*, BCL-2 and B-cell lymphoma-extra large (BCL-xL)], prevent the release of cytochrome c and support cell survival [60]. The perforin/granzyme pathway is used by cytotoxic lymphocytes to destroy virus-infected cells [61].

**Autophagy** is a programmed self-degradative process that could be triggered by nutrition deficiency and wherein lysosomes remove malfunctioning components [62]. There are following forms exist: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) and crinophagy [63,64]. During autophagy, cytoplasmic components such as damaged organelles and/or proteins are sequestered into vesicles with double-membrane which is called autophagosomes [65].

**Necrosis** is an uncontrolled cell death caused by various factors such as damage, infection, toxins and hypoxia [66]. It typically releases proinflammatory signals that affects nearby cells and could also promote angiogenesis, proliferation, and inflammation. Necrotic cell could exhibit characteristics such as cell size enlargement, disrupted cellular membrane, and cytoplasm release [57,67].

### 1.1.5 Cytoskeleton modulation

Studies have revealed that the shape of a cell and the tension in its cytoskeleton play crucial roles in cell function [68]. Researchers have shown that controlling cell shape by using micropatterned substrates coated with adhesive islands of extracellular matrix, can switch cells between proliferation, apoptosis and differentiation, alter cell migration speed, and promote tissue formation [69,70].

The cytoskeleton consists of three components: microtubules, intermediate filaments, and microfilaments. The microtubules are assembled by the polymerization of  $\alpha$ -tubulin and  $\beta$ -tubulin which formed a hollow pipe-like structure [71,72]. Microtubules are essential for motor driven intracellular transport, structural support against tension, as well as chromosomes separation during cell division [73]. Actin filaments are also known as microfilaments due to their assembly from thin and flexible actin fibers that have a diameter of approximately seven nanometers. Microfilaments provide cell with a flexibility that is vital for cell adhesion, cell

shape maintenance, and cell membrane mobility [74]. Intermediate filaments have a diameter of approximately ten nanometers, and are responsible for maintaining cell integrity and providing mechanical strength support [75]. These components interact with each other to support cellular morphogenesis processes like vesicular transport, vesicular shedding, and differentiation [76,77].

During the metastatic process, the cytoskeleton require to be dynamically remodeled and reorganized to support the mobility and shape modifications of cancer cells [78]. Reorganization of the actin cytoskeleton is important for epithelial-mesenchymal transition (EMT) by dynamically regulating cell elongation and directed motility [79-81].

Cytoskeleton modulations are also important in relation to the immune response. For example, phagocytosis utilizes actin polymerization to envelop and wrap the target particle [82-84]. The rearrangement of cortical actin is associated with the close interaction between T-cells and antigen-presenting cells (APCs), and the CTL-mediated killing process [85]. Additionally, microtubule repositioning towards the cellular interface is required for polarized vesicle secretion [86]. The cytoskeleton plays a critical role in NK cell effector functions, including their motility, infiltration, conjugation with target cells and assembly of immunological synapse [87].

There are key regulators that are essential for modulating cytoskeletal dynamics. For instance, small guanosine triphosphate binding proteins (i.e. Rho GTPase) belonging to the Ras superfamily and are essential for controlling the actin cytoskeleton, cell migration, and stem cell development [88]. One important factor that mediate Rho GTPase activation is the Rho-associated kinase (ROCK), which is essential for controlling the arrangement and interactions that modulate the cytoskeleton. ROCK phosphorylates proteins for instance, myosin light chain [89], and actin binding protein such as Lin-11, Isl-1, and Mec-3 (LIM) and profilin. This phosphorylation process plays a key role in modulating the structure and stability of actin filaments [90,91].

Studies have demonstrated that neural crest progenitors originally reside within the dorsal neural tube (NT). These progenitors, which begins as epithelial cells, undergo an EMT phase to acquire cellular motility and further migrate. The successful delamination process also requires the involvement of effector proteins, which play a role in reorganizing the actin cytoskeleton, altering adhesive properties, and leading subsequently to the loss of epithelial polarity [92-94]. For example, the downregulation of active RhoA and RhoB in the cell membrane of epithelial progenitors is an important step during delamination [95,96].

Formins (formin homology proteins) are a group of conserved actin polymerization factors that are essential for the assembly of actin filaments, by binding to their rapidly elongating region [97]. The comprehensive information of Formin family is shown in **Table 1**. Formins are important for the formation of cellular structures like filopodia, lamellipodia, and stress fibers [98]. Filopodia serve as cellular antennae enabling cells to explore and sense their surroundings [99]. The lamellipodium are involved in firmly adhering to the underlying substrate [100]. Stress fibers are contractile bundles of actin [101]. During development events, formins also play a vital role in gastrulation during which the embryo transforms from one layer to multi-layer and are also important in neural tube closure which eventually forms the brain and spinal cord [102-104].

**Table 1. Comprehensive information on the Formin proteins family** [105]. The table shows the detailed gene names, full names, UniProt accession numbers, chromosome locations, and Gene Ontology (GO) terms of each member. The GO terms provided in the table represent Molecular Functions, as sourced from GeneCards (<https://www.genecards.org/>).

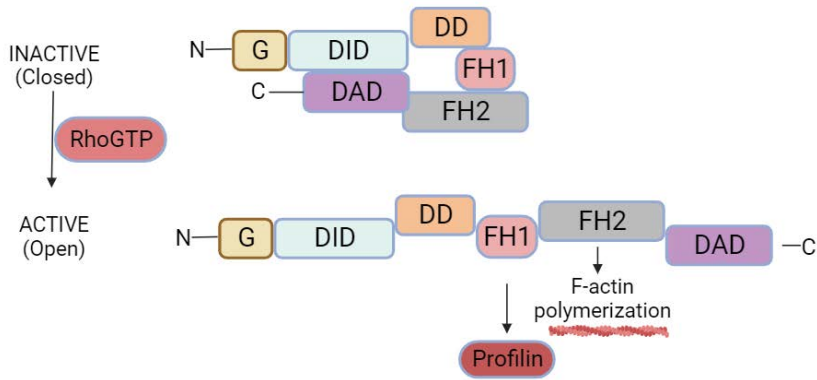
Gene name	Full name	UniProt accession numbers	Chromosome location	Qualified GO term (GeneCards)
<i>DAAMI</i>	Disheveled-associated activators of morphogenesis 1	Q9Y4D1	14q23.1	actin binding protein binding small GTPase binding
<i>DAAM2</i>	Disheveled-associated activators of morphogenesis 2	Q86T65	6p21.2	actin binding protein binding small GTPase binding
<i>DIAPH1</i>	Diaphanous related formin 1	O60610	5q31.3	RNA binding actin binding signaling receptor binding
<i>DIAPH2</i>	Diaphanous related formin 2	O60879	Xq21.33	actin binding signaling receptor binding small GTPase binding
<i>DIAPH3</i>	Diaphanous related formin 3	Q9NSV4	13q21.2	actin binding small GTPase binding cadherin binding

<i>FHDC1 (INF1)</i>	FH2 domain containing 1		4q31.3	actin binding microtubule binding
<i>FHOD1</i>	Formin homology domain containing proteins 1	Q9Y613	16q22.1	actin binding protein binding protein domain specific binding
<i>FHOD3</i>	Formin homology domain containing proteins 3	Q2V2M9	18q12.2	actin binding protein binding actin filament binding
<i>FMNL1</i>	Formin Like 1	O95466	17q21.31	actin binding protein binding small GTPase binding GTPase activating protein binding
<i>FMNL2</i>	Formin Like 2	Q96PY5	2q23.3	actin binding small GTPase binding cadherin binding actin filament binding
<i>FMNL3</i>	Formin Like 3	Q8IVF7	12q13.12	actin binding protein binding small GTPase binding GTPase activating protein binding actin filament binding
<i>FMN1</i>	Formin 1	Q68DA7	15q13.3	actin binding microtubule binding SH3 domain binding
<i>FMN2</i>	Formin 2	Q9NZ56	1q43	actin binding small GTPase binding cadherin binding actin filament binding
<i>GRID2IP</i>	Delphilin/Grid2 Interacting Protein	A4D2P6	7p22.1	protein binding
<i>INF2</i>	Inverted Formin 2	Q27J81	14q32.33	actin binding protein binding small GTPase binding

DIAPH3, or Diaphanous Homolog 3, is a member of the Formin family subgroup of the Diaphanous family. This protein is involved in the assembly of filopodia, essential for cell migration [106], as well as in the creation of the contractile ring and the cleavage furrow, essential for cytokinesis [107,108]. DIAPH3 was found to be a diagnostic biomarker by the pan-cancer analysis, and pathway enrichment analysis showed it is involved in tumor expansion, movement, programmed cell death, and alterations in the tumor microenvironment [109]. The function of DIAPH3 in the development of tumors varies based on the specific type of cancer, as shown in **Table 2**. The structure of diaphanous and activation program is shown as **Figure 2**.

**Table 2. Role of DIAPH3 in various cancer types.**

Cancer Type	Role of DIAPH3
Cervical Cancer	Its expression was associated with poor prognosis and described to have positive correlation with angiogenesis and TGF- $\beta$ signaling [109].
Colorectal Cancer (CRC)	<i>DIAPH3</i> knockdown increased the ability of CRCs to proliferate and migrate. DIAPH3 was suggested to decrease the progression of CRC by preserving EGFR degradation [110].
Hepatocellular Carcinoma	DIAPH3 facilitated the proliferation, migration, EMT, and metastasis of hepatocellular carcinoma cells [111].
Lung Adenocarcinoma	Expression of DIAPH3 increased in lung adenocarcinoma (LUAD) and stimulated LUAD cell proliferation and colony formation [112].
Osteosarcoma	<i>DIAPH3</i> knockdown inhibited osteosarcoma cell growth and metastasis [113].
Pancreatic cancer	Knocking down <i>DIAPH3</i> reduced ROS levels, TrxR1 expression, and tumor development [114].

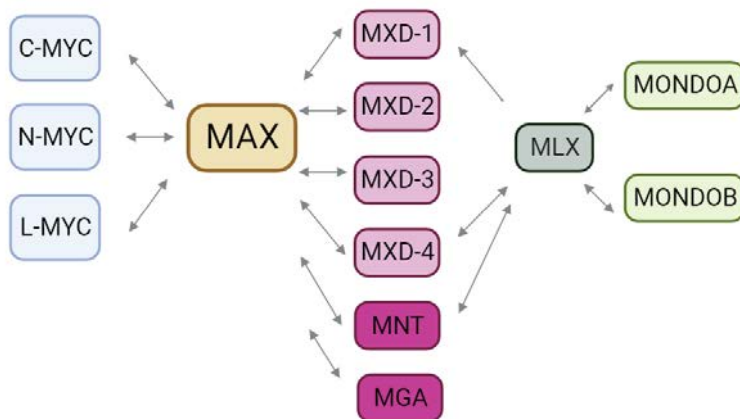


**Figure 2. Structure of diaphanous-related formins.** The interaction between the DID (Diaphanous Inhibitory Domain) and the DAD (Diaphanous Autoregulatory Domain) causes the inactive form of formin in a closed conformation. When Rho-GTPase interact with GTPase-binding domain (GBD), it lead to disruption of the DID-DAD binding. This disruption enables the molecule to adopt an active, open conformation. In this activated state, the FH1 (Formin Homology 1) domain interactives Profilin. Profilin then supplies G-actin (globular actin) to the FH2 (Formin Homology 2) domain, enabling the assembly of actin filaments. Created with Biorender.com

### 1.1.6 MYC Proteins

The Myelocytomatosis (MYC) family comprises three members: c-MYC (encoded by *MYC*), L-MYC (encoded by *MYCL*), and N-MYC (encoded by *MYCN*) [115,116]. The c-MYC gene was the first identified in this family, due to its similarity to the viral gene *v-myc* in the chick retrovirus [117]. MYC acts as a transcription factor and belongs to the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family residing in the cell nucleus [118]. Its 439 amino acid structure includes a C-terminal domain (CTD) and an N-terminal transactivation domain (TAD). Three highly conserved sections called MYC Boxes (MB0, MBI, and MBII) are found in the N-terminal region and play a crucial role in the regulation of transcription and protein degradation [119]. The bHLH-Zip motif found in the C-terminal domain is also necessary for the protein's ability to regulate transcription. This is done by binding of a specific region named “E-box” (enhancer boxes), with a canonical sequence “CACGTG” [118,120]. Located in the center of the protein is the PEST domain that has proline (P), glutamate (E), serine (S), and threonine (T), and three additional conserved MYC boxes, MBIIIa, MBIIIb, and MBIV, associated with transcription [121].

Via the HLH-Zip domain, MYC (*i.e.* c-MYC, MYCN, L-MYC) forms a heterodimer with the protein MYC-associated factor X (MAX). The MYC-MAX complex then binds to the specific DNA regions (*i.e.* E-boxes), thereby activate the transcription of genes involved in cell proliferation, metabolism and apoptosis. On the other hand, MXD proteins (encoded by *MXD1-4*), MNT, and MGA are also act as partners for MAX through the formation of heterodimers. They achieve this by competing with MYC for binding to MAX, resulting in the inhibition of MYC's transcriptional activity, and by binding to target genes on the DNA [122]. Thus, the balance between MYC-MAX and MXD-MAX complexes is critical for cell function. MYC is predominantly expressed in proliferating cells, while MXD proteins are present in differentiating cells. This spatial separation in expression patterns ensures that there is no direct competition between MYC and MXD proteins, highlighting the specialized roles of these complexes in distinct cellular processes. Studies suggest that dimerization of c-MYC with MAX increases c-MYC stability by reducing its ubiquitination and subsequent proteasomal degradation, and result in increased c-MYC levels upon MAX overexpression [123]. MAX-like protein (MLX) binds to MXD-1 and -4 as well as with MONDOA and MONDOB [124]. **Figure 3** illustrates the intricate network of interactions involving the MYC family.



**Figure 3. MYC interaction network.** The MYC family form dimers with MAX and on the other side, MAX form dimers with MXDs. MLX binds to MNT, MXD-1 and -4 as well as with MONDOA and MONDOB. Created with Biorender.com

In normal cells, *MYC* expression is governed by mitogenic signals. Due to the rapid degradation of both the protein and mRNAs, the balance between these elements sustains cell proliferation. However the activation or overexpression of *MYC* alone in normal cells does not cause cancer, because of the existence of balanced control mechanisms encompassing proliferative halt,

apoptosis, and senescence [125]. In tumor cells, the aberrant expression of *MYC* is rarely due to mutation but to chromosome amplification and translocation, or driven by upstream pathways such as the Ras and Notch pathways and also super enhancer activation [126]. Post-translational modification, such as the phosphorylation of serine at position 62, helps in reducing the degradation of *MYC* and enhancing its stability [127,128]. In cancer cells, *MYC* activation is the trigger for various ‘hallmarks’ of cancer, including proliferation and growth, protein or RNA synthesis, genomic instability, metabolism reprogramming, or angiogenesis [129-132].

c-MYC is an intrinsically disordered protein, it resides in the nucleus and lacks enzymatic activity or a particular binding pocket for traditional small molecules, making it difficult to target [133]. Since c-MYC also plays a vital role in normal cells, targeting c-MYC might cause side effects that disrupt normal cell function. *MYC* is abnormally expressed in over 70% of human cancers and strongly associated with poor prognosis, thus making it an interesting gene to target [119]. Currently, strategies targeting c-MYC include small molecules like IIA6B17, 10058-F4, 10074-G5, Mycro1, 3jc483 and MYCMI-6, which inhibit the dimerization of c-MYC/MAX or prevent their binding to DNA, yet they lack the specificity to target c-MYC effectively [119]. Notably, 10058-F4 [5-(4-ethyl-benzylidene)-2-thioxo-thiazolidin-4-one]] and 10075-G5 [1-(3-Chloro-phenyl)-3-diethylamino-pyrrolidine-2,5-dioneand] were initially identified through a high-throughput drug screening approach utilizing a yeast two-hybrid system [134]. To interact with DNA, c-MYC needs to form a heterodimer with MAX. The compounds 10058-F4 and 10075-G5 interact with c-MYC while it is in a disordered state, thereby preventing the formation of a heterodimer with MAX and thus inhibiting the transactivation of c-MYC target genes [134]. To enhance the binding efficiency and affinity, these two inhibitors were designed covalently connected, thus potentially engaging with multiple sites on the target proteins [135]. Modified versions of 10075-G5, such as 3jc48-3 and JY-3-094, have demonstrated higher potency than the initial generation [136,137].

Unlike small molecules or large proteins, peptides are smaller and can often penetrate cells more easily and can interact with biological targets in multiple ways and on a broader scale, which could result in better treatment outcomes [138]. Omomyc is a peptide that acts as a mimic of *MYC*’s bHLH-Zip domain thus could bind to c-MYC, MYCN, MAX and Miz-1, but not MXD1 or other bHLH-Zip proteins [139]. This selective binding prevents *MYC* interacting on E-boxes to transactivate target genes and at the same time repress other genes by binding to Miz-1. Omomyc also leads to a reduced acetylation and increased methylation at a specific site on histone H3, a protein that helps package DNA [139]. Moreover, Omomyc has shown its effect on various cell lines or animal model, for example, renal carcinoma, pancreatic ductal

adenocarcinoma (PDAC), and non-small cell lung cancers [140-142]. The encouraging development of Omomyc (OMO-103), has successfully passed the phase I clinical trial which involved 22 patients, encompassing pancreatic, bowel, and non-small cell lung cancers (<https://www.peptomyc.com/>, released in November 2023). There are also ongoing studies (NCT06059001 and NCT04808362) that investigate the safety, efficacy, and pharmacological aspects of OMO-103, one in combination with standard chemotherapy in metastatic pancreatic cancer and the other as a monotherapy in various advanced solid tumors (<https://clinicaltrials.gov>, access during December 2023).

c-MYC works as a global transcriptional factor, regulating 10-15% of the genome [143]. It targets genes involved in processes like DNA replication, the creation of ribosomes, the translation of mRNA, controlling the cell cycle, and managing stress responses, affecting key biological activities including cell proliferation, differentiation, programmed cell death, and the regulation of immune functions [144]. By attaching the hormone-binding domain of the ER to c-MYC, researchers can turn the c-MYC protein on and off in cells by regulated exposure to estrogen or anti-estrogen compounds [145]. For example, this technique was employed to discover  $\alpha$ -prothymosin (*PTMA*) and ornithine decarboxylase 1 (*ODCI*) as target genes of c-MYC [146,147].

The introduction of expression microarrays facilitated the extensive analysis of c-MYC regulated genes on a large scale. Nonetheless, these studies faced with difficulties due to the small changes in mRNA expression levels of genes influenced by c-MYC, which resulted in signal-to-noise ratio problems in the initial analyses [143]. More recently, chromatin immunoprecipitation (ChIP) assays have significantly improved the process of identification of direct c-MYC targets. ChIP revealed that c-MYC binding to genomic loci depends on chromatin structure and modifications, such as histone methylation [148]. ChIP-sequencing (Chromatin Immunoprecipitation followed by sequencing) sequences all the DNA fragments bound to the protein after ChIP which provides a high-resolution map of protein-DNA interactions across the genome [149]. ChIP-chip, also known as Chromatin Immunoprecipitation followed by DNA microarray, is a method that involves the extraction of DNA-protein complexes and subsequent hybridization of the DNA onto a microarray chip loaded with numerous genomic DNA sequences [150]. One study that utilized ChIP and promoter microarrays identified 1469 genes as direct targets of c-MYC, which are mostly involved in mitochondrial biogenesis [151].

## 1.2 Pediatric cancers and their features

Childhood cancer presents an incidence rate of 15.3 per 100,000 children each year [152]. The most common types of cancer in children are leukemia [especially acute lymphoblastic leukemia (ALL)], brain tumors, (*e.g.*, medulloblastomas, gliomas), lymphoma, neuroblastoma, Wilms tumor, rhabdomyosarcoma, and retinoblastoma [153].

Childhood cancer arises during development when cells that expected to undergo maturation instead remain in a proliferate state [154]. Childhood cancer is rare compared to adult cancer and has lower mutations rates. This difference could be attributed to less exposure to environmental carcinogens and the effects of aging over time [155]. Adult cancer comprises malignancies like breast, lung, prostate, colorectal cancers, and others [156]. It is influenced both by endogenous factors like aging, inflammation, and exogenous factors like radiation, carcinogens, environmental variables, occupational factors, and lifestyle decisions [157].

Apart from large doses of radiation and chemotherapy, childhood cancer appears to be associated with inherent factors like congenital abnormalities, weight at delivery, and maternal age [158].

## 1.3 Pathology and classification of peripheral neuroblastic tumors

Peripheral neuroblastic tumors (PNTs) have a neural crest origin and are thought to be embryonal remnants from the developing sympathetic nervous system [159]. These malignancies are common extracranial solid tumors that account for 7-10% of cancer in children [160]. Based on microscopic features, including neuroblast differentiation grade and Schwannian stromal development quantity [161], the International Neuroblastoma Pathology Committee defines four groups of peripheral neuroblastic tumors: 1) Neuroblastoma (Schwannian stroma-poor), 2) Ganglioneuroblastoma, intermixed (Schwannian stroma-rich), 3) Ganglioneuroma (Schwannian stroma-dominant), and 4) Ganglioneuroblastoma, nodular (Schwannian stroma-dominant) [162].

Neuroblastoma is the most common and the least mature/differentiated form among these categories. Following the tumor histology, this disease can be classified in three subgroups: undifferentiated, poorly differentiated, and differentiating [161,163]. The undifferentiated neuroblastoma subtype presents unfavorable histology and rare occurrence [164]. There are small to medium size tumors, presenting diffuse cytoplasmic limits, few hetero-chromatin and round to elongated nuclei, granular chromatin, and a distinctively prominent nucleolar

formation [165]. This subtype presents unfavorable outcomes in association with *MYCN*-amplification, but MYC-protein enrichment may provide a better predictor of more aggressive clinical behavior [164].

The poorly differentiated subtype of neuroblastomas shows differentiation features in less than 5% of the tumor cells [161]. Within this subtype, favorable tumors present more pronounced differentiation, higher expression of TrkA, and larger content of Schwannian stromal cells, compared with biologically unfavorable undifferentiated tumors [163,166]. Histologically, the differentiating subtype of neuroblastomas usually shows abundant axon outgrowth and neurite production and have a favorable prognosis. More than 5% of tumor cells in this subtype present differentiating neuroblasts [163,167].

Ganglioneuroblastoma is a neoplasm with miscellaneous forms of neuroblasts and ganglion cells in different proportions. Tumors in this category have contrasting differentiation levels and stroma abundance and can be classified as stroma-rich (well-differentiated, intermixed, nodular) or stroma-poor [168]. Ganglioneuroblastoma, nodular (Schwannian dominant) GNB-N is a rare subtype of neuroblastic tumors. The tumors of this subtype have traditionally been considered as either biologically and clinically non-aggressive (Schwannian stroma-rich and stroma-dominant) or aggressive nodular (Schwannian stroma-poor). Stroma-poor component usually shows hemorrhagic and/or necrotic [161].

Ganglioneuroma (Schwannian stroma-dominant) are biologically benign malignancies from the neural crest, that can be classified as maturing and mature tumors. The former consists of a mixture of maturing and mature ganglion cells, whereas the latter contains only mature ganglion cells. The stromal tissue is usually well organized and displays Schwann cell fasciculi. The composite subgroup is composed of different clones. The stroma-poor component is usually hemorrhagic and/or necrotic.

## **1.4 Neuroblastoma**

### **1.4.1 Incidence and Epidemiological Features of Neuroblastoma**

Neuroblastoma (NB) is the third most common pediatric cancer after leukemia and primary central nervous system (CNS) tumors. It has an incidence of 11 to 13 per million children in the USA, which accounts for 15% of childhood cancer deaths [169-171]. Each year approximately 20 new cases occur in Sweden, 1500 cases in Europe, and 700 cases in the USA

and Canada [172]. In recent years, remarkable improvements in the treatment of NB patients have been achieved with 5-year survival increasing from 52% (1975-1977) to 82 % (2011-2017), as reported by the Surveillance, Epidemiology, and End Results databases for the USA ([www.seer.cancer.gov](http://www.seer.cancer.gov)). Nevertheless, half of the newly registered cases display metastases, which result in a low quality of postoperative life [173]. In the Nordic region, the five-year survival rate for NB improved from 61% in 1999-2001 to 80% in 2005-2007 [174]. One single center in Beijing, China performed a retrospective study on 1041 cases during 2007-2019. The result revealed that the five-year overall survival rate was 97.5%, 96.7%, and 48.9% in low-risk, intermediate-risk and high-risk group, respectively [175].

### 1.4.2 Origin and development of Neuroblastoma

Neuroblastoma usually arises in the adrenal medulla, abdomen, or chest [176]. It is thought to have origin in neural crest derive cells that fail to properly migrate or differentiate during the sympathetic nervous system development [177]. Neural crest cells form at the edges of the neural plate, which is also precursor to the CNS [178-180].

In recent years, single-cell RNA sequencing (scRNA-seq) has been utilized to study the phenotypic variability of the neural-crest-derived cell populations giving origin to the sympathoadrenal system. scRNA-seq is a powerful technology for analysis of complex tissues or mixed cells, that also allows to track the gene expression changes during development [181]. Single-cell transcriptomic analyses conducted by Jansky *et al.* (2021) showed that the transcriptional characteristics of NBs are similar to those of normal fetal adrenal neuroblasts [182]. In addition, NBs with different clinical features resemble distinct differentiation trajectories and statuses in association with their clinical outcome [182]. Single-nuclei analysis performed by Bedoya-Reina *et al.* (2021) identified that NBs with low-risk resemble more committed sympathoblast and chromaffin cells, while high-risk subtype resembles a neurotrophic tyrosine kinase receptor B (TRKB) cholinergic progenitor population [183]. Kildisiute *et al.* (2021) demonstrated that the similarities in gene transcription between NB cells and sympathoblasts could imply that neuroblastoma originates directly from sympathoblasts [184]. Olsen *et al.* (2020) have identified a role of SCP in the origin of NB [185]. For a comprehensive overview of recent studies applying single cell/nucleus analyses, please see **Table 3**.

**Table 3. Summary of single cell/nucleus analysis on NB.** The table presents author's last name, publication year, and key methodologies. Studies employing either single cell or single nucleus analysis are shown in the table. "Yes" denotes the use of such analysis, and "-" denotes that it was not utilized. The sequencing

platforms of each study, including 10x, CEL-seq2, Smart-seq2, or GEXSCOPE, are specified. The number of patients involved in each study is listed, along with data availability. Datasets with "GSE" prefixes are entries in the Gene Expression Omnibus (GEO) database, asterisk-marked (\*) numbers showcase other databases, or "Not available" if the data is not publicly accessible.

1\*: <http://neuroblastomacellatlas.org>.

2\*: [https://adrenal.kitz-heidelberg.de/developmental\\_programs\\_NB\\_viz/](https://adrenal.kitz-heidelberg.de/developmental_programs_NB_viz/)

3\*: [https://oxygen.mtc.ki.se/nc\\_nb\\_2021.html](https://oxygen.mtc.ki.se/nc_nb_2021.html)

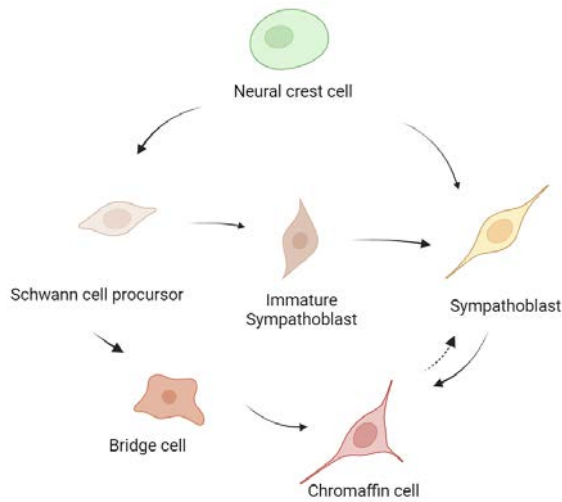
4\*: European Genome-Phenome Archive (EGA), EGAS00001004781

5\*: EGA, under EGAS00001004781 and EGAS00001005322.

Study	Slyper	Dong	Kildisiute	Jansky	Reina	Verhoeven	Costa	Yuan	Janoueix-Lerosey
Year	2020	2020	2021	2021	2021	2022	2022	2022	2023
Single cell	Yes	Yes	Yes	-	-	Yes	Yes	Yes	Yes
Single nucleus	Yes	-	-	Yes	Yes	-	-	-	-
Platform	10x	10x	10x/CEL-seq2	10x	Smart-seq2	10x	10x	Smart-seq2	10x
Patient number	4	16	20	14	11	19	10	5	18
Access	GSE 140819	GSE 137804	1*	2*	3*	GSE 147766	4*	GSE 192906	5*

**Schwann cell precursors** (SCPs) is a nerve-associated cell originated from a second wave of migrating neural crest cells. They are important for myelination and axonal homeostasis. Adameyko's group (2022) has found evidence suggesting that early-stage SCPs and later-stage neural crest cells share a common, multipotent state where they can develop into different cell types. The "hub" gene *Sox8*, for example, impacts how SCPs develop, particularly along the nerves and in their choice to become sympathoadrenal cells [186]. The group also identified with the usage of lineage tracing experiments that SCPs originates both sympathoblasts and chromaffin cells in mouse and observed a similar pattern in humans [187,188]. Jansky *et al.* (2021) investigated the entropy of single cells from adrenal medulla and found that SCPs have the highest differentiation potential and therefore they are at the root of the adrenal medullary differentiation hierarchy. The authors identified that in human, SCPs led to the formation of three lineages: chromaffin cells; sympathoblast/neuroblasts; and late SCPs [182,188]. **Chromaffin cells** of the adrenal gland medulla produce catecholamines include epinephrine (adrenaline) and norepinephrine (noradrenaline) that help triggering the "fight-or-flight"

response [189]. It is believed that during the later stages of adrenal medulla development, bridge cells serve as a transitional stage from SCPs to chromaffin cells [188]. Phenylethanolamine N-methyltransferase (*PNMT*) is one of the signature gene of chromaffin as PNMT catalyzes the conversion of norepinephrine to epinephrine [190,191]. **Sympathoblasts**, also known as neuroblast, originate from neural crest cells and serve as precursors to sympathetic neurons. Kildisiute *et al.* (2021) performed sc-RNA showing that NB cancer cells resemble the fetal sympathoblast state, and NB cells share transcriptional characteristics with sympathoblasts by using transcriptional featured signature validated by bulk data [184]. **Figure 4** show human sympathoadrenal lineage development. Arguably, during development chromaffin cells could originate sympathoblast/neuroblast and vice versa [182,187,192].



**Figure 4: Human Sympathoadrenal Lineage Development.** The differentiation of Schwann Cell Precursors into Chromaffin Cells and Sympathoblasts. Adapted from KI Lundberg *et al*, 2022. With CC BY licence.

**Adrenergic and Mesenchymal Subtypes:** NB can be categorized into adrenergic (ADR /ADRN or formally NOR) and Mesenchymal (MES) types. **ADR-type neuroblastoma** features neuronal traits and neuroblastic characteristics. Cells in the ADR state are committed to a sympathetic noradrenergic lineage and have the potential to partial neuronal differentiation when exposed to RA. **MES-type neuroblastoma**, resemble neural crest derived precursor cells [193]. MES-type neuroblastoma cells are resilient to treatments with Anaplastic Lymphoma Kinase inhibitors (ALKi) even when carrying mutations in the tumor-promoting *ALK* oncogene [194]. The activation of *PRRX1* [193], or an inducible NOTCH-IC transgene reprogram the

ADRN cells into a MES state [195]. It is considered that ADR is a differentiated status while MES is an immature form. However it is challenging to define an immature MES-like state, primarily due to heterogeneity of NB, the use of diverse ADR/MES cell state markers, and the employment of various sample sources like cell lines, mouse models, and patient derived xenografts (PDXs) [196].

**Morphological Subtypes of NB Cells:** NB cell lines have three different subtypes based on morphologies and behaviors. The first type is neuronal (N-type) neuroblastoma cells that display characteristics similar of neurons from the sympathoadrenal lineage and a transcriptional profile of ADR cells. This type of cell has the expression of neuron-specific neurofilaments, morphological differentiation, and the ability to secrete neurotransmitters, and are highly invasive. For example, SH-SY-5Y, SK-N-BE(1), SK-N-BE(2), BE(2)-M17, SMS-KCN, IMR-32, SK-N-FI. The second type is substrate adhesive (S-type) neuroblastoma cells that showed the unique flat and adherent cellular morphology as they spread. They display a transcriptional profile of MES cells. For example, SH-EP, SK-N-AS and SK-N-MC. The third type is intermediate (I-type), showed a mixed phenotype and could differentiate into both types [197]. For example, SK-N-SH is an I-type cell line that was subcloned to SH-SY5Y and SH-EP. The initial clone derived from metal cylinders isolation and culture was named SH-SY. This was then subcloned to generate SH-SY5, and finally SH-SH5Y. On the other hand, epithelial-like cells subgroup from SK-N-SH were also subcloned to generate the SH-EP and SH-FE cell lines [198].

### 1.4.3 Classification and Risk Stratification of Neuroblastoma

The "International NB Staging System" (INSS) was initially proposed in 1988 and further updated in 1993, to stratify the NB cases into five different stages (**Table 4**), according to the anatomical location at diagnosis and assessment after the initial surgery [199,200]. In this classification, stage 1 presents localized tumors that can be surgically removed, and stage 2 localized tumors that cannot be entirely removed. Higher stages 3 and 4 classify spread tumors with regional and distant lymph node infiltration. There is a special stage 4S, which may have potentially spread to lymph nodes, liver, skin, and/or bone marrow, patients in this group tend to undergo spontaneous regression [201].

The International NB Risk Group (INRG) classification system was proposed in 2005 and analyzed by an international consensus on risk assignment of 8800 patients diagnosed between 1990–2002. Compared to the INSS, the INRG stage was determined by the results of imaging tests performed prior to surgery but not by surgery results or lymph node spread (**Table 5**).

INRGSS was developed to help surgeons and physicians determine the resectability or unresectable of loco-regional disease depending on the image-defined risk factors. In this system, stages L1 and L2 are defined as localized malignancies without- and with image defined risk factors, respectively. The stage M and MS group are metastatic cases, in which S means "special" to children under 18 months old with metastases restricted to skin, liver, and/or bone marrow [202].

**Table 4. International Neuroblastoma Staging System Committee (INSS) system.** Adapted from Lucena et al, 2018, Rev Paul Pediatr [203].

Stage	Description
Stage 1	The tumor and its adjacent lymph nodes can be fully excised through surgery. While these removed nodes may have cancer, other nearby lymph nodes do not.
Stage 2A	The tumor is confined to its original area and cannot be fully removed surgically. Nearby lymph nodes are cancer-free.
Stage 2B	The tumor, restricted to its initial area, might not be fully removable in surgery, and nearby lymph nodes are cancerous.
Stage 3	The tumor cannot be removed and has spread to nearby lymph nodes and adjacent areas, but not to distant body parts.
Stage 4	The initial tumor has metastasized to distant lymph nodes, bones, bone marrow, liver, skin, and other organs, excluding those specified in stage 4S.
Stage 4S	The initial tumor remains at its origin (as in stages 1, 2A, or 2B) and has only spread to the skin, liver, and/or bone marrow in infants under one year old, with minimal bone marrow involvement (typically under 10% cancerous cells).

**Table 5. The International Neuroblastoma Risk Group Staging System (INRGSS).** Adapted from Monclair *et al*, J Clin Oncol. 2009 [204].

Stage	Description
Stage L1	The tumor is located only in the area where it started; no image-defined risk factors (IDRFs) are found on imaging scans, such as a CT or MRI scan.
Stage L2	The tumor has not spread beyond the area where it started and the nearby tissue; IDRFs are found on imaging scans, such as a CT or MRI scan.
Stage M	The tumor has spread to other parts of the body (except stage MS).
Stage MS	The tumor has spread to only the skin, liver, and/or bone marrow (less than 10% bone marrow involvement) in a patient under 18 months.

A better understanding of NB biology has led to the implementation of risk stratification methodologies used to predict prognosis and more precise treatments. One widely used classification system is the Children's Oncology Group (COG) classification, which divides patients into three risk groups based on the International Neuroblastoma Staging System (INSS): low, intermediate, and high-risk groups [205]. INRG also developed a pretreatment classifier (**Table 6**, on next page) by using INRGSS and based on seven prognostic risk factors; these factors were then used to categorize 16 groups and 4 categories: very low, low, intermediate, and high-risk groups [206,207].

#### **1.4.4 Genetic Alterations in Neuroblastoma**

##### **Spontaneous *versus* familial NB**

Spontaneous NB arises sporadically, and the genetic alterations are acquired, which contribute to the majority of NBs. On the other hand, familial NB is less common and comes from inherited genetic changes [208]. In familial NB, there are two main genes often found to be altered, *anaplastic lymphoma kinase (ALK)* and *paired-like homeobox 2b (PHOX2B)* [208].

##### ***MYCN* amplification**

The *MYCN* oncogene is located on chromosome 2p24 and is amplified in approximately 20-25% of all NB and in 40% of high-risk patients with stage 3 and 4 diseases. *MYCN* amplification is regarded to be one of the strongest hallmarks of high-risk NB with poor prognosis [209]. Besides NB, *MYCN* amplification can also occur in medulloblastoma, retinoblastoma, glioblastoma, Wilms tumor, prostate cancer, and lung cancer [210-214]. *MYCN* belongs to the *MYC* family, which contains a specific domain known as a bHLH-Zip domain. In 1983, Schwab *et al.* identified *MYCN* as an amplified gene homologous to *v-myc* but different from *MYC* in NB. As a result, a different name was given to this gene [215]. The analysis of high-throughput pharmacogenomic data reveals *MYCN*-amplification as a potential predictor of response to bromodomain inhibitors, which function by displacing the Bromodomain containing protein 4 (BRD4) from the promoter of the *MYCN* gene [216]. *MYCN* overexpression has been implicated in promoting malignancy and an aggressive phenotype, characterized by a stem-like/undifferentiated state, increased proliferation, migration, which are observed in approximately 50% of patients with NB at the time of diagnosis [169,217].

**Table 6. The International Neuroblastoma Risk Group Pretreatment Classification Schema [202].**

Abbreviations: GN, ganglioneuroma; GNB, ganglioneuroblastoma; INRG, International Neuroblastoma Risk Group; NA, not amplified. Pretreatment risk group, A,B,C: very low risk; D,E,F: low risk; G,H,I,J :intermediate risk; K,O,P,Q,R,N: high risk; Adapted from Susan L *et al*, J Clin Oncol 2009.

INR G stage	Age (months)	Histologic category	Grade of tumor differentiation	MYCN- amplification	11q aberration	Ploidy	Risk group
L1/L 2		GN maturing; GNB intermixed					A
L1		Any except GN maturing or GNB intermixed		NA			B
				Amplified			K
L2	<18	Any except GN maturing or GNB intermixed	Differentiating	NA	No		D
					Yes		G
					No		E
	≥ 18	GNB nodular; neuroblastoma	Poorly differentiated or undifferentiated	NA	Yes		H
							N
M	<18			NA		Hyper- diploid	F
	<12			NA		Diploid	I
	12 to <18			NA		Diploid	J
	<18			Amplified			O
	≥ 18						P
MS	<18			NA	No		C
					Yes		Q
				Amplified			R

This phenotype is characterized by an increase in matrix metalloproteinase (MMP) activity [218] and inhibition of E-cadherin, resulting in epithelial to mesenchymal transition (EMT) [219].

Neuroblastoma is believed to be derived from neural crest cells. Neural crest cells undergo maturation, migration, and differentiation during this developmental phase [177]. *MYCN* is highly expressed in migration and post migration cells, as these cells mature into sympathetic neurons, *MYCN* expression gradually decreases [220]. Excessive *MYCN* expression in neuroblasts prevents them from differentiating, which lowers the number of chromaffin cells [220,221].

### ***ALK* mutation**

The *ALK* is located on the 2p23 chromosomal segment and encodes for the ALK receptor tyrosine kinase and is a member of the insulin receptor superfamily [222,223]. *ALK* which was originally identified as a fusion kinase found in non-Hodgkin's lymphoma [224], is mutated in approximately all familial NB and in 10% of the spontaneous cases [225,226]. The majority of all ALK variants in NB are concentrated at three sites (R1275, F1174, and F1245) located in the tyrosine kinase domain [227]. Over-expression is significantly associated with high-risk and unfavorable prognosis [228]. When recurrence of disease occurred, *ALK* alterations experienced a 70% rise in prevalence [229]. Note that amplification of *ALK* is associated with adverse outcome of NB [229]. *ALK* is a *MYCN* target gene and *MYCN*-amplification is associated with *ALK* mutation, these aberrations synergistically drive disease progression and enhanced lethality [230,231].

### ***PHOX2B* mutation**

*PHOX2B* is located on chromosome 4p12, a locus frequently altered in NB and for which mutations are involved in the initiation of tumorigenesis [232]. Mutations in *PHOX2B* have been reported in a group of NB patients with either sporadic or familial NB, implicating its potential role in NB predisposition [232]. Jansky *et al.* (2021) identified and characterized the primary cell types within the sympathoadrenal lineage in humans by using scRNA sequencing, from which the result showed that *PHOX2B* was expressed during SCPs to bridge cells transition. Dubreuil *et al.* (2000) demonstrated that when the *PHOX2B* gene is overexpressed in the developing spinal cord of chicks, it encourages neural crest cells to exit the cell cycle and differentiate [233]. *PHOX2B* is also responsible for the specific transcription of the dopamine beta-hydroxylase gene in noradrenergic cells [234]. In alignment with its function as a crucial gene in neurodevelopment, the overexpression of the not-mutated (wild type) *PHOX2B* gene

in NB cell lines led to a reduction in cell growth [235]. A study by Naftali *et al.* (2016) suggested that it functions as a suppressor of NB progression [236].

PHOX2B was also reported as a specific indicator of minimal residual disease in NB [237]. Fan *et al.* (2020) found that the presence of PHOX2B in bone marrow and peripheral blood at diagnosis was associated with worse event-free survival (EFS) and overall survival (OS) in non-high-risk NB patients, suggesting that PHOX2B could be a potential biomarker for predicting metastasis and prognosis in these patients [238]. Further research is required to completely understand the role PHOX2B plays in NB.

### ***ATRX* mutation**

The *ATRX* gene is located on the long arm of X chromosome (Xq13.3) [239]. *ATRX* is a transcriptional regulator that contains an ATPase/helicase domain, sometimes referred to as ATP-dependent helicase *ATRX* [240]. As part of its function, it recognizes guanine-rich DNA segments and deposits the H3.3 histone variant to prevent G-quadruplex (G4) formation, then DNA replication or transcription can be hindered. *ATRX* mutation is implicated in tumorigenesis but does not show a positive correlation with elevated *MYCN* [241]. *ATRX* mutations are usually found in NB tumors in children older than 5 years with a progressive clinical character or with a chronic disease [242].

### **Rearrangements of the Telomerase Reverse Transcriptase (*TERT*) gene**

*TERT* rearrangements derived from genomic translocations occurring at chromosome 5p15 [243]. The telomerase enzyme *TERT* comprise the most important unit within the telomerase complex [244]. Rearrangements of the *TERT* gene are common in NB and predict aggressive tumor behavior and identify a subgroup of high-risk tumors [243,245].

### **Loss of chromosome 1p**

One homozygous deleted region has been identified at 1p36, which is associated with *MYCN*-amplification [246,247]. Loss of heterozygosity (LOH) of this region might harbor tumor suppressor genes like *CHD5* or *KIF1B* and is related to poor prognosis in various kinds of tumors including NB [248-250].

### **Gain of 17q chromosome**

Chromosome 17q gain caused by the recurrent unbalanced translocations has been detected in more than 90% of untreated high-grade NBs [251,252]. This gain has been associated with

deletion of 1p and *MYCN*-amplification which indicates a poor outcome [253-255]. A genomic instability study had identified Protein Tyrosine Phosphatase Receptor Type D (*PTPRD*) as a candidate tumor suppressor gene in NB [256].

### **Loss of chromosome 11q**

Prevalence of 11q LOH in primary NBs is 44% [257]. Loss of chromosome 11q is a marker of high-risk tumors and correlates with features of metastatic relapse and unfavorable outcome [258]. Contrary to 1p LOH, 11q allelic deletion is negatively correlated with *MYCN*-amplification [257]. There has been some evidence that calmodulin 1 (*CAMI*) is a potential tumor suppressor gene at 11q [259].

### **Gain of chromosome 2p**

Javanmardi *et al.* (2019) reported that 30% of 365 NB cases showed gain of chromosome 2p which is located close to *MYCN*, *ALK*, and *ALKAL2* (ALK ligand) [260]. All these three loci are located in the region of 2p-gain, which was reported to be associated with poor event free survival in a study comprising 177 NB patients [261].

## **1.4.5 Treatment of Neuroblastoma**

Treatment options for NB depend on various pretreatment prognostic factors, which include the size and location of the tumor, whether it has spread, the risk group classification of the tumor, along with additional relevant criteria. In the **low and intermediate risk** groups, the treatment strategy includes observation, surgical resection and moderate chemotherapy, and survival rate is larger than 90% with or without chemotherapy [80]. Infants with localized NB may not need treatment, including surgery or chemotherapy, and could spontaneously regress. In a prospective trial conducted by the Children's Oncology Group (COG) to study infants under six months of age with small adrenal masses and no indication of metastasis beyond the primary tumor site, it was found that around 80% of the participants were successfully spared resection through closely monitored expectant observation, and achieve favorable event free survival outcome in a large majority of the patients [262].

**High-risk NB** is defined by patients with *MYCN* amplification, except for those with completely resected L1 *MYCN* amplified disease, and encompasses any patient aged 18 months or older with metastatic disease, regardless of *MYCN* amplification status [202,263]. High-risk NB contributes to about half of all NB patients, remains difficult to treat with poor outcomes. Large cooperative group studies report long-term survival rates of approximately 40-50% [264];

In most North American institutions, the current treatment for this condition is divided into three phases: induction, consolidation, and post-consolidation or maintenance therapy.

**Induction therapy** involves multi-agent chemotherapy, surgical resection of the primary tumor and followed by peripheral blood stem cell (PBSC) therapy. The induction regimens utilized in North America currently consist of vincristine, doxorubicin, cyclophosphamide, cisplatin, and etoposide. The COJEC regimen employed by the Society of Pediatric Oncology Europe Neuroblastoma Group (SIOPEN), incorporates cisplatin [C], vincristine [O], carboplatin [J], etoposide [E], and cyclophosphamide [C] for treatment [265]. The final response after induction therapy shows similarities across different international regimens, with less than 50 percent of patients achieving complete remission (CR) and over ten percent of patients experiencing progressive disease (PD). Furthermore, the end induction response serves as a predictor of the overall treatment response [266]. **Consolidation therapy** includes high-dose chemotherapy (HDC), autologous stem cell transplantation (ASCT), radiation therapy (XRT), and PBSC infusion [267-269]. Busulfan and melphalan have demonstrated improved event-free survival in high-risk NB patients who respond well to initial treatment, with fewer severe adverse events compared to carboplatin, etoposide, and melphalan, making them potential standard high-dose chemotherapy agents [270]. For individuals with high-risk NB, tandem transplant demonstrated a notably superior event-free survival (EFS) outcome compared to single transplant [271]. Finally, the **post-consolidation** therapy consists of the anti-ganglioside 2 (GD2) antibody (and cytokines) and 13-*cis*-retinoic acid (isotretinoin) [272,273].

The utilization of 13-*cis*-retinoic acid following chemotherapy or transplantation showed beneficial effects in patients without progressive disease [267]. Compared to standard therapy, immunotherapy incorporating ch14.18, a monoclonal antibody targeting the tumor-associated disialoganglioside, along with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2, exhibited a better outcome in patients with high-risk NB [274].

High-risk NB standard treatment protocol involves four elements, induction therapy, tumor management, integration, and maintenance therapy. Currently, conventional induction chemotherapy includes platinum, alkylating, and topoisomerase agents. local surgery, radiotherapy, immunotherapy including antibodies against GD2, followed by 13-*cis*-retinoic acid treatment [275-278]. It has been shown that one or two autologous stem cell transplantations using healthy blood stem cells to supplant diseased bone marrow result in better outcome [279]. For children with localized, low-risk disease, surgical removal of the tumor gains beneficial prognosis, however, aggressive surgical resection of the high-risk NB is controversial [280].

#### 1.4.6 Neural differentiation in Neuroblastoma

**Nuclear Hormone Receptors and their role in NB differentiation:** The nuclear hormone receptors (NHRs) are commonly referred to as intracellular receptors. They are a family of transcription factors that bind to thyroid and steroid hormones, retinoids, vitamin D, and other ligands, usually located in the cytoplasm [281,282]. Humans and mice have 48 and 49 NHRs, respectively [283]. Upon activation by their ligands, these receptors recognize and bind specifically to hormone response elements (HRE) to regulate gene transcription. NHRs play a vital role in neural stem cells fate decision, balance of proliferation, differentiation, and are of particular relevance for cancer cells [284,285]. Estrogen receptors (ERs) significantly influence the development and progression of breast cancer. Breast tumors are classified based on ER alpha (ER $\alpha$ ) status as either ER-positive or ER-negative. The ER-positive tumors can be treated with tamoxifen to downregulate the hormone levels and reduce tumor burden [286], while estrogen receptor beta (ER $\beta$ ) has been associated with tumor suppressive effect. However, the splice variant ER $\beta$ 2 has been linked to prostate cancer initiation and progression [287,288]. Contrary to its oncogenic role in breast cancer, ER $\alpha$  expression has been connected to growth arrest and differentiation in NB [289,290].

Our research group has demonstrated that MYCN promotes upregulation of the *miR-17~92* cluster, which in turn represses the expression of several NHRs including ER $\alpha$  and the glucocorticoid receptor (GR) inhibiting NB cell differentiation. In addition, with ER $\alpha$  overexpression, an increase of neuronal features and inhibition of mouse tumor progression were observed [291,292]. Additionally, our lab showed that MYCN inhibition or GR overexpression could induce neural differentiation and reduce tumor burden [293].

Retinoic acid (RA) exists in three isomers: all-*trans*-RA (ATRA), 9-*cis* RA, and 13-*cis* RA. Among the three RA isoforms, 9-*cis* RA appears to be the most potent *in vitro* inducer of NB cell differentiation [294]. Furthermore, 13-*cis* RA is used for residual minimal disease differentiation therapy in high-risk NB [295]. Retinoic acid receptors (RARs) are crucial for early embryonic development, and the ligand retinoic acid (RA) can activate it to trigger differentiation and stop NB cell proliferation [296-299]. Incubation of certain NB cell lines with retinoic acid causes visible morphological changes that resemble differentiated cells. However, clinical treatment has shown ineffective for many high-risk patients due to the resistance caused by MYCN [300]. Our recent data has shown that combined expression and activation of GR, ER $\alpha$ , and RAR $\alpha$  promote neuronal differentiation, and high expression of these receptors was associated with a favorable outcome in several NB cohorts (**Paper I**). Understanding the

interactions between retinoic acid isomers, nuclear receptors, and MYCN could guide more effective therapeutic strategies for high-risk NB patients.

#### **1.4.7 Metabolism in Neuroblastoma**

Cellular metabolism provides energy and carries out biosynthetic process that are necessary for cell function. Certain metabolites in cancer cells not only create energy and mass, but also control genes and impact nearby healthy cells [301].

Cells use glycolysis to convert glucose into pyruvate. Then, under normal oxygen conditions, pyruvate enters the mitochondria to the tricarboxylic acid cycle for further energy production. However, in the absence of oxygen, pyruvate can stay in the cytoplasm and turn into lactate. Notably, cancer cells exhibit a unique metabolic behavior, they can carry out aerobic glycolysis for rapid energy production and formation of building blocks, even in the presence of oxygen [302].

*MYCN* amplification induces metabolic reprogramming in NB cells, leading to increased fatty acid uptake and accumulation of neutral lipids [303]. Lipid accumulation in NB tumors is caused mainly by disruptions in  $\beta$ -oxidation, and we have previously described that *MYCN*-amplified NB cells prefer fatty acids as an energy source [304,305]. Inhibition of fatty acid synthesis promotes differentiation and lower tumor burden in NB [306].

Reactive Oxygen Species (ROS) are chemical molecules containing oxygen atom and unpaired electrons [307]. They are created naturally as a consequence of oxygen metabolism and are crucial for maintaining homeostasis and cell signaling. ROS includes free radicals such as superoxide and hydroxyl radical, and non-radical species like hydrogen peroxide that can be converted to radicals [308]. External factors like tobacco, pollution, smoke, drugs, xenobiotics, or ionizing radiation can trigger the production of ROS [309]. They can be generated in various cellular locations including the mitochondria, peroxisomes, and endoplasmic reticulum [310]. Cancer cells adjust their metabolism to balance internal ROS levels, promoting their growth and survival while reducing their likelihood of undergoing programmed cell death, or apoptosis [311,312]. Moderate levels of ROS play important roles in physiological processes. For instance, they are involved in macrophages, influencing both innate and adaptive immunity, as well as modulating the immune response [313]. However, if ROS levels increase dramatically, it can be harmful to cells and result in a process of oxidative stress, causing damage to DNA, RNA, proteins, and lipids [314].

Our bodies use antioxidants to guard against the harmful effects of ROS. These antioxidants neutralize ROS, preventing them from causing cell damage and maintaining cell homeostasis [315].

#### **1.4.8 Hypoxia in the Initiation and Progression of Neuroblastoma**

Hypoxia, an oxygen deficient microenvironment, is a major feature of solid tumors and is commonly used as a prognostic hallmark because of its association with vasculogenesis, angiogenesis, invasiveness, metastasis, and metabolic reprogramming [316,317]. To adapt to hypoxia environments and sustain continuous growth and proliferation, cancer cells altered their metabolism often increasing glycolytic capacity and antioxidant capacity [318,319].

The hypoxia inducible factor 1 alpha (HIF-1 $\alpha$ ) is the most important transcription factor typically activated under hypoxic conditions. It functions by dimerizing with HIF-1 $\beta$  [encoded by *Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)*], and binding to the hypoxia response elements of the promoter of target genes [320,321]. HIF-1 $\alpha$  has been proposed to be a fundamental requirement for normal embryonic development and *HIF-1 $\alpha$*  knock down mice present severe neural tube defects [322]. Correlating with *MYCN*-amplification, HIF1- $\alpha$  expression shows an antiapoptotic effect via Survivin upregulation [323]. Hypoxia mediates every step of the metastatic progression, from initial epithelial-mesenchymal transition to migration and colonization, which occurs in majority of newly diagnosed patients [324,325]. Low oxygen condition also affects neural crest development, including deficiency of neural crest cells migration and differentiation which could result in initiation of NB [317,322,326].

#### **1.4.9 Cytoskeletal Modulation in Neuroblastoma**

Studies have shown that the state of the cytoskeletal network has a direct impact on the severity and capacity for tumor formation of SK-N-BE(2) cells, by using a sensitive single-cell force spectroscopy technique to assessing this status [327]. According to another study, disruption of the cytoskeleton leads to mitochondrial membrane collapse and induces apoptosis in NB cells treated with okadaic acid [328]. In a NB cell line, it was found that cell attachment to matrix components is facilitated by integrins. When these integrins bind to their ligands, they promote the reorganization of cytoskeletal proteins, enabling the cell to spread out on the surface and encouraging neurite outgrowth and differentiation [329]. The actin cytoskeleton and intermediated filaments showed significantly changes during 1-Methyl cyclohexane carboxylic acid (CCA) triggering morphological differentiation of NB, which is observed by neurite extension and synthesis of tubulin subunits [330]. Moreover, unique changes in the synthesis of

cytoskeletal associated proteins were observed after RA induced NB differentiation [331], and it has been reported that high expression of the *Transient Receptor Potential Cation Channel Subfamily M Member 7 (TRPM7)*, involved in cytoskeletal reorganization and adhesion remodeling, contributes to NB progression [332].

### 1.5 Methods for statistical analysis

Regression models such as the Cox proportional hazards model are often used in healthcare studies to examine and determine prognostic variables. The model simultaneously assesses the impact of several parameters on survival. Proportional hazards assumptions state that the risk of an event in any group present the same proportion over time. According to this assumption, the groups' hazard curves require to be proportional and unable to intersect. A number of techniques can be employed to evaluate the viability of the Cox model assumptions, such as evaluating the proportional hazards assumption by Schoenfeld residuals, estimating for nonlinearity using Martingale residuals, and examining significant observations with a symmetrical transformation of these residuals (i.e. Deviance residuals) [333,334].

Least Absolute Shrinkage and Selection Operator (LASSO)-Cox regression is a statistical approach that merges the Cox proportional hazards model with LASSO regularization. This method is useful in survival analysis, especially when study multiple factors relate to the timing of an event, such as failure or death [335]. LASSO aims at finding the best fitting model measured by “log partial likelihood” under a specific constraint. Under this constraint, the sum of the absolute values of the coefficient score must be less or equal to a certain constant which is the tuning parameter log Lambda.

For high-dimensional datasets in which the number of variables is significantly more than the number of observations, LASSO-Cox regression works well. It can effectively manage such data while avoiding overfitting [336].

The LASSO regression is called L1 normalization while Ridge regression is called L2 normalization. Ridge Regression is a technique that modifies the ordinary least squares (OLS) method by adding a penalty to the sum of the squared coefficients. Ridge regression lessens the influence of less important predictors rather than removing any predictors from the model, which is different from LASSO regression, so it does not do feature selection [337].

Elastic Net Regression is a computation method combined with both LASSO regression and Ridge regression; thus, it balances the power of feature extraction and feature preservation. When working with strongly associated predictor variables, Elastic Net regularization is useful [338].

# 1 Research aims

## **Study I:**

To examine differentiation induction as a therapeutic approach for patients with neuroblastoma (NB), focusing on simultaneously activating the glucocorticoid receptor (GR), the estrogen receptor (ER $\alpha$ ), and retinoic acid receptor alpha (RAR $\alpha$ ). We aimed to determine the effects of combining their expression and activation on neuronal differentiation, cell viability, metabolic changes, lipid droplet accumulation, and tumor burden.

## **Study II:**

To develop a multigene risk score model for NB patients by analyzing genetic and clinical data from 498 individuals, particularly examining MYCN and c-MYC target genes. We aimed to understand how these genes correlate with clinical outcome and overall survival, thereby enhancing prognostic predictions for high-risk NB cases to shed light on their roles in tumorigenesis. In addition, we examined their expression during development of the peripheral nervous system.

## **Study III:**

To investigate *DIAPH3* expression levels in NB patient cohorts, assessing its impact on survival and contribution to poor clinical outcome. We also explored effects of *DIAPH3* inhibition on cell proliferation, cell cycle regulation, and lipid metabolism *in vitro*.



## 2 Materials and methods

### **Paper I: Expression and activation of nuclear hormone receptors result in neuronal differentiation and favorable prognosis in neuroblastoma.**

We used previously generated GR overexpressing SK-N-BE(2) cells [293], hereafter named “BE(2)-GR”. To generate cells co-expressing GR [encoded by *Nuclear Receptor subfamily 3 group C member 1, (NR3C1)*] and ER $\alpha$  [encoded by *Estrogen Receptor 1, (ESR1)*], BE(2)-GR cells were transduced with a lentivirus carrying the *ER $\alpha$*  cDNA or a control vector, resulting in BE(2)-GR + ER $\alpha$  and BE(2)-GR + EV cell lines, respectively. The cell lines with stable expression were selected using puromycin or hygromycin. Similarly, KCN69n and IMR32 cells with overexpression of GR and ER $\alpha$  were generated were produced. Cells were treated with the corresponding ligands for these NHRs, 17- $\beta$ -estradiol (E2), DEX, and/or ATRA, with optimized concentrations. WST1 was used to assess cell viability. GR, ER $\alpha$ , RAR $\alpha$ , and MYCN protein expression levels were evaluated by Western blot, together with the differentiation markers, p75<sup>NTR</sup>, secretogranin 2 (SCG2),  $\beta$ III-tubulin, tyrosine hydroxylase (TH), and Glial Fibrillary Acidic Protein (GFAP).

The development of neurites was observed using bright field microscopy. In addition, differentiation markers such as  $\beta$ III-tubulin, vimentin, and SCG2 were visualized by immunofluorescence to further study this process. Other markers *TRKA*, *Neurofilament Light Chain (NEFL)*, *Neuroepithelial Stem Cell Protein*, *Nestin (NES)*, *MYCN*, and *Discs Large MAGUK Scaffold Protein 2 (DLG2)* mRNA were analyzed by RT-qPCR. For the detection of lipid droplets within cells, Nile red staining was applied.

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were analyzed using the Seahorse XF Analyzer as estimation of glycolytic capacity and mitochondrial respiration.

For xenograft studies, we employed BE(2)-EV or BE(2)-GR, as well as BE(2)-GR + EV or BE(2)-GR + ER $\alpha$  cells which were injected subcutaneously into one flank region of the mice. Tumor growth was followed and at endpoint when tumors in the control group reached the maximum allowable volume of 1 cm<sup>3</sup>, the animals were sacrificed. The tumors collected and fixed with formaldehyde for Immunohistochemistry staining. Expression of Ki67, GR, ER $\alpha$ , SCG2,  $\beta$ III tubulin, p75<sup>NTR</sup>, and endomucin were analyzed.

We then explored the mRNA expression level of the three NHRs genes on publicly available NB cell line RNA sequencing data. Furthermore, we examined the expression profiles of these genes in two patient cohorts, SEQC (498 patients) and Kocak (649 patients). Patients were divided in low and high expression groups based on the expression score of the first and last quartile of GR, ER $\alpha$ , and RAR $\alpha$  expression levels. We named the intersection of low GR and low ER $\alpha$  group as Low <sup>GR+ER $\alpha$</sup> , and similarly, High <sup>GR+ER $\alpha$</sup> . We examined the expression level of the genes encoding TH, SCG2, p75<sup>NTR</sup>. By using gene set enrichment analysis performed on GSEA software, pathways or processes enriched differently between High<sup>GR+ER $\alpha$</sup>  and Low<sup>GR+ER $\alpha$</sup>  were identified. The Suntsova dataset was analyzed to examine the expression levels of available genes of interest, *NR3C1*, *NGFR*, *Tubulin Beta 3 class III (TUBB3)*, and Receptor Tyrosine-Protein Kinase erbB-3 (*ERBB3*) in normal and embryonic adrenal tissue.

By examining the single nuclei data from Bedoya-Reina *et al.* (2021), we explored the expression pattern of these three NHRs in the clusters of post-natal adrenal glands. Additionally, we analyzed gene expression statuses using single cell sequencing data from Dong *et al.* (2020), which includes data on human fetal adrenal gland. In this study, we also applied pseudo-time progression analysis to investigate the alterations in gene expression levels throughout the early and late phases of development.

#### Ethical Considerations:

All procedures involving animal experiments were conducted in compliance with the ethical guidelines and regulations of Karolinska Institutet and the Swedish law. The Ethical Committee at the Northern Court of Stockholm granted approval for these procedures, under the ethical permit numbers N71/15 and 10579-2020.

#### **Paper II: Target Genes of c-MYC and MYCN with Prognostic Power in Neuroblastoma Exhibit Different Expressions during Sympathoadrenal Development.**

This project aimed to build a prognosis model based on the gene expression on c-MYC and MYCN targets.

For our study, we selected three well defined c-MYC/MYCN target gene sets. First, we took from the GSEA molecular signature database two specific gene sets: “HALLMARK\_MYC\_TARGETS\_V1” and “HALLMARK\_MYC\_TARGETS\_V2”. These sets were originally created through computational methods and refined with manual expert curation. Following the removal of duplicate entries, the total of unique genes in these c-MYC target sets were 240. The second gene set was derived from a ChIP-Seq and promoter

microarray results conducted on Hela cells and human fibroblasts. After removing duplicates, 1459 c-MYC target genes were identified [151]. The third gene contained 157 genes affected after MYCN knockdown mediated by shRNA silencing, with validation obtained through chip-on-chip analysis [339]. These gene sets were labeled as “c-MYC”, “c-MYC ChIP”, and “MYCN”, respectively.

Expression data and patient information including clinical parameters and survival of patients were obtained for the different neuroblastoma cohorts from the R2 database. The SEQC dataset which contains 498 samples was used as a training cohort and the Kocak and Versteeg datasets including 649 and 88 samples, respectively, were used as validation cohorts. We evaluated different approaches to filter target gene set and select those with biological and clinical relevance. In detail, we examined the normalized gene expression and determined significant differences between patients with contrasting clinical features by using Wilcoxon rank-sum tests, corrected with the Benjamini-Hochberg approach. These clinical features were provided for each patient in the SEQC cohort, and include high risk, INSS stage, *MYCN*-amplification, and disease progression status. Genes were further filtered by selecting those with prognostic power using Univariate Cox regression. Additional filtering was conducted by selecting genes encoding proteins that have similar biological functions by using protein-protein interaction (*i.e.* PPI) network analysis. For this, we used data extracted from the STRING database which is a public database including protein interaction data computed by co-expression data and supported by text mining or experiments. With these genes, we used a least absolute shrinkage and selection operator (LASSO) method with Cox regression to build models. Each patient got a risk score as a bi-product of these models, and was grouped into high or low scoring.

We used different methods to evaluate the efficiency of this new risk score. First, the Log-rank survival analysis was used to compare the difference between the high and low risk-score groups. Second, the area under curve (AUC) values by the receiver operating characteristic (ROC) to evaluate the accuracy of the risk score. Third, *t*-distributed stochastic neighbor embedding (*t*-SNE) was employed to examine whether risk scores could classify patients into distinct groups. Fourth, we investigated the risk score distributions across various clinical category groups, to analyze if the risk score was high or low in patients with different clinical features including INSS stages, *MYCN*-amplification, progression, favorable outcome, age, and gender. We also investigated the molecular function of c-MYC and MYCN target genes by using gene ontology (GO) analysis.

We then investigate the expression of the target genes with prognostic power in different cell populations during sympathoblast development in human and mice, as well as in human neuroblastoma. The data utilized for the developing sympathoadrenal system was obtained from Furlan *et al.* (2017) and Jansky *et al.* (2021) and the tumor data was obtained from van Groningen *et al.* (2017), Kildisiute *et al.* (2021), and Bedoya-Reina *et al.* (2021). Note that the genes included in these steps are “Full gene set” which consist of target genes without any filtering such as differential expression analysis/Univariate Cox regression and protein-protein network analysis and were instead generated directly by the LASSO-Cox model.

Using the gene sets identified in each cluster from the single-cell data, we applied the same method as used for c-MYC/MYCN target genes to derive a risk score for each patient. Subsequently, we computed the overlap of patients classified as high-risk based on both the gene sets from the single-cell data and those identified by c-MYC/MYCN targets. We also used orthologue c-MYC/MYCN target genes from mouse adrenal anlagen at E12.5 and E13.5 to generate a signature score. This signature score computed as the mean expression of orthologous c-MYC/MYCN target genes per 10,000 cells, incremented by 1, and then by subtracting the mean expression of a set of randomly selected genes (per 10,000 cells, incremented by 1).

#### Ethical Considerations:

This study does not need any ethical permits since it relies solely on the analysis of existing, publicly available datasets, which were collected and shared in accordance with the ethical standards and permissions stipulated by the original researchers.

### **Paper III: DIAPH3 is expressed during sympathoadrenal development and correlated with poor prognosis in neuroblastoma.**

We generated SK-N-BE(2) NB cells with stable *DIAPH3* downregulation by shRNA lentiviral transduction. Real-time monitoring of cell behavior, including proliferation, was evaluated using the IncuCyte live cell imaging system. Cells were seeded at 96 well plates and images were taken every 4 hours for a period of 7 days. Cells were grown on coverslips fixed and then stained with a 0.3% Oil Red O solution diluted in 60% isopropanol, to examine the lipid droplet accumulation. Knockdown efficiency of *DIAPH3* and expression levels of p21 were evaluated by Western blot.

Expression and clinical data were extracted from the SEQC, Kocak, Versteeg, and NRC cohorts using the R2 database. The expression scores were normalized, and differential

expression analysis between high and low expression of *DIAPH3* was conducted utilizing the "limma" package. The expression levels of *DIAPH3* across different clinical groups were analyzed using non-parametric estimation, and Kaplan-Meier curves were utilized to demonstrate the prognostic value of *DIAPH3* in determining survival outcomes. To execute the gene set enrichment analysis, the hallmark gene set, collection 2 which contains curated online pathways and literature, and collection 5 contains gene ontology resources, and human phenotype ontology were incorporated in the analysis. After conducting 1000 permutations, a significant gene set between comparison of high and low *DIAPH3* was considered as  $FDR < 0.25$  and normalized enrichment score  $> 1.25$ , which is recommended by GSEA group.

Single cell sequencing was utilized to investigate the differential expression of *DIAPH3* across various cell clusters. The study incorporated multiple datasets: mouse adrenal anlagen E12.5 and E13.5 from Furlan *et al.* (2017), human adrenal gland from Jansky *et al.* (2021), another postnatal dataset from Bedoya-Reina *et al.* (2021), NB tumors were derived from Kildisiute *et al.* (2021) and Bedoya-Reina *et al.* (2021).

#### Ethical Considerations:

The Bulk-Seq data were sourced from the R2 database, and the single-cell/single-nuclei data were acquired as detailed in the original publication where the data were first presented. Wet lab experiments were carried out on established commonly used human cell lines. Ethical permit is not needed since our study did not involve any clinical data or animal work.



### 3 Results

#### **Paper I: Expression and activation of nuclear hormone receptors results in neuronal differentiation and favorable prognosis in neuroblastoma.**

Summary of results:

We employed the BE(2)-GR and BE(2)-EV cell lines previously established by transfection containing *NR3C1* vector or an empty vector, respectively [293]. These cells were then treated with ATRA, DEX, a synthetic ligand for GR, or the combination (DEX+ATRA). We found that DEX+ATRA treatment induced significant neurite outgrowth compared with DEX alone. Additionally, with DEX treatment, the BE(2)-GR cells showed a higher expression of the neuronal differentiation marker TH, and a minor increase in p75<sup>NTR</sup> levels. However no noticeable alterations were observed in the levels of secretogranin 2 (SCG2) or  $\beta$ III-tubulin by Western blot. The expression of SCG2 was found to be upregulated in both cell lines following treatment with ATRA, whether applied independently or in combination with DEX. Additionally, we conducted a xenograft experiment using both BE(2)-EV or BE(2)-GR cells to investigate potential differences in tumor growth. We found that tumor size was decreased in mice which injected with cells that overexpressing GR compared to control. Analysis of tumor sections confirmed the higher expression of GR protein and low Ki67 expression in BE(2)-GR-derived tumors.

We next generated the BE(2)-GR + EV and BE(2)-GR + ER $\alpha$  cell lines, by introducing lentiviral vectors carrying the ER $\alpha$  gene or an empty vector into the previously established BE(2)-GR cells. Our aim was to investigate the potential of simultaneous activation of ER $\alpha$ , GR, and RAR $\alpha$  in promoting cell differentiation and reducing tumor proliferation. Initially, we observed a decrease in the number of colonies when comparing BE(2)-GR + ER $\alpha$  cells to control cells. We also found that neurite outgrowth was induced by the combination E2 + DEX, and that neuronal differentiation was further promoted by the triple combination of E2 + DEX + ATRA. In control cells, E2 + DEX treatment led to a modest rise in TH levels. Furthermore, ATRA by itself or in conjunction with E2 + DEX increased the levels of TH, SCG2, p75<sup>NTR</sup>, and  $\beta$ III-tubulin. In BE(2)-GR + EV cells, DEX or ATRA were associated with increased *NEFL* and *TRKA*, whereas ATRA, the double (E2 + DEX), and triple combination (E2 + DEX + ATRA) resulted in a downregulation of *SOX2* and *NES*. Similarly, in KCN69n and IMR32 cells, triple activation of GR, ER $\alpha$ , and RAR $\alpha$  enhanced neuronal differentiation. These

findings indicate that the combined activation of ER $\alpha$ , GR, and RAR $\alpha$ , effectively promote neuronal differentiation and inhibit proliferation.

We also investigated the impact of combination treatments on metabolism, including glycolysis, oxidative phosphorylation, and lipid accumulation. Our results showed that the GR and ER $\alpha$  co-expressing cells were less glycolytic and had a lower oxidative phosphorylation capacity than GR-expressing cells. In the SK-N-BE(2)-GR+ER $\alpha$  cells, glycolysis was increased by independent E2 and DEX treatments, and this effect was further amplified when E2 and DEX were combined. Moreover, a more significant increase in glycolysis was observed with ATRA alone or in the triple combination treatment. Furthermore, these combination treatments also led to an accumulation of lipid droplets in the cells. Our findings showed that co-activation of GR and ER $\alpha$  in BE(2)-GR+ER $\alpha$  caused strong differentiation along with reduced tumor burden and angiogenesis in mice xenograft model.

By examining three public NB patient cohorts, SEQC, Kocak, and Oberthuer, we studied the role of *GR*, *ER $\alpha$* , and *RAR $\alpha$*  in the survival and prognosis. Patients were divided into high and low *GR*, *ER $\alpha$* , and/or *RAR $\alpha$*  based on the quartile score of RNA expression level. Our analysis revealed that high expression level of these genes is associated with favorable survival in the SEQC, Kocak, and Oberthuer cohort dataset, with the exception of *GR* in the Kocak cohort.

Furthermore, individuals with *MYCN*-amplification exhibited reduced expression level of these genes. We then considered the combination score of these patients and defined the High<sup>GR+ER $\alpha$</sup>  versus Low<sup>GR+ER $\alpha$</sup>  patient groups based on the intersection of the GR and ER $\alpha$  groups. We found that the High<sup>GR+ER $\alpha$</sup>  patients with a non-*MYCN* amplification status showed a better survival than the patients with Low<sup>GR+ER $\alpha$</sup>  carrying *MYCN*-amplification. Interestingly, there was no patient with *MYCN*-amplification with High<sup>GR+ER $\alpha$</sup> . By using GSEA, we investigated the gene sets or pathways enriched differently in High<sup>GR+ER $\alpha$</sup>  versus Low<sup>GR+ER $\alpha$</sup> , and we identified pathways related with differentiation and metabolism.

Notably, through single-nuclei analysis we identified genes expressed in the process of sympathoadrenal differentiation. Our findings revealed that GR (*NR3C1*) was significantly highly expressed in chromaffin cell clusters and *RARA* in non-cycling chromaffin cells. Using pseudotime reconstruction on human adrenal glands, our results showed that *ESR1*, *NR3C1*, and *RARA* are expressed sequentially during the stage from early progenitor and late chromaffin cells.

## **Paper II: Target Genes of c-MYC and MYCN with Prognostic Power in Neuroblastoma Exhibit Different Expressions during Sympathoadrenal Development.**

We first performed differential expression analysis of c-MYC and MYCN targets across various risk groups, INSS stages, *MYCN* amplification and progression status, as well as utilized Univariate Cox regression analysis and protein-protein interaction network analysis to select the genes with prognosis value and biological relevance. Following this, a LASSO-Cox regression analysis was applied to derive a beta coefficient score. This score was then used to develop a signature risk score model, which also incorporated gene expression levels. To test the accuracy of this model, we performed survival analysis comparing the patients with high and low risk scores and found that patients with a high c-MYC/MYCN signature have poor survival when compared to the low signature group. This was also confirmed by ROC curves, where the AUC values were all significantly high indicating that this model has a good performance. The *t*-SNE plots showed NB patients from the SEQC cohort could be distinctively separated based on clinical risk classification and on the risk score produced by the model.

We subsequently examined the functions of the gene lists generated from the original c-MYC, c-MYC ChIP, and MYCN target sets. The gene ontology analysis revealed that methods incorporating differential expression analysis and Univariate Cox regression analysis provided a greater number of ontology items (“PPI” and “Without PPI”) compared to methods without any screening (“Full gene set”). We found that c-MYC targets are mostly enriched in protein and RNA binding molecular functions, while MYCN targets were enriched in small nucleolar RNAs (snoRNA) and telomerase RNA ontologies.

To explore the binding position at the transcription, start sites on the identified c-MYC/MYCN target genes, we investigated the public ChIP-seq data on NB cells from R2 database. We found a significant enrichment of c-MYC ChIP-seq peaks at the TSS of c-MYC targets genes, and evident enrichment of MYCN ChIP-seq peaks at the TSS of MYCN targets genes.

We then studied the expression patterns of the identified genes (the “Full gene set” which was only screened with LASSO-Cox) during the sympathoadrenal development and in neuroblastoma samples. c-MYC targets genes were found to be highly expressed in mouse developing sympathoblasts at both E12.5 and E13.5, as well as in Schwann cell precursors (SCPs) at E12.5. MYCN targets genes were upregulated in SCPs at E12.5 and in developing sympathoblasts at E13.5. Both c-MYC targets and MYCN targets were expressed in the cluster of malignant tumors.

We next explored if the LASSO-Cox risk stratification strategy could be applied on the marker genes from each cluster in sympathoadrenal development and neuroblastoma. We found that the high-risk scoring patients identified from each of these cell clusters, strongly overlapped with high-risk scoring patients computed by the c-MYC, c-MYC ChIP, and MYCN target genes, with an overlap over 80%. Note that the c-MYC ChIP targets model has a stronger overlap with murine adrenal anlagen. Further, we noticed that the overlap of c-MYC and MYCN targets genes was higher for marker genes in mouse adrenal anlagen and human tumor tissue.

We then explored in depth the expression levels of the c-MYC and MYCN genes, as well as the target genes regulated by c-MYC/MYCN, during stage E12.5 and E13.5 of the mouse adrenal anlagen. The *MYC* gene was highly expressed in chromaffin clusters at E12.5 and in chromaffin and bridging cells at E13.5. The *MYCN* gene was highly expressed in sympathoblast during E12.5 and E 13.5 as well as in SCPs at E12.5. Furthermore, we found that c-MYC and MYCN targets with poor prognosis were significantly expressed in the murine developing sympathoblast cluster at E12.5 and E13.5, except for the c-MYC ChIP targets which did not show any significance. In addition, MYCN target genes with good prognosis had lower expression in SCPs.

We then examined the expression of the identified genes in mouse sympathoadrenal development. Several of these genes have already been reported in existing literature. Specifically, genes including *DKC1*, *PLK1*, *FBXO8*, *ODC1*, and *CDK4* were similarly noted for their roles in poor prognosis in various studies.

### **Paper III: DIAPH3 is expressed during sympathoadrenal development and correlated with poor survival in neuroblastoma.**

By analyzing the expression on bulk-seq data matrix with clinical variables from four different NB cohorts, SEQC, Kocak, NRC, and Versteeg, we found that the high expression level of *DIAPH3* is associated with reduced overall survival rate and/or event free survival. High expression was also related to a high-risk status, higher INSS stage (stage 3 and 4), *MYCN*-amplification, disease progression, and age at diagnosis.

We further investigated the single cell/single nuclei data, to explore the expression pattern of *DIAPH3* during sympathoadrenal development in mice and human, as well as within post-natal adrenal gland and tumor tissue in human. The results showed that *Diaph3* is highly expressed in sympathoblast clusters in mouse at E12.5 and E13.5. In the human embryo, *DIAPH3* was

elevated in cycling SCPs and cycling neuroblasts. In human post-natal tissue, *DIAPH3* was associated with progenitor clusters while in NB tumor tissue, expression was abundant in the undifferentiated cluster 3 which is associated with high risk. Furthermore, *DIAPH3* expression was shown to be upregulated in tumor clusters 2 and 3 in the biopsy or tumor samples from the Great Ormond Street Hospital (GOSH) cohort.

Next, we performed differential expression analysis based on the median expression score of *DIAPH3* in SEQC cohort. This allowed us to identify elevated genes such as *TOP2A*, *MKI67*, and *CENPF*, which have previously been reported to be linked to poor prognosis. Moreover, the downregulated gene *NTRK1* is associated with a good prognosis in NB. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that enriched pathways are involved in cell cycle, senescence, and mitosis. By using GSEA, we discovered key pathways enriched in terms of "Mitotic," "DNA repair," "G2/M checkpoint," "Cytoskeleton," and others.

To validate those bioinformatics results, we established *DIAPH3* stable knockdown SK-N-BE(2) cells. We detected a slight upregulation of the cell cycle inhibitor p21 and a slower, although not significant, growth rate in the knockdown cell lines. Accumulation of lipid droplets was also observed upon *DIAPH3* silencing.



## 4 Discussion

### **Paper I: Expression and activation of nuclear hormone receptors result in neuronal differentiation and favorable prognosis in neuroblastoma.**

The therapeutic application of hormone modulation has shown considerable promise in the treatment of various cancers, as evidenced by several strategies targeting hormonal pathways. For example, DEX is a synthetic glucocorticoid that has potent anti-inflammatory and immunosuppressive properties, which can be a benefit when treating cancers such as lymphoma and leukemia [340]. Additionally, androgen suppression therapy is utilized to inhibit the progression of prostate cancer [341] and blocking estrogens is common treatment in ER expressing breast cancer. In addition, high ER $\alpha$  expression is thought to be beneficial for the management of endometrial and ovarian malignancies [342,343].

Compared to a single drug treatment, combinatory treatments are more effective as they target important pathways in a way that is additive or synergistic. Retinoic acid and histone deacetylase (HDAC) inhibitors alone cannot produce effective results, however, combination of HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA) and retinoids, or SAHA with N-4-hydroxyphenyl retinamide (4-HPR), which is a semisynthetic retinoid, has a synergistic anti-tumor effect, and reduced toxicity [344,345].

Earlier research in our lab showed that MYCN maintains an undifferentiated state in NB cells by suppressing nuclear hormone receptors, including ER $\alpha$  and GR. However, this approach did not lead to full differentiation. Therefore, we studied the co-expression and co-activation of these nuclear hormone receptors, potentially leading to more effective differentiation.

In our study, we used previously generated GR-overexpressing SK-N-BE(2) cells, creating SK-N-BE (2)-GR, which we then activated using DEX [293]. This led to visible differentiation in the cells and reduced tumor volume in the xenograft model. To explore the effects of combined treatment with ER $\alpha$ , we established ER $\alpha$ -overexpressing cell lines based on the SK-N-BE (2)-GR model. When ER $\alpha$  and GR were activated simultaneously, these cells exhibited more pronounced differentiation, as evidenced by morphological changes and the expression of neural differentiation markers, compared to cells treated with a single agent. Furthermore, the addition of the ligand of RAR $\alpha$ , ATRA, in a triple treatment regimen resulted in a further enhanced differentiation phenotype. In our mouse model with overexpression of combined GR

and ER $\alpha$ , we observed a significant increase in markers indicative of neural differentiation, also obtained a decrease in tumor volume and weight, as well as reduced vasculature.

In endometrial cancer, elevated levels of ER $\alpha$  may limit the progression of the malignancy via regulating angiogenic factors [346,347]. In our mouse model, the activation of ER $\alpha$  led to a diminished angiogenic appearance in tumors. However, additional research is required, for instance, examining the angiogenesis factors to understand their interaction with ER $\alpha$ .

Upon evaluating patients with High<sup>GR + ER $\alpha$</sup>  versus Low<sup>GR + ER $\alpha$</sup> , and High<sup>GR + ER $\alpha$  + RAR $\alpha$</sup>  versus those with Low<sup>GR + ER $\alpha$  + RAR $\alpha$</sup>  mRNA expression group, we found that patients with high expression levels of *NHRs* showed better prognosis. This aligned with our Lab's prior research, which demonstrated that activating GR signaling promote neural differentiation and decreases tumor burden [293]. Overexpressing ER $\alpha$  effectively triggers NGF-stimulated neuronal differentiation in *MYCN*-amplified NB cells [292]. ATRA was identified as one of the strongest agents for inducing differentiation in human neuroblastoma cells [297]. In addition, our findings indicates that a higher expression level of neuronal differentiation markers in patients are associated with high expression of these receptors as mentioned above. It hints a potential differentiation-inducing role of GR, ER $\alpha$ , and RAR $\alpha$  in tumor cells. The negative correlation between *NHR* gene expression and *MYCN* amplification is a critical observation in our results. Since *MYCN* amplification is typically associated with aggressive and undifferentiated cancer phenotypes, the inverse relationship suggests that high GR, ER $\alpha$ , and RAR $\alpha$  expression might be protective or indicative of less aggressive tumor behavior.

These findings propose a possible therapeutic approach for NB directed to the GR, ER $\alpha$ , and RAR $\alpha$  receptors. The synergistic effect observed with combined receptors activation opens avenues for developing multi-targeted therapies. Further research is needed to understand the underlying molecular mechanisms and to evaluate the long-term efficacy and safety of such treatments.

## **Paper II: Target Genes of c-MYC and MYCN with Prognostic Power in Neuroblastoma Exhibit Different Expressions during Sympathoadrenal Development.**

Neuroblastoma requires a multi-modal treatment approach, particularly in the high-risk group, where, despite the aggressive interventions, the survival rate remains disappointingly low, less than 50% [263]. Typically, side effects come after the multiple aggressive treatments.

Accurately identifying solid target genes and biomarkers is crucial for efficient risk stratification in NB. This process can significantly impact treatment plans and outcomes,

tailoring therapies more precisely to individual patient needs. Emerging studies utilize computational methods that incorporate gene expression scores into prediction models, yielding better performance than traditional classifications. Considering the significant roles that c-MYC and MYCN play in NB, we aimed to explore the targets of c-MYC and MYCN to investigate whether utilizing these targets could help in building a more efficient model. By doing so, we used the LASSO-Cox model which allows an accessible result interpretation, selecting relevant variables (genes, in this scenario) that have the survival prediction power.

We choose targets genes of biological and clinical relevance by three distinct approaches, Differential expression analysis determined genes that have significant differential expression between clinically high risk and non-high-risk groups, across higher (stage 3 and 4) and lower (stage 1, 2, and 4S) INSS stages, and between cases with progression or without, as well as with different *MYCN* amplification status. Therefore, these clinical parameters enabled us to select the genes that have clinical relevance. Further, we used the PPI network analysis by utilizing data from the STRING database, to select the genes with a potential functional interaction within each other. We observed no significant distinction between employing or not employing PPI screening, which might be attributed to the LASSO-Cox model selection power to identify genes and formulate a robust predictive model through its regularization. However, choosing genes based on PPI yield a set of genes with greater biological relevance as evidenced by gene ontology enrichment.

After the different selection steps tested, we found that there was minimal overlap between the final generated c-MYC and MYCN targets. A study on spontaneous regression and aggressive cases in NB showed different patterns of gene activity regulated by MYCN and c-MYC [348]. Specifically, c-MYC is responsible for the aggressive behavior of stage 4-non-*MYCN* amplified tumors. Conversely, there is only a moderate increase in MYCN activity in stage 4S NB without *MYCN* amplification. This results in the activation of few genes, a pattern that aligns with the potential for the tumors to undergo spontaneous regression [348].

Our findings identified multiple genes previously researched in NB and other types of cancer. For instance, inhibiting *Polo-like kinase 1 (PLK1)* has been observed to decrease the growth of tumor xenografts, including those from NB [349]. Additionally, inhibiting *ODC1* and *CDK4* has demonstrated advantages for NB patients and in cell culture models [350,351].

Of relevance, genes targeted by c-MYC and MYCN exhibit varied expression patterns in the development sympathoadrenal system. Targets of c-MYC and MYCN associated with poorer prognoses tended to be more frequently expressed in sympathoblasts, which are precursor cells

in the sympathetic nervous system. This could suggest that sympathoblasts pathologies might be potentially driven by aberrant expression of c-MYC and MYCN targets. This could offer a new perspective on targeting sympathoblasts for therapeutic interventions. Conversely, the less frequent expression of MYCN targets associated with favorable outcomes in SCPs than in other cell clusters hints at a potentially aggressive role of these cells in disease progression. The oncogenic targets activated by c-MYC and MYCN in sympathoblasts might involve complex regulatory networks that promote cell differentiation. Understanding these targets could provide valuable insights into how these oncogenes contribute to disease progression.

**Paper III: *DIAPH3* is expressed during sympathoadrenal development and correlated with poor survival in neuroblastoma.**

Our study showed that *DIAPH3* expression is associated with poor survival and indicates poor prognosis encompassing various clinical parameters. Its expression is also found to be active in clusters of sympathoblasts during the sympathoadrenal development of mice, as well as in cycling neuroblasts and cycling SCPs in human embryos, progenitors within the postnatal human adrenal gland, and in clusters exhibiting undifferentiation within NB tumors. Thus, these results reflect its crucial role in the regulatory pathways that control the differentiation.

As cells undergo differentiation and organ formation, they experience alterations in their form and dimensions that are mediated by cytoskeleton dynamics, which are crucial for the development of their new specialized functions [352]. For instance, cell shape and cytoskeletal tension that influenced by RhoA signaling, are critical determinants of human mesenchymal stem cells' differentiation into adipocytes or osteoblasts, revealing that mechanical cues are essential in stem cell fate determination. Cytoskeleton disruption with Cytochalasin D affected the morphology and mechanical stiffness of the human mesenchymal stem cells (hMSCs), with notable differences between cell types in response and recovery, suggesting that hMSCs undergo cytoskeletal changes during differentiation [353].

Gene set enrichment analysis showed that *DIAPH3* expression is associated with proliferation, cell cycle and lipid accumulation processes. In addition, preliminary experimental work indicated a slowdown in growth rate following knockdown, alongside with elevated levels of p21, an inhibitor of the cell cycle. The cytoskeleton undergoes significant dynamic changes to meet the mechanical demands of different cellular processes like chromosome separation and cell division during mitosis. Specifically, spindle microtubules, originating from the centrosomes, seek kinetochores that connect to the replicated chromosomes during metaphase and then separate them, drawing them to opposite ends of the nucleus in anaphase [354]. One

study showed that DIAPH3 is important for the spindle assembly and bipolarity and that DIAPH3 inhibition led to mitotic errors [355].

Nonetheless, to conclusively determine cell cycle dynamics, fluorescence-activated cell sorting (FACS) should be employed, and the expression levels of cyclins, cyclin-dependent kinases or other cell cycle proteins needs to be measured. Further research will be required to confirm our preliminary wet lab data and provide insights into the roles of DIAPH3 in NB tumorigenesis.



## 5 Conclusions

### **Paper I: Expression and activation of nuclear hormone receptors result in neuronal differentiation and favorable prognosis in neuroblastoma.**

*In silico* analysis suggested that a high expression of genes encoding GR, ER $\alpha$ , and RAR $\alpha$  is associated with a positive prognosis. Our research has demonstrated that when these receptors are simultaneously activated, it greatly enhances the process of neuronal differentiation and reduces tumor burden in mouse models. Through single-nuclei RNA sequencing analysis, we have discovered that these receptors are expressed in a specific sequence during the differentiation of chromaffin cells in the post-natal human adrenal gland. These findings provide a foundation for further exploration of the potential therapeutic benefits of activating NHRs as a means of inducing differentiation for high-risk NB treatment. Additionally, combination treatment approach might be effective in combination therapies with lower ligand concentrations, potentially reducing adverse side effects. However, both measuring and administering estradiol present challenges in children, and even more so in boys than in girls. Overall, this study represents a notable progression in understanding and potentially treating high-risk NB, highlighting the importance of targeted receptor stimulation and combination therapies in the field of cancer treatment.

### **Paper II: Target Genes of c-MYC and MYCN with Prognostic Power in Neuroblastoma Exhibit Different Expressions during Sympathoadrenal Development.**

We have developed a robust pipeline that summarizes a set of genes into a risk score. This pipeline can be utilized for various marker genes associated with NB and can also be extended to include other types of tumors. We discovered that high-risk scores associated with c-MYC and MYCN targets are indicative of lower survival rates and unfavorable prognoses across a range of clinical parameters. Significantly, the target genes that hold prognostic significance vary between c-MYC and MYCN, showing unique patterns of expression within the developing sympathoadrenal system. During sympathoadrenal development, genes linked to adverse outcomes are predominantly observed in sympathoblasts instead of chromaffin cells. Our study opens up the potential to investigate a cluster of genes instead of a single gene for cancer stratification and could offer insight into employing customized treatments for specific patient risk signatures.

**Paper III: DIAPH3 is expressed during sympathoadrenal development and correlated with poor prognosis in neuroblastoma.**

Our preliminary data provides evidence of the potential role of DIAPH3 in the development and progression of NB. Our analysis suggests that DIAPH3 may play a critical role in cytoskeletal regulation, cell cycle dynamics, and lipid metabolism in NB cells. We also observed a correlation between elevated *DIAPH3* expression and adverse clinical outcomes in NB patients, highlighting the potential of exploring DIAPH3 as a therapeutic target for high-risk NB cases.

## 6 Points of perspective

### **Paper I: Expression and activation of nuclear hormone receptors result in neuronal differentiation and favorable prognosis in neuroblastoma.**

Future research should focus on the clinical translation of these findings, exploring the potential of personalized medicine approaches. This could involve stratifying NB patients based on the expression levels of GR, ER $\alpha$ , and/or RAR $\alpha$ , tailoring treatment regimens to individual receptor profiles for optimized efficacy. Additionally, the intriguing role of lipid metabolism in NB progression and differentiation, as highlighted by this study, warrants further investigation. Understanding the interplay between metabolic reprogramming, differentiation, and tumorigenesis could uncover new metabolic targets for therapy. The potential for NHR activation to induce differentiation in other cancer types, leveraging similar receptor interplays, also presents an exciting area for exploration. Ultimately, this research paves the way for developing more effective, less toxic treatments for high-risk NB, potentially improving survival rates and quality of life for affected children.

### **Paper II: Target Genes of c-MYC and MYCN with Prognostic Power in Neuroblastoma Exhibit Different Expressions during Sympathoadrenal Development.**

The present study has laid a foundational framework for refining the prognostic stratification of NB by incorporating c-MYC and MYCN target gene analysis. The next step would be to develop a more comprehensive list of MYC/MYCN target genes. This involves the integration of ChIP-seq data and experimental work that shows the direct or indirect regulation of genes by c-MYC/MYCN. Our future efforts would also aim to integrate broader genetic and molecular datasets, possibly incorporating machine learning algorithms that can handle multi-dimensional data, such as genomics, transcriptomics, proteomics, and metabolomics data that derived from NB cell lines or patient samples. Thus, could generate a more complete c-MYC/MYCN target gene set serving for research. Further, this could improve risk stratification and individualize patient treatment plans further, leading to more targeted and effective interventions. Additionally, the future research will be to detail the specific roles of the identified genes in the pathogenesis and progression of NB.

**Paper III: DIAPH3 is expressed during sympathoadrenal development and correlated with poor prognosis in neuroblastoma.**

Further research is needed to investigate the underlying mechanisms by which DIAPH3 affects cell cycle, proliferation, and lipid metabolism. The potential synergistic effects of targeting DIAPH3 along with c-MYC/MYCN should also be explored. Ultimately, our study aims to provide a fundamental theory for the identification and exploration of novel therapeutic targets to enhance the prospects of successful treatment outcomes for NB patients, particularly those who are considered high-risk and currently face limited curative options.

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## 8 References

1. Hanahan, D.; Weinberg, R.A. The hallmarks of cancer. *Cell* **2000**, *100*, 57–70, doi:10.1016/s0092-8674(00)81683-9.
2. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **2011**, *144*, 646–674, doi:10.1016/j.cell.2011.02.013.
3. Hanahan, D. Hallmarks of Cancer: New Dimensions. *Cancer Discov* **2022**, *12*, 31–46, doi:10.1158/2159-8290.CD-21-1059.
4. Perona, R. Cell signalling: growth factors and tyrosine kinase receptors. *Clin Transl Oncol* **2006**, *8*, 77–82, doi:10.1007/s12094-006-0162-1.
5. Bhowmick, N.A.; Neilson, E.G.; Moses, H.L. Stromal fibroblasts in cancer initiation and progression. *Nature* **2004**, *432*, 332–337, doi:10.1038/nature03096.
6. Burkhart, D.L.; Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer* **2008**, *8*, 671–682, doi:10.1038/nrc2399.
7. Sherr, C.J.; McCormick, F. The RB and p53 pathways in cancer. *Cancer Cell* **2002**, *2*, 103–112, doi:10.1016/s1535-6108(02)00102-2.
8. Adams, J.M.; Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **2007**, *26*, 1324–1337, doi:10.1038/sj.onc.1210220.
9. Shay, J.W.; Wright, W.E. Role of telomeres and telomerase in cancer. *Semin Cancer Biol* **2011**, *21*, 349–353, doi:10.1016/j.semcancer.2011.10.001.
10. Gupta, M.K.; Qin, R.Y. Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* **2003**, *9*, 1144–1155, doi:10.3748/wjg.v9.i6.1144.
11. Fan, X.; Jin, S.; Li, Y.; Khadaroo, P.A.; Dai, Y.; He, L.; Zhou, D.; Lin, H. Genetic And Epigenetic Regulation Of E-Cadherin Signaling In Human Hepatocellular Carcinoma. *Cancer Manag Res* **2019**, *11*, 8947–8963, doi:10.2147/CMAR.S225606.
12. Vander Heiden, M.G.; Cantley, L.C.; Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **2009**, *324*, 1029–1033, doi:10.1126/science.1160809.
13. Liu, C.; Jin, Y.; Fan, Z. The Mechanism of Warburg Effect-Induced Chemoresistance in Cancer. *Front Oncol* **2021**, *11*, 698023, doi:10.3389/fonc.2021.698023.
14. Vander Heiden, M.G.; DeBerardinis, R.J. Understanding the Intersections between Metabolism and Cancer Biology. *Cell* **2017**, *168*, 657–669, doi:10.1016/j.cell.2016.12.039.
15. Kim, R.; Emi, M.; Tanabe, K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* **2007**, *121*, 1–14, doi:10.1111/j.1365-2567.2007.02587.x.
16. Pages, F.; Galon, J.; Dieu-Nosjean, M.C.; Tartour, E.; Sautes-Fridman, C.; Fridman, W.H. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* **2010**, *29*, 1093–1102, doi:10.1038/onc.2009.416.
17. Sabapathy, K.; Lane, D.P. Therapeutic targeting of p53: all mutants are equal, but some mutants are more equal than others. *Nat Rev Clin Oncol* **2018**, *15*, 13–30, doi:10.1038/nrclinonc.2017.151.
18. Zhou, W.; Yang, L.; Nie, L.; Lin, H. Unraveling the molecular mechanisms between inflammation and tumor angiogenesis. *Am J Cancer Res* **2021**, *11*, 301–317.
19. Jang, J.H.; Kim, D.H.; Surh, Y.J. Dynamic roles of inflammasomes in inflammatory tumor microenvironment. *NPJ Precis Oncol* **2021**, *5*, 18, doi:10.1038/s41698-021-00154-7.
20. Grivennikov, S.I.; Greten, F.R.; Karin, M. Immunity, inflammation, and cancer. *Cell* **2010**, *140*, 883–899, doi:10.1016/j.cell.2010.01.025.
21. Ordóñez-Moran, P.; Dafflon, C.; Imajo, M.; Nishida, E.; Huelsken, J. HOXA5 Counteracts Stem Cell Traits by Inhibiting Wnt Signaling in Colorectal Cancer. *Cancer Cell* **2015**, *28*, 815–829, doi:10.1016/j.ccell.2015.11.001.
22. Krah, N.M.; De La, O.J.; Swift, G.H.; Hoang, C.Q.; Willet, S.G.; Chen Pan, F.; Cash, G.M.; Bronner, M.P.; Wright, C.V.; MacDonald, R.J.; et al. The acinar differentiation determinant PTF1A inhibits

- initiation of pancreatic ductal adenocarcinoma. *Elife* **2015**, *4*, doi:10.7554/eLife.07125.
23. Kaufman, C.K.; Mosimann, C.; Fan, Z.P.; Yang, S.; Thomas, A.J.; Ablain, J.; Tan, J.L.; Fogley, R.D.; van Rooijen, E.; Hagedorn, E.J.; et al. A zebrafish melanoma model reveals emergence of neural crest identity during melanoma initiation. *Science* **2016**, *351*, aad2197, doi:10.1126/science.aad2197.
24. Costa, P.; Sales, S.L.A.; Pinheiro, D.P.; Pontes, L.Q.; Maranhao, S.S.; Pessoa, C.D.O.; Furtado, G.P.; Furtado, C.L.M. Epigenetic reprogramming in cancer: From diagnosis to treatment. *Front Cell Dev Biol* **2023**, *11*, 1116805, doi:10.3389/fcell.2023.1116805.
25. Boesch, M.; Horvath, L.; Baty, F.; Pircher, A.; Wolf, D.; Spahn, S.; Straussman, R.; Tilg, H.; Brutsche, M.H. Compartmentalization of the host microbiome: how tumor microbiota shapes checkpoint immunotherapy outcome and offers therapeutic prospects. *J Immunother Cancer* **2022**, *10*, doi:10.1136/jitc-2022-005401.
26. Di Micco, R.; Krizhanovsky, V.; Baker, D.; d'Adda di Fagagna, F. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat Rev Mol Cell Biol* **2021**, *22*, 75–95, doi:10.1038/s41580-020-00314-w.
27. Park, S.S.; Choi, Y.W.; Kim, J.H.; Kim, H.S.; Park, T.J. Senescent tumor cells: an overlooked adversary in the battle against cancer. *Exp Mol Med* **2021**, *53*, 1834–1841, doi:10.1038/s12276-021-00717-5.
28. Brown, G. Oncogenes, Proto-Oncogenes, and Lineage Restriction of Cancer Stem Cells. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22189667.
29. Torry, D.S.; Cooper, G.M. Proto-oncogenes in development and cancer. *Am J Reprod Immunol* **1991**, *25*, 129–132, doi:10.1111/j.1600-0897.1991.tb01080.x.
30. Haigis, K.M.; Kendall, K.R.; Wang, Y.; Cheung, A.; Haigis, M.C.; Glickman, J.N.; Niwa-Kawakita, M.; Sweet-Cordero, A.; Sebolt-Leopold, J.; Shannon, K.M.; et al. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. *Nat Genet* **2008**, *40*, 600–608, doi:10.1038/ng.115.
31. Schaub, F.X.; Dhankani, V.; Berger, A.C.; Trivedi, M.; Richardson, A.B.; Shaw, R.; Zhao, W.; Zhang, X.; Ventura, A.; Liu, Y.; et al. Pan-cancer Alterations of the MYC Oncogene and Its Proximal Network across the Cancer Genome Atlas. *Cell Syst* **2018**, *6*, 282–300 e282, doi:10.1016/j.cels.2018.03.003.
32. Iwakawa, R.; Kohno, T.; Kato, M.; Shiraishi, K.; Tsuta, K.; Noguchi, M.; Ogawa, S.; Yokota, J. MYC amplification as a prognostic marker of early-stage lung adenocarcinoma identified by whole genome copy number analysis. *Clin Cancer Res* **2011**, *17*, 1481–1489, doi:10.1158/1078-0432.CCR-10-2484.
33. Iqbal, N.; Iqbal, N. Human Epidermal Growth Factor Receptor 2 (HER2) in Cancers: Overexpression and Therapeutic Implications. *Mol Biol Int* **2014**, *2014*, 852748, doi:10.1155/2014/852748.
34. Lee, E.Y.; Muller, W.J. Oncogenes and tumor suppressor genes. *Cold Spring Harb Perspect Biol* **2010**, *2*, a003236, doi:10.1101/cshperspect.a003236.
35. Steele, R.J.; Thompson, A.M.; Hall, P.A.; Lane, D.P. The p53 tumour suppressor gene. *Br J Surg* **1998**, *85*, 1460–1467, doi:10.1046/j.1365-2168.1998.00910.x.
36. Hernandez Borrero, L.J.; El-Deiry, W.S. Tumor suppressor p53: Biology, signaling pathways, and therapeutic targeting. *Biochim Biophys Acta Rev Cancer* **2021**, *1876*, 188556, doi:10.1016/j.bbcan.2021.188556.
37. Kennedy, M.C.; Lowe, S.W. Mutant p53: it's not all one and the same. *Cell Death Differ* **2022**, *29*, 983–987, doi:10.1038/s41418-022-00989-y.
38. Matthews, H.K.; Bertoli, C.; de Bruin, R.A.M. Cell cycle control in cancer. *Nat Rev Mol Cell Biol* **2022**, *23*, 74–88, doi:10.1038/s41580-021-00404-3.
39. Nurse, P.; Masui, Y.; Hartwell, L. Understanding the cell cycle. *Nat Med* **1998**, *4*, 1103–1106, doi:10.1038/2594.
40. Foster, D.A.; Yellen, P.; Xu, L.; Saqcena, M. Regulation of G1 Cell Cycle Progression: Distinguishing the Restriction Point from a Nutrient-Sensing Cell Growth Checkpoint(s). *Genes Cancer* **2010**, *1*, 1124–1131, doi:10.1177/1947601910392989.

41. Stark, G.R.; Taylor, W.R. Control of the G2/M transition. *Mol Biotechnol* **2006**, *32*, 227–248, doi:10.1385/MB:32:3:227.
42. Evans, T.; Rosenthal, E.T.; Youngblom, J.; Distel, D.; Hunt, T. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **1983**, *33*, 389–396, doi:10.1016/0092-8674(83)90420-8.
43. Draetta, G.; Luca, F.; Westendorf, J.; Brizuela, L.; Ruderman, J.; Beach, D. Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell* **1989**, *56*, 829–838, doi:10.1016/0092-8674(89)90687-9.
44. Riabowol, K.; Draetta, G.; Brizuela, L.; Vandre, D.; Beach, D. The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* **1989**, *57*, 393–401, doi:10.1016/0092-8674(89)90914-8.
45. Romeiro Motta, M.; Zhao, X.; Pastuglia, M.; Belcram, K.; Roodbarkelari, F.; Komaki, M.; Harashima, H.; Komaki, S.; Kumar, M.; Bulankova, P.; et al. B1-type cyclins control microtubule organization during cell division in Arabidopsis. *EMBO Rep* **2022**, *23*, e53995, doi:10.15252/embr.202153995.
46. Murray, A.W. Recycling the cell cycle: cyclins revisited. *Cell* **2004**, *116*, 221–234, doi:10.1016/s0092-8674(03)01080-8.
47. Bartek, J.; Bartkova, J.; Lukas, J. The retinoblastoma protein pathway in cell cycle control and cancer. *Exp Cell Res* **1997**, *237*, 1–6, doi:10.1006/excr.1997.3776.
48. Dyson, N. The regulation of E2F by pRB-family proteins. *Genes Dev* **1998**, *12*, 2245–2262, doi:10.1101/gad.12.15.2245.
49. Smith, J.; Tho, L.M.; Xu, N.; Gillespie, D.A. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res* **2010**, *108*, 73–112, doi:10.1016/B978-0-12-380888-2.00003-0.
50. Bartek, J.; Lukas, J. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol* **2007**, *19*, 238–245, doi:10.1016/j.ceb.2007.02.009.
51. Chen, J. The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression. *Cold Spring Harb Perspect Med* **2016**, *6*, a026104, doi:10.1101/cshperspect.a026104.
52. Li, J.; Poi, M.J.; Tsai, M.D. Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. *Biochemistry* **2011**, *50*, 5566–5582, doi:10.1021/bi200642e.
53. Wang, Z. Regulation of Cell Cycle Progression by Growth Factor-Induced Cell Signaling. *Cells* **2021**, *10*, doi:10.3390/cells10123327.
54. Shtivelman, E.; Sussman, J.; Stokoe, D. A role for PI 3-kinase and PKB activity in the G2/M phase of the cell cycle. *Curr Biol* **2002**, *12*, 919–924, doi:10.1016/s0960-9822(02)00843-6.
55. Blajeski, A.L.; Phan, V.A.; Kottke, T.J.; Kaufmann, S.H. G(1) and G(2) cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *J Clin Invest* **2002**, *110*, 91–99, doi:10.1172/JCI13275.
56. Shen, S.; Shao, Y.; Li, C. Different types of cell death and their shift in shaping disease. *Cell Death Discov* **2023**, *9*, 284, doi:10.1038/s41420-023-01581-0.
57. Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **2007**, *35*, 495–516, doi:10.1080/01926230701320337.
58. Shi, Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* **2002**, *9*, 459–470, doi:10.1016/s1097-2765(02)00482-3.
59. Guicciardi, M.E.; Gores, G.J. Life and death by death receptors. *FASEB J* **2009**, *23*, 1625–1637, doi:10.1096/fj.08-111005.
60. Qian, S.; Wei, Z.; Yang, W.; Huang, J.; Yang, Y.; Wang, J. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. *Front Oncol* **2022**, *12*, 985363, doi:10.3389/fonc.2022.985363.
61. Trapani, J.A.; Smyth, M.J. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* **2002**, *2*, 735–747, doi:10.1038/nri911.
62. Yang, Z.; Klionsky, D.J. An overview of the molecular mechanism of autophagy. *Curr Top Microbiol Immunol* **2009**, *335*, 1–32, doi:10.1007/978-3-642-00302-8\_1.
63. Liu, B.; Bao, J.K.; Yang, J.M.; Cheng, Y. Targeting autophagic pathways for cancer drug discovery.

- Chin J Cancer* **2013**, *32*, 113-120, doi:10.5732/cjc.012.10010.
64. Csizmadia, T.; Juhasz, G. Crinophagy mechanisms and its potential role in human health and disease. *Prog Mol Biol Transl Sci* **2020**, *172*, 239-255, doi:10.1016/bs.pmbts.2020.02.002.
  65. Yorimitsu, T.; Klionsky, D.J. Autophagy: molecular machinery for self-eating. *Cell Death Differ* **2005**, *12 Suppl 2*, 1542-1552, doi:10.1038/sj.cdd.4401765.
  66. Golstein, P.; Kroemer, G. Cell death by necrosis: towards a molecular definition. *Trends Biochem Sci* **2007**, *32*, 37-43, doi:10.1016/j.tibs.2006.11.001.
  67. Galluzzi, L.; Kroemer, G. Necroptosis: a specialized pathway of programmed necrosis. *Cell* **2008**, *135*, 1161-1163, doi:10.1016/j.cell.2008.12.004.
  68. LeDuc, P.; Ostuni, E.; Whitesides, G.; Ingber, D. Use of micropatterned adhesive surfaces for control of cell behavior. *Methods Cell Biol* **2002**, *69*, 385-401, doi:10.1016/s0091-679x(02)69024-7.
  69. Dike, L.E.; Chen, C.S.; Mrksich, M.; Tien, J.; Whitesides, G.M.; Ingber, D.E. Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *In Vitro Cell Dev Biol Anim* **1999**, *35*, 441-448, doi:10.1007/s11626-999-0050-4.
  70. Corey, J.M.; Gertz, C.C.; Sutton, T.J.; Chen, Q.; Mycek, K.B.; Wang, B.S.; Martin, A.A.; Johnson, S.L.; Feldman, E.L. Patterning N-type and S-type neuroblastoma cells with Pluronic F108 and ECM proteins. *J Biomed Mater Res A* **2010**, *93*, 673-686, doi:10.1002/jbm.a.32485.
  71. Weisenberg, R.C. Microtubule formation in vitro in solutions containing low calcium concentrations. *Science* **1972**, *177*, 1104-1105, doi:10.1126/science.177.4054.1104.
  72. de Pablo, P.J.; Schaap, I.A.; MacKintosh, F.C.; Schmidt, C.F. Deformation and collapse of microtubules on the nanometer scale. *Phys Rev Lett* **2003**, *91*, 098101, doi:10.1103/PhysRevLett.91.098101.
  73. Ilan, Y. Microtubules: From understanding their dynamics to using them as potential therapeutic targets. *J Cell Physiol* **2019**, *234*, 7923-7937, doi:10.1002/jcp.27978.
  74. Logan, C.M.; Menko, A.S. Microtubules: Evolving roles and critical cellular interactions. *Exp Biol Med (Maywood)* **2019**, *244*, 1240-1254, doi:10.1177/1535370219867296.
  75. Herrmann, H.; Bar, H.; Kreplak, L.; Strelkov, S.V.; Aebi, U. Intermediate filaments: from cell architecture to nanomechanics. *Nat Rev Mol Cell Biol* **2007**, *8*, 562-573, doi:10.1038/nrm2197.
  76. Hall, A. Rho GTPases and the actin cytoskeleton. *Science* **1998**, *279*, 509-514, doi:10.1126/science.279.5350.509.
  77. Burns, S.; Thrasher, A.J.; Blundell, M.P.; Machesky, L.; Jones, G.E. Configuration of human dendritic cell cytoskeleton by Rho GTPases, the WAS protein, and differentiation. *Blood* **2001**, *98*, 1142-1149, doi:10.1182/blood.v98.4.1142.
  78. Fife, C.M.; McCarroll, J.A.; Kavallaris, M. Movers and shakers: cell cytoskeleton in cancer metastasis. *Br J Pharmacol* **2014**, *171*, 5507-5523, doi:10.1111/bph.12704.
  79. Thiery, J.P.; Aclouque, H.; Huang, R.Y.; Nieto, M.A. Epithelial-mesenchymal transitions in development and disease. *Cell* **2009**, *139*, 871-890, doi:10.1016/j.cell.2009.11.007.
  80. Aclouque, H.; Adams, M.S.; Fishwick, K.; Bronner-Fraser, M.; Nieto, M.A. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* **2009**, *119*, 1438-1449, doi:10.1172/JCI38019.
  81. Lamouille, S.; Xu, J.; Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* **2014**, *15*, 178-196, doi:10.1038/nrm3758.
  82. Dustin, M.L.; Olszowy, M.W.; Holdorf, A.D.; Li, J.; Bromley, S.; Desai, N.; Widder, P.; Rosenberger, F.; van der Merwe, P.A.; Allen, P.M.; et al. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* **1998**, *94*, 667-677, doi:10.1016/s0092-8674(00)81608-6.
  83. Kunkel, E.J.; Butcher, E.C. Chemokines and the tissue-specific migration of lymphocytes. *Immunity* **2002**, *16*, 1-4, doi:10.1016/s1074-7613(01)00261-8.
  84. Swanson, J.A.; Johnson, M.T.; Beningo, K.; Post, P.; Mooseker, M.; Araki, N. A contractile activity that closes phagosomes in macrophages. *J Cell Sci* **1999**, *112 ( Pt 3)*, 307-316, doi:10.1242/jcs.112.3.307.

85. Lyubchenko, T.A.; Wurth, G.A.; Zweifach, A. The actin cytoskeleton and cytotoxic T lymphocytes: evidence for multiple roles that could affect granule exocytosis-dependent target cell killing. *J Physiol* **2003**, *547*, 835–847, doi:10.1113/jphysiol.2002.033522.
86. Monks, C.R.; Freiberg, B.A.; Kupfer, H.; Sciaky, N.; Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **1998**, *395*, 82–86, doi:10.1038/25764.
87. Ben-Shmuel, A.; Sabag, B.; Biber, G.; Barda-Saad, M. The Role of the Cytoskeleton in Regulating the Natural Killer Cell Immune Response in Health and Disease: From Signaling Dynamics to Function. *Front Cell Dev Biol* **2021**, *9*, 609532, doi:10.3389/fcell.2021.609532.
88. Boureux, A.; Vignal, E.; Faure, S.; Fort, P. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Mol Biol Evol* **2007**, *24*, 203–216, doi:10.1093/molbev/msl145.
89. Totsukawa, G.; Yamakita, Y.; Yamashiro, S.; Hartshorne, D.J.; Sasaki, Y.; Matsumura, F. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J Cell Biol* **2000**, *150*, 797–806, doi:10.1083/jcb.150.4.797.
90. Ohashi, K.; Nagata, K.; Maekawa, M.; Ishizaki, T.; Narumiya, S.; Mizuno, K. Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. *J Biol Chem* **2000**, *275*, 3577–3582, doi:10.1074/jbc.275.5.3577.
91. Shao, J.; Welch, W.J.; Diprospero, N.A.; Diamond, M.I. Phosphorylation of profilin by ROCK1 regulates polyglutamine aggregation. *Mol Cell Biol* **2008**, *28*, 5196–5208, doi:10.1128/MCB.00079-08.
92. Nakagawa, S.; Takeichi, M. Neural crest emigration from the neural tube depends on regulated cadherin expression. *Development* **1998**, *125*, 2963–2971, doi:10.1242/dev.125.15.2963.
93. Nakagawa, S.; Takeichi, M. Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* **1995**, *121*, 1321–1332, doi:10.1242/dev.121.5.1321.
94. Duband, J.L.; Monier, F.; Delannet, M.; Newgreen, D. Epithelium-mesenchyme transition during neural crest development. *Acta Anat (Basel)* **1995**, *154*, 63–78, doi:10.1159/000147752.
95. Nakaya, Y.; Sukowati, E.W.; Wu, Y.; Sheng, G. RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. *Nat Cell Biol* **2008**, *10*, 765–775, doi:10.1038/ncb1739.
96. Sigsby, L.M. Crisis and ethical dilemmas: who will care for the rural nurse? *Heart Lung* **1991**, *20*, 523–525.
97. Evangelista, M.; Zigmond, S.; Boone, C. Formins: signaling effectors for assembly and polarization of actin filaments. *J Cell Sci* **2003**, *116*, 2603–2611, doi:10.1242/jcs.00611.
98. Breitsprecher, D.; Goode, B.L. Formins at a glance. *J Cell Sci* **2013**, *126*, 1–7, doi:10.1242/jcs.107250.
99. Mattila, P.K.; Lappalainen, P. Filopodia: molecular architecture and cellular functions. *Nat Rev Mol Cell Biol* **2008**, *9*, 446–454, doi:10.1038/nrm2406.
100. Innocenti, M. New insights into the formation and the function of lamellipodia and ruffles in mesenchymal cell migration. *Cell Adh Migr* **2018**, *12*, 401–416, doi:10.1080/19336918.2018.1448352.
101. Kreis, T.E.; Birchmeier, W. Stress fiber sarcomeres of fibroblasts are contractile. *Cell* **1980**, *22*, 555–561, doi:10.1016/0092-8674(80)90365-7.
102. Lai, S.L.; Chan, T.H.; Lin, M.J.; Huang, W.P.; Lou, S.W.; Lee, S.J. Diaphanous-related formin 2 and profilin I are required for gastrulation cell movements. *PLoS One* **2008**, *3*, e3439, doi:10.1371/journal.pone.0003439.
103. Ghimire, S.; Mantziou, V.; Moris, N.; Martinez Arias, A. Human gastrulation: The embryo and its models. *Dev Biol* **2021**, *474*, 100–108, doi:10.1016/j.ydbio.2021.01.006.
104. Greene, N.D.; Copp, A.J. Neural tube defects. *Annu Rev Neurosci* **2014**, *37*, 221–242, doi:10.1146/annurev-neuro-062012-170354.
105. Pruyne, D. Revisiting the Phylogeny of the Animal Formins: Two New Subtypes, Relationships with Multiple Wing Hairs Proteins, and a Lost Human Formin. *PLoS One* **2016**, *11*, e0164067, doi:10.1371/journal.pone.0164067.

106. Stastna, J.; Pan, X.; Wang, H.; Kollmannsperger, A.; Kutscheidt, S.; Lohmann, V.; Grosse, R.; Fackler, O.T. Differing and isoform-specific roles for the formin DIAPH3 in plasma membrane blebbing and filopodia formation. *Cell Res* **2012**, *22*, 728–745, doi:10.1038/cr.2011.202.
107. Chen, A.; Arora, P.D.; McCulloch, C.A.; Wilde, A. Cytokinesis requires localized beta-actin filament production by an actin isoform specific nucleator. *Nat Commun* **2017**, *8*, 1530, doi:10.1038/s41467-017-01231-x.
108. Watanabe, S.; De Zan, T.; Ishizaki, T.; Yasuda, S.; Kamijo, H.; Yamada, D.; Aoki, T.; Kiyonari, H.; Kaneko, H.; Shimizu, R.; et al. Loss of a Rho-regulated actin nucleator, mDia2, impairs cytokinesis during mouse fetal erythropoiesis. *Cell Rep* **2013**, *5*, 926–932, doi:10.1016/j.celrep.2013.10.021.
109. Chen, X.; Xie, L.; Qiao, K.; Zhu, X.; Ren, J.; Tan, Y. The pan-cancer analysis identified DIAPH3 as a diagnostic biomarker of clinical cancer. *Aging (Albany NY)* **2023**, *15*, 689–704, doi:10.18632/aging.204459.
110. Huang, R.; Wu, C.; Wen, J.; Yu, J.; Zhu, H.; Yu, J.; Zou, Z. DIAPH3 is a prognostic biomarker and inhibit colorectal cancer progression through maintaining EGFR degradation. *Cancer Med* **2022**, *11*, 4688–4702, doi:10.1002/cam4.4793.
111. Dong, L.; Li, Z.; Xue, L.; Li, G.; Zhang, C.; Cai, Z.; Li, H.; Guo, R. DIAPH3 promoted the growth, migration and metastasis of hepatocellular carcinoma cells by activating beta-catenin/TCF signaling. *Mol Cell Biochem* **2018**, *438*, 183–190, doi:10.1007/s11010-017-3125-7.
112. Xiang, G.; Weiwei, H.; Erji, G.; Haitao, M. DIAPH3 promotes the tumorigenesis of lung adenocarcinoma. *Exp Cell Res* **2019**, *385*, 111662, doi:10.1016/j.yexcr.2019.111662.
113. Zhang, Z.; Dai, F.; Luo, F.; Wu, W.; Zhang, S.; Zhou, R.; Xu, J.; Zhou, Q.; Song, L. Diaphanous related formin 3 knockdown suppresses cell proliferation and metastasis of osteosarcoma cells. *Discov Oncol* **2021**, *12*, 20, doi:10.1007/s12672-021-00415-8.
114. Rong, Y.; Gao, J.; Kuang, T.; Chen, J.; Li, J.A.; Huang, Y.; Xin, H.; Fang, Y.; Han, X.; Sun, L.Q.; et al. DIAPH3 promotes pancreatic cancer progression by activating selenoprotein TrxR1-mediated antioxidant effects. *J Cell Mol Med* **2021**, *25*, 2163–2175, doi:10.1111/jcmm.16196.
115. Kohl, N.E.; Gee, C.E.; Alt, F.W. Activated expression of the N-myc gene in human neuroblastomas and related tumors. *Science* **1984**, *226*, 1335–1337, doi:10.1126/science.6505694.
116. Nau, M.M.; Brooks, B.J.; Battey, J.; Sausville, E.; Gazdar, A.F.; Kirsch, I.R.; McBride, O.W.; Bertness, V.; Hollis, G.F.; Minna, J.D. L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* **1985**, *318*, 69–73, doi:10.1038/318069a0.
117. Vennstrom, B.; Sheiness, D.; Zabielski, J.; Bishop, J.M. Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. *J Virol* **1982**, *42*, 773–779, doi:10.1128/JVI.42.3.773-779.1982.
118. Blackwood, E.M.; Eisenman, R.N. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **1991**, *251*, 1211–1217, doi:10.1126/science.2006410.
119. Madden, S.K.; de Araujo, A.D.; Gerhardt, M.; Fairlie, D.P.; Mason, J.M. Taking the Myc out of cancer: toward therapeutic strategies to directly inhibit c-Myc. *Mol Cancer* **2021**, *20*, 3, doi:10.1186/s12943-020-01291-6.
120. Pan, Y.; van der Watt, P.J.; Kay, S.A. E-box binding transcription factors in cancer. *Front Oncol* **2023**, *13*, 1223208, doi:10.3389/fonc.2023.1223208.
121. Beaulieu, M.E.; Castillo, F.; Soucek, L. Structural and Biophysical Insights into the Function of the Intrinsically Disordered Myc Oncoprotein. *Cells* **2020**, *9*, doi:10.3390/cells9041038.
122. Ayer, D.E.; Kretzner, L.; Eisenman, R.N. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell* **1993**, *72*, 211–222, doi:10.1016/0092-8674(93)90661-9.
123. Sun, X.X.; Li, Y.; Sears, R.C.; Dai, M.S. Targeting the MYC Ubiquitination-Proteasome Degradation Pathway for Cancer Therapy. *Front Oncol* **2021**, *11*, 679445, doi:10.3389/fonc.2021.679445.
124. Diolaiti, D.; McFerrin, L.; Carroll, P.A.; Eisenman, R.N. Functional interactions among members of the MAX and MLX transcriptional network during oncogenesis. *Biochim Biophys Acta* **2015**, *1849*, 484–500, doi:10.1016/j.bbagr.2014.05.016.
125. Gabay, M.; Li, Y.; Felsher, D.W. MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harb Perspect Med* **2014**, *4*, doi:10.1101/cshperspect.a014241.

126. Soucek, L.; Evan, G.I. The ups and downs of Myc biology. *Curr Opin Genet Dev* **2010**, *20*, 91-95, doi:10.1016/j.gde.2009.11.001.
127. Raman, D.; Chong, S.J.F.; Iskandar, K.; Hirpara, J.L.; Pervaiz, S. Peroxynitrite promotes serine-62 phosphorylation-dependent stabilization of the oncoprotein c-Myc. *Redox Biol* **2020**, *34*, 101587, doi:10.1016/j.redox.2020.101587.
128. Henriksson, M.; Bakardjiev, A.; Klein, G.; Luscher, B. Phosphorylation sites mapping in the N-terminal domain of c-myc modulate its transforming potential. *Oncogene* **1993**, *8*, 3199-3209.
129. van Riggelen, J.; Yetil, A.; Felsner, D.W. MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat Rev Cancer* **2010**, *10*, 301-309, doi:10.1038/nrc2819.
130. Dong, Y.; Tu, R.; Liu, H.; Qing, G. Regulation of cancer cell metabolism: oncogenic MYC in the driver's seat. *Signal Transduct Target Ther* **2020**, *5*, 124, doi:10.1038/s41392-020-00235-2.
131. Popay, T.M.; Wang, J.; Adams, C.M.; Howard, G.C.; Codreanu, S.G.; Sherrod, S.D.; McLean, J.A.; Thomas, L.R.; Lorey, S.L.; Machida, Y.J.; et al. MYC regulates ribosome biogenesis and mitochondrial gene expression programs through its interaction with host cell factor-1. *Elife* **2021**, *10*, doi:10.7554/eLife.60191.
132. Denis, N.; Kitzis, A.; Kruh, J.; Dautry, F.; Corcos, D. Stimulation of methotrexate resistance and dihydrofolate reductase gene amplification by c-myc. *Oncogene* **1991**, *6*, 1453-1457.
133. Wang, C.; Zhang, J.; Yin, J.; Gan, Y.; Xu, S.; Gu, Y.; Huang, W. Alternative approaches to target Myc for cancer treatment. *Signal Transduct Target Ther* **2021**, *6*, 117, doi:10.1038/s41392-021-00500-y.
134. Yin, X.; Giap, C.; Lazo, J.S.; Prochownik, E.V. Low molecular weight inhibitors of Myc-Max interaction and function. *Oncogene* **2003**, *22*, 6151-6159, doi:10.1038/sj.onc.1206641.
135. Wanner, J.; Romashko, D.; Werner, D.S.; May, E.W.; Peng, Y.; Schulz, R.; Foreman, K.W.; Russo, S.; Arnold, L.D.; Pingle, M.; et al. Reversible linkage of two distinct small molecule inhibitors of Myc generates a dimeric inhibitor with improved potency that is active in myc over-expressing cancer cell lines. *PLoS One* **2015**, *10*, e0121793, doi:10.1371/journal.pone.0121793.
136. Wang, H.; Chauhan, J.; Hu, A.; Pendleton, K.; Yap, J.L.; Sabato, P.E.; Jones, J.W.; Perri, M.; Yu, J.; Cione, E.; et al. Disruption of Myc-Max heterodimerization with improved cell-penetrating analogs of the small molecule 10074-G5. *Oncotarget* **2013**, *4*, 936-947, doi:10.18632/oncotarget.1108.
137. Chauhan, J.; Wang, H.; Yap, J.L.; Sabato, P.E.; Hu, A.; Prochownik, E.V.; Fletcher, S. Discovery of methyl 4'-methyl-5-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)-[1,1'-biphenyl]-3-carboxylate, an improved small-molecule inhibitor of c-Myc-max dimerization. *ChemMedChem* **2014**, *9*, 2274-2285, doi:10.1002/cmdc.201402189.
138. Mason, J.M. Design and development of peptides and peptide mimetics as antagonists for therapeutic intervention. *Future Med Chem* **2010**, *2*, 1813-1822, doi:10.4155/fmc.10.259.
139. Savino, M.; Annibali, D.; Carucci, N.; Favuzzi, E.; Cole, M.D.; Evan, G.I.; Soucek, L.; Nasi, S. The action mechanism of the Myc inhibitor termed Omomyc may give clues on how to target Myc for cancer therapy. *PLoS One* **2011**, *6*, e22284, doi:10.1371/journal.pone.0022284.
140. Li, W.; Bano, F.; Khan, A.; Wei, D.Q.; Alshammari, A.; Xu, B.; Wang, Y. Inhibition of cMYC-MAX transcription factors hetero-dimerization with structurally engineered omoMYC to downregulate oncogenic pathways in renal carcinoma. *Comput Biol Med* **2023**, *164*, 107257, doi:10.1016/j.combiomed.2023.107257.
141. Sodir, N.M.; Kortlever, R.M.; Barthet, V.J.A.; Campos, T.; Pellegrinet, L.; Kupczak, S.; Anastasiou, P.; Swigart, L.B.; Soucek, L.; Arends, M.J.; et al. MYC Instructs and Maintains Pancreatic Adenocarcinoma Phenotype. *Cancer Discov* **2020**, *10*, 588-607, doi:10.1158/2159-8290.CD-19-0435.
142. Beaulieu, M.E.; Jauset, T.; Masso-Valles, D.; Martinez-Martin, S.; Rahl, P.; Maltais, L.; Zacarias-Fluck, M.F.; Casacuberta-Serra, S.; Serrano Del Pozo, E.; Fiore, C.; et al. Intrinsic cell-penetrating activity propels Omomyc from proof of concept to viable anti-MYC therapy. *Sci Transl Med* **2019**, *11*, doi:10.1126/scitranslmed.aar5012.
143. Dang, C.V.; O'Donnell, K.A.; Zeller, K.I.; Nguyen, T.; Osthus, R.C.; Li, F. The c-Myc target gene network. *Semin Cancer Biol* **2006**, *16*, 253-264, doi:10.1016/j.semcancer.2006.07.014.

144. Meyer, N.; Penn, L.Z. Reflecting on 25 years with MYC. *Nat Rev Cancer* **2008**, *8*, 976-990, doi:10.1038/nrc2231.
145. Eilers, M.; Picard, D.; Yamamoto, K.R.; Bishop, J.M. Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* **1989**, *340*, 66-68, doi:10.1038/340066a0.
146. Eilers, M.; Schirm, S.; Bishop, J.M. The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J* **1991**, *10*, 133-141, doi:10.1002/j.1460-2075.1991.tb07929.x.
147. Bello-Fernandez, C.; Packham, G.; Cleveland, J.L. The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci U S A* **1993**, *90*, 7804-7808, doi:10.1073/pnas.90.16.7804.
148. Guccione, E.; Martinato, F.; Finocchiaro, G.; Luzi, L.; Tizzoni, L.; Dall'Olio, V.; Zardo, G.; Nervi, C.; Bernard, L.; Amati, B. Myc-binding-site recognition in the human genome is determined by chromatin context. *Nat Cell Biol* **2006**, *8*, 764-770, doi:10.1038/ncb1434.
149. Furey, T.S. ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions. *Nat Rev Genet* **2012**, *13*, 840-852, doi:10.1038/nrg3306.
150. Chen, X.; Xu, H.; Yuan, P.; Fang, F.; Huss, M.; Vega, V.B.; Wong, E.; Orlov, Y.L.; Zhang, W.; Jiang, J.; et al. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* **2008**, *133*, 1106-1117, doi:10.1016/j.cell.2008.04.043.
151. Kim, J.; Lee, J.H.; Iyer, V.R. Global identification of Myc target genes reveals its direct role in mitochondrial biogenesis and its E-box usage in vivo. *PLoS One* **2008**, *3*, e1798, doi:10.1371/journal.pone.0001798.
152. Gatta, G.; Capocaccia, R.; Coleman, M.P.; Ries, L.A.; Berrino, F. Childhood cancer survival in Europe and the United States. *Cancer* **2002**, *95*, 1767-1772, doi:10.1002/cncr.10833.
153. Ward, E.; DeSantis, C.; Robbins, A.; Kohler, B.; Jemal, A. Childhood and adolescent cancer statistics, 2014. *CA Cancer J Clin* **2014**, *64*, 83-103, doi:10.3322/caac.21219.
154. Behjati, S.; Gilbertson, R.J.; Pfister, S.M. Maturation Block in Childhood Cancer. *Cancer Discov* **2021**, *11*, 542-544, doi:10.1158/2159-8290.CD-20-0926.
155. Rahal, Z.; Abdulhai, F.; Kadara, H.; Saab, R. Genomics of adult and pediatric solid tumors. *Am J Cancer Res* **2018**, *8*, 1356-1386.
156. Mettlin, C.J.; Menck, H.R.; Winchester, D.P.; Murphy, G.P. A comparison of breast, colorectal, lung, and prostate cancers reported to the National Cancer Data Base and the Surveillance, Epidemiology, and End Results Program. *Cancer* **1997**, *79*, 2052-2061, doi:10.1002/(sici)1097-0142(19970515)79:10<2052::aid-cncr29>3.0.co;2-s.
157. Wu, S.; Zhu, W.; Thompson, P.; Hannun, Y.A. Evaluating intrinsic and non-intrinsic cancer risk factors. *Nat Commun* **2018**, *9*, 3490, doi:10.1038/s41467-018-05467-z.
158. Spector, L.G.; Pankratz, N.; Marcotte, E.L. Genetic and nongenetic risk factors for childhood cancer. *Pediatr Clin North Am* **2015**, *62*, 11-25, doi:10.1016/j.pcl.2014.09.013.
159. De Bernardi, B.; Pistoia, V.; Gambini, C.; Granata, C. Peripheral Neuroblastic Tumors. In *Clinical Endocrine Oncology: Second Edition*; Blackwell Publishing, Ltd: 2009; pp. 360-369.
160. Brodeur, G.M.J.N.r.c. Neuroblastoma: biological insights into a clinical enigma. **2003**, *3*, 203-216.
161. Shimada, H.; Ambros, I.M.; Dehner, L.P.; Hata, J.; Joshi, V.V.; Roald, B. Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. *Cancer* **1999**, *86*, 349-363.
162. Peuchmaur, M.; d'Amore, E.S.; Joshi, V.V.; Hata, J.; Roald, B.; Dehner, L.P.; Gerbing, R.B.; Stram, D.O.; Lukens, J.N.; Matthay, K.K.; et al. Revision of the International Neuroblastoma Pathology Classification: confirmation of favorable and unfavorable prognostic subsets in ganglioneuroblastoma, nodular. *Cancer* **2003**, *98*, 2274-2281, doi:10.1002/cncr.11773.
163. Shimada, H.; Ambros, I.M.; Dehner, L.P.; Hata, J.; Joshi, V.V.; Roald, B.; Stram, D.O.; Gerbing, R.B.; Lukens, J.N.; Matthay, K.K.; et al. The International Neuroblastoma Pathology Classification (the Shimada system). *Cancer* **1999**, *86*, 364-372.
164. Wang, L.L.; Sukanuma, R.; Ikegaki, N.; Tang, X.; Naranjo, A.; McGrady, P.; London, W.B.; Hogarty, M.D.; Gastier-Foster, J.M.; Look, A.T.; et al. Neuroblastoma of undifferentiated subtype, prognostic significance of prominent nucleolar formation, and MYC/MYCN protein expression:

- a report from the Children's Oncology Group. *Cancer* **2013**, *119*, 3718-3726, doi:10.1002/cncr.28251.
165. Tornoczky, T.; Kalman, E.; Kajtar, P.G.; Nyari, T.; Pearson, A.D.; Tweddle, D.A.; Board, J.; Shimada, H. Large cell neuroblastoma: a distinct phenotype of neuroblastoma with aggressive clinical behavior. *Cancer* **2004**, *100*, 390-397, doi:10.1002/cncr.20005.
  166. Tornoczky, T.; Semjen, D.; Shimada, H.; Ambros, I.M. Pathology of peripheral neuroblastic tumors: significance of prominent nucleoli in undifferentiated/poorly differentiated neuroblastoma. *Pathol Oncol Res* **2007**, *13*, 269-275, doi:10.1007/BF02940304.
  167. Shimada, H.; Ikegaki, N. Genetic and Histopathological Heterogeneity of Neuroblastoma and Precision Therapeutic Approaches for Extremely Unfavorable Histology Subgroups. *Biomolecules* **2022**, *12*, doi:10.3390/biom12010079.
  168. Coco, S.; Defferrari, R.; Scaruffi, P.; Cavazzana, A.; Di Cristofano, C.; Longo, L.; Mazzocco, K.; Perri, P.; Gambini, C.; Moretti, S.; et al. Genome analysis and gene expression profiling of neuroblastoma and ganglioneuroblastoma reveal differences between neuroblastic and Schwannian stromal cells. *J Pathol* **2005**, *207*, 346-357, doi:10.1002/path.1843.
  169. Maris, J.M.; Hogarty, M.D.; Bagatell, R.; Cohn, S.L. Neuroblastoma. *Lancet* **2007**, *369*, 2106-2120, doi:10.1016/S0140-6736(07)60983-0.
  170. Kaatsch, P.; Steliarova-Foucher, E.; Crocetti, E.; Magnani, C.; Spix, C.; Zambon, P.J.E.J.o.C. Time trends of cancer incidence in European children (1978-1997): report from the Automated Childhood Cancer Information System project. **2006**, *42*, 1961-1971.
  171. Hsieh, M.H.; Meng, M.V.; Walsh, T.J.; Matthay, K.K.; Baskin, L.S. Increasing incidence of neuroblastoma and potentially higher associated mortality of children from nonmetropolitan areas: analysis of the surveillance, epidemiology, and end results database. *J Pediatr Hematol Oncol* **2009**, *31*, 942-946, doi:10.1097/MPH.0b013e3181bcc809.
  172. Spix, C.; Pastore, G.; Sankila, R.; Stiller, C.A.; Steliarova-Foucher, E. Neuroblastoma incidence and survival in European children (1978-1997): report from the Automated Childhood Cancer Information System project. *Eur J Cancer* **2006**, *42*, 2081-2091, doi:10.1016/j.ejca.2006.05.008.
  173. Vo, K.T.; Matthay, K.K.; Neuhaus, J.; London, W.B.; Hero, B.; Ambros, P.F.; Nakagawara, A.; Miniati, D.; Wheeler, K.; Pearson, A.D.; et al. Clinical, biologic, and prognostic differences on the basis of primary tumor site in neuroblastoma: a report from the international neuroblastoma risk group project. *J Clin Oncol* **2014**, *32*, 3169-3176, doi:10.1200/JCO.2014.56.1621.
  174. Gatta, G.; Botta, L.; Rossi, S.; Aareleid, T.; Bielska-Lasota, M.; Clavel, J.; Dimitrova, N.; Jakab, Z.; Kaatsch, P.; Lacour, B.; et al. Childhood cancer survival in Europe 1999-2007: results of EUROCare-5--a population-based study. *Lancet Oncol* **2014**, *15*, 35-47, doi:10.1016/S1470-2045(13)70548-5.
  175. Su, Y.; Qin, H.; Chen, C.; Wang, S.; Zhang, S.; Zhang, D.; Jin, M.; Peng, Y.; He, L.; Wang, X.; et al. Treatment and outcomes of 1041 pediatric patients with neuroblastoma who received multidisciplinary care in China. *Pediatr Investig* **2020**, *4*, 157-167, doi:10.1002/ped4.12214.
  176. Newman, E.A.; Abdessalam, S.; Aldrink, J.H.; Austin, M.; Heaton, T.E.; Bruny, J.; Ehrlich, P.; Dasgupta, R.; Baertschiger, R.M.; Lautz, T.B.; et al. Update on neuroblastoma. *J Pediatr Surg* **2019**, *54*, 383-389, doi:10.1016/j.jpedsurg.2018.09.004.
  177. Ponzoni, M.; Bachetti, T.; Corrias, M.V.; Brignole, C.; Pastorino, F.; Calarco, E.; Bensa, V.; Giusto, E.; Ceccherini, I.; Perri, P. Recent advances in the developmental origin of neuroblastoma: an overview. *J Exp Clin Cancer Res* **2022**, *41*, 92, doi:10.1186/s13046-022-02281-w.
  178. Bhatt, S.; Diaz, R.; Trainor, P.A. Signals and switches in Mammalian neural crest cell differentiation. *Cold Spring Harb Perspect Biol* **2013**, *5*, doi:10.1101/cshperspect.a008326.
  179. Jiang, M.; Stanke, J.; Lahti, J.M. The connections between neural crest development and neuroblastoma. *Curr Top Dev Biol* **2011**, *94*, 77-127, doi:10.1016/B978-0-12-380916-2.00004-8.
  180. Bronner, M.E. Formation and migration of neural crest cells in the vertebrate embryo. *Histochem Cell Biol* **2012**, *138*, 179-186, doi:10.1007/s00418-012-0999-z.
  181. Tang, F.; Barbacioru, C.; Wang, Y.; Nordman, E.; Lee, C.; Xu, N.; Wang, X.; Bodeau, J.; Tuch, B.B.; Siddiqui, A.; et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* **2009**,

- 6, 377–382, doi:10.1038/nmeth.1315.
182. Jansky, S.; Sharma, A.K.; Korber, V.; Quintero, A.; Toprak, U.H.; Wecht, E.M.; Gartlgruber, M.; Greco, A.; Chomsky, E.; Grunewald, T.G.P.; et al. Single-cell transcriptomic analyses provide insights into the developmental origins of neuroblastoma. *Nat Genet* **2021**, *53*, 683–693, doi:10.1038/s41588-021-00806-1.
183. Bedoya-Reina, O.C.; Li, W.; Arceo, M.; Plescher, M.; Bullova, P.; Pui, H.; Kaucka, M.; Kharchenko, P.; Martinsson, T.; Holmberg, J.; et al. Single-nuclei transcriptomes from human adrenal gland reveal distinct cellular identities of low and high-risk neuroblastoma tumors. *Nat Commun* **2021**, *12*, 5309, doi:10.1038/s41467-021-24870-7.
184. Kildisiute, G.; Kholosy, W.M.; Young, M.D.; Roberts, K.; Elmentaite, R.; van Hooff, S.R.; Pacyna, C.N.; Khabirova, E.; Piapi, A.; Thevanesan, C.; et al. Tumor to normal single-cell mRNA comparisons reveal a pan-neuroblastoma cancer cell. *Sci Adv* **2021**, *7*, doi:10.1126/sciadv.abd3311.
185. Olsen, T.K.; Otte, J.; Mei, S.; Kameneva, P.; Björklund, Å.; Kryukov, E.; Hou, Z.; Johansson, A.; Sundström, E.; Martinsson, T.; et al. Malignant Schwann cell precursors mediate intratumoral plasticity in human neuroblastoma. *bioRxiv* **2020**, 2020.2005.2004.077057, doi:10.1101/2020.05.04.077057.
186. Kastriti, M.E.; Faure, L.; Von Ahsen, D.; Boudierlique, T.G.; Bostrom, J.; Solovieva, T.; Jackson, C.; Bronner, M.; Meijer, D.; Hadjab, S.; et al. Schwann cell precursors represent a neural crest-like state with biased multipotency. *EMBO J* **2022**, *41*, e108780, doi:10.15252/embj.2021108780.
187. Kameneva, P.; Artemov, A.V.; Kastriti, M.E.; Faure, L.; Olsen, T.K.; Otte, J.; Erickson, A.; Semsch, B.; Andersson, E.R.; Ratz, M.; et al. Single-cell transcriptomics of human embryos identifies multiple sympathoblast lineages with potential implications for neuroblastoma origin. *Nat Genet* **2021**, *53*, 694–706, doi:10.1038/s41588-021-00818-x.
188. Furlan, A.; Dyachuk, V.; Kastriti, M.E.; Calvo-Enrique, L.; Abdo, H.; Hadjab, S.; Chontorotzea, T.; Akkuratova, N.; Usoskin, D.; Kamenev, D.; et al. Multipotent peripheral glial cells generate neuroendocrine cells of the adrenal medulla. *Science* **2017**, *357*, doi:10.1126/science.aal3753.
189. Carbone, E.; Borges, R.; Eiden, L.E.; Garcia, A.G.; Hernandez-Cruz, A. Chromaffin Cells of the Adrenal Medulla: Physiology, Pharmacology, and Disease. *Compr Physiol* **2019**, *9*, 1443–1502, doi:10.1002/cphy.c190003.
190. Kameneva, P.; Kastriti, M.E.; Adameyko, I. Neuronal lineages derived from the nerve-associated Schwann cell precursors. *Cell Mol Life Sci* **2021**, *78*, 513–529, doi:10.1007/s00018-020-03609-5.
191. Bedoya-Reina, O.C.; Schlisio, S. Chromaffin Cells with Sympathoblast Signature: Too Similar to Keep Apart? *Cancer Cell* **2021**, *39*, 134–135, doi:10.1016/j.ccell.2020.12.009.
192. Thirant, C.; Peltier, A.; Durand, S.; Kramdi, A.; Louis-Brennetot, C.; Pierre-Eugene, C.; Gautier, M.; Costa, A.; Grellet, A.; Zaidi, S.; et al. Reversible transitions between noradrenergic and mesenchymal tumor identities define cell plasticity in neuroblastoma. *Nat Commun* **2023**, *14*, 2575, doi:10.1038/s41467-023-38239-5.
193. van Groningen, T.; Koster, J.; Valentijn, L.J.; Zwijnenburg, D.A.; Akogul, N.; Hasselt, N.E.; Broekmans, M.; Haneveld, F.; Nowakowska, N.E.; Bras, J.; et al. Neuroblastoma is composed of two super-enhancer-associated differentiation states. *Nat Genet* **2017**, *49*, 1261–1266, doi:10.1038/ng.3899.
194. Westerhout, E.M.; Hamdi, M.; Stroeken, P.; Nowakowska, N.E.; Lakeman, A.; van Arkel, J.; Hasselt, N.E.; Bleijlevens, B.; Akogul, N.; Haneveld, F.; et al. Mesenchymal-Type Neuroblastoma Cells Escape ALK Inhibitors. *Cancer Res* **2022**, *82*, 484–496, doi:10.1158/0008-5472.CAN-21-1621.
195. van Groningen, T.; Akogul, N.; Westerhout, E.M.; Chan, A.; Hasselt, N.E.; Zwijnenburg, D.A.; Broekmans, M.; Stroeken, P.; Haneveld, F.; Hooijer, G.K.J.; et al. A NOTCH feed-forward loop drives reprogramming from adrenergic to mesenchymal state in neuroblastoma. *Nat Commun* **2019**, *10*, 1530, doi:10.1038/s41467-019-09470-w.
196. Manas, A.; Aaltonen, K.; Andersson, N.; Hansson, K.; Adamska, A.; Seger, A.; Yasui, H.; van den Bos, H.; Radke, K.; Esfandyari, J.; et al. Clinically relevant treatment of PDX models reveals patterns of neuroblastoma chemoresistance. *Sci Adv* **2022**, *8*, eabq4617, doi:10.1126/sciadv.abq4617.

197. Qi, S.; Zheng, J.; Zhu, H.; Yang, L.; Xiao, X. Identification of neuroblastoma stem cells by characterization of side population cells in the human neuroblastoma SK-N-SH cell line. *J Pediatr Surg* **2010**, *45*, 2305–2311, doi:10.1016/j.jpedsurg.2010.08.022.
198. Biedler, J.L.; Roffler-Tarlov, S.; Schachner, M.; Freedman, L.S. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res* **1978**, *38*, 3751–3757.
199. Brodeur, G.M.; Seeger, R.C.; Barrett, A.; Berthold, F.; Castleberry, R.P.; D'Angio, G.; De Bernardi, B.; Evans, A.E.; Favrot, M.; Freeman, A.I.; et al. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. *J Clin Oncol* **1988**, *6*, 1874–1881, doi:10.1200/JCO.1988.6.12.1874.
200. Brodeur, G.M.; Pritchard, J.; Berthold, F.; Carlsen, N.L.; Castel, V.; Castleberry, R.P.; De Bernardi, B.; Evans, A.E.; Favrot, M.; Hedborg, F.; et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* **1993**, *11*, 1466–1477, doi:10.1200/JCO.1993.11.8.1466.
201. Kawano, A.; Hazard, F.K.; Chiu, B.; Naranjo, A.; LaBarre, B.; London, W.B.; Hogarty, M.D.; Cohn, S.L.; Maris, J.M.; Park, J.R.; et al. Stage 4S Neuroblastoma: Molecular, Histologic, and Immunohistochemical Characteristics and Presence of 2 Distinct Patterns of MYCN Protein Overexpression—A Report From the Children's Oncology Group. *Am J Surg Pathol* **2021**, *45*, 1075–1081, doi:10.1097/PAS.0000000000001647.
202. Cohn, S.L.; Pearson, A.D.; London, W.B.; Monclair, T.; Ambros, P.F.; Brodeur, G.M.; Faldut, A.; Hero, B.; Ichihara, T.; Machin, D.; et al. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J Clin Oncol* **2009**, *27*, 289–297, doi:10.1200/JCO.2008.16.6785.
203. Lucena, J.N.; Alves, M.T.S.; Abib, S.C.V.; Souza, G.O.; Neves, R.P.C.; Caran, E.M.M. Clinical and Epidemiological Characteristics and Survival Outcomes of Children with Neuroblastoma: 21 Years of Experience at the Instituto De Oncologia Pediatrica, in Sao Paulo, Brazil. *Rev Paul Pediatr* **2018**, *36*, 254–260, doi:10.1590/1984-0462/2018;36;3;00007.
204. Monclair, T.; Brodeur, G.M.; Ambros, P.F.; Brisse, H.J.; Cecchetto, G.; Holmes, K.; Kaneko, M.; London, W.B.; Matthay, K.K.; Nuchtern, J.G.; et al. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. *J Clin Oncol* **2009**, *27*, 298–303, doi:10.1200/JCO.2008.16.6876.
205. Irwin, M.S.; Naranjo, A.; Zhang, F.F.; Cohn, S.L.; London, W.B.; Gastier-Foster, J.M.; Ramirez, N.C.; Pfau, R.; Reshmi, S.; Wagner, E.; et al. Revised Neuroblastoma Risk Classification System: A Report From the Children's Oncology Group. *J Clin Oncol* **2021**, *39*, 3229–3241, doi:10.1200/JCO.21.00278.
206. Ambros, P.F.; Ambros, I.M.; Brodeur, G.M.; Haber, M.; Khan, J.; Nakagawara, A.; Schleiermacher, G.; Speleman, F.; Spitz, R.; London, W.B.; et al. International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. *Br J Cancer* **2009**, *100*, 1471–1482, doi:10.1038/sj.bjc.6605014.
207. Liang, W.H.; Federico, S.M.; London, W.B.; Naranjo, A.; Irwin, M.S.; Volchenboum, S.L.; Cohn, S.L. Tailoring Therapy for Children With Neuroblastoma on the Basis of Risk Group Classification: Past, Present, and Future. *JCO Clin Cancer Inform* **2020**, *4*, 895–905, doi:10.1200/CCI.20.00074.
208. Ritenour, L.E.; Randall, M.P.; Bosse, K.R.; Diskin, S.J. Genetic susceptibility to neuroblastoma: current knowledge and future directions. *Cell Tissue Res* **2018**, *372*, 287–307, doi:10.1007/s00441-018-2820-3.
209. Bagatell, R.; Cohn, S.L. Genetic discoveries and treatment advances in neuroblastoma. *Curr Opin Pediatr* **2016**, *28*, 19–25, doi:10.1097/MOP.0000000000000296.
210. Swartling, F.J.; Grimmer, M.R.; Hackett, C.S.; Northcott, P.A.; Fan, Q.W.; Goldenberg, D.D.; Lau, J.; Masic, S.; Nguyen, K.; Yakovenko, S.; et al. Pleiotropic role for MYCN in medulloblastoma. *Genes Dev* **2010**, *24*, 1059–1072, doi:10.1101/gad.1907510.
211. Aldosari, N.; Bigner, S.H.; Burger, P.C.; Becker, L.; Kepner, J.L.; Friedman, H.S.; McLendon, R.E. MYCC and MYCN oncogene amplification in medulloblastoma. A fluorescence in situ hybridization study on paraffin sections from the Children's Oncology Group. *Arch Pathol Lab Med* **2002**, *126*, 540–544, doi:10.5858/2002-126-0540-MAMOA1.

212. Williams, R.D.; Chagtai, T.; Alcaide-German, M.; Apps, J.; Wegert, J.; Popov, S.; Vujanic, G.; van Tinteren, H.; van den Heuvel-Eibrink, M.M.; Kool, M.; et al. Multiple mechanisms of MYCN dysregulation in Wilms tumour. *Oncotarget* **2015**, *6*, 7232–7243, doi:10.18632/oncotarget.3377.
213. Mosquera, J.M.; Beltran, H.; Park, K.; MacDonald, T.Y.; Robinson, B.D.; Tagawa, S.T.; Perner, S.; Bismar, T.A.; Erbersdobler, A.; Dhir, R.; et al. Concurrent AURKA and MYCN gene amplifications are harbingers of lethal treatment-related neuroendocrine prostate cancer. *Neoplasia* **2013**, *15*, 1–10, doi:10.1593/neo.121550.
214. Nau, M.M.; Brooks, B.J., Jr.; Carney, D.N.; Gazdar, A.F.; Battey, J.F.; Sausville, E.A.; Minna, J.D. Human small-cell lung cancers show amplification and expression of the N-myc gene. *Proc Natl Acad Sci U S A* **1986**, *83*, 1092–1096, doi:10.1073/pnas.83.4.1092.
215. Schwab, M.; Alitalo, K.; Klempnauer, K.H.; Varmus, H.E.; Bishop, J.M.; Gilbert, F.; Brodeur, G.; Goldstein, M.; Trent, J. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* **1983**, *305*, 245–248, doi:10.1038/305245a0.
216. Puissant, A.; Frumm, S.M.; Alexe, G.; Bassil, C.F.; Qi, J.; Chanthery, Y.H.; Nekritz, E.A.; Zeid, R.; Gustafson, W.C.; Greninger, P.; et al. Targeting MYCN in neuroblastoma by BET bromodomain inhibition. *Cancer Discov* **2013**, *3*, 308–323, doi:10.1158/2159-8290.CD-12-0418.
217. Goodman, L.A.; Liu, B.C.; Thiele, C.J.; Schmidt, M.L.; Cohn, S.L.; Yamashiro, J.M.; Pai, D.S.; Ikegaki, N.; Wada, R.K. Modulation of N-myc expression alters the invasiveness of neuroblastoma. *Clin Exp Metastasis* **1997**, *15*, 130–139, doi:10.1023/a:1018448710006.
218. Noujaim, D.; van Golen, C.M.; van Golen, K.L.; Grauman, A.; Feldman, E.L. N-Myc and Bcl-2 coexpression induces MMP-2 secretion and activation in human neuroblastoma cells. *Oncogene* **2002**, *21*, 4549–4557, doi:10.1038/sj.onc.1205552.
219. Ma, L.; Young, J.; Prabhala, H.; Pan, E.; Mestdag, P.; Muth, D.; Teruya-Feldstein, J.; Reinhardt, F.; Onder, T.T.; Valastyan, S.; et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* **2010**, *12*, 247–256, doi:10.1038/ncb2024.
220. Otte, J.; Dyberg, C.; Pepich, A.; Johnsen, J.I. MYCN Function in Neuroblastoma Development. *Front Oncol* **2020**, *10*, 624079, doi:10.3389/fonc.2020.624079.
221. Zhu, S.; Lee, J.S.; Guo, F.; Shin, J.; Perez-Atayde, A.R.; Kutok, J.L.; Rodig, S.J.; Neuberg, D.S.; Helman, D.; Feng, H.; et al. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. *Cancer Cell* **2012**, *21*, 362–373, doi:10.1016/j.ccr.2012.02.010.
222. Lemmon, M.A.; Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **2010**, *141*, 1117–1134, doi:10.1016/j.cell.2010.06.011.
223. Janoueix-Lerosey, I.; Lequin, D.; Brugieres, L.; Ribeiro, A.; de Pontual, L.; Combaret, V.; Raynal, V.; Puisieux, A.; Schleiermacher, G.; Pierron, G.; et al. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* **2008**, *455*, 967–970, doi:10.1038/nature07398.
224. Morris, S.W.; Kirstein, M.N.; Valentine, M.B.; Dittmer, K.; Shapiro, D.N.; Look, A.T.; Saltman, D.L. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* **1995**, *267*, 316–317, doi:10.1126/science.267.5196.316-b.
225. Mossé, Y.P.; Laudenslager, M.; Longo, L.; Cole, K.A.; Wood, A.; Attiyeh, E.F.; Laquaglia, M.J.; Sennett, R.; Lynch, J.E.; Perri, P.J.N. Identification of ALK as a major familial neuroblastoma predisposition gene. **2008**, *455*, 930–935.
226. Kiyonari, S.; Kadomatsu, K.J.E.o.o.d.d. Neuroblastoma models for insights into tumorigenesis and new therapies. **2015**, *10*, 53–62.
227. Javanmardi, N.; Fransson, S.; Djos, A.; Sjöberg, R.M.; Nilsson, S.; Truve, K.; Kogner, P.; Martinsson, T. Low Frequency ALK Hotspots Mutations In Neuroblastoma Tumours Detected By Ultra-deep Sequencing: Implications For ALK Inhibitor Treatment. *Sci Rep* **2019**, *9*, 2199, doi:10.1038/s41598-018-37240-z.
228. Bellini, A.; Potschger, U.; Bernard, V.; Lapouble, E.; Baulande, S.; Ambros, P.F.; Auger, N.; Beiske, K.; Bernkopf, M.; Betts, D.R.; et al. Frequency and Prognostic Impact of ALK Amplifications and Mutations in the European Neuroblastoma Study Group (SIOPEN) High-Risk Neuroblastoma Trial (HR-NBL1). *J Clin Oncol* **2021**, *39*, 3377–3390, doi:10.1200/JCO.21.00086.

229. Rosswog, C.; Fassunke, J.; Ernst, A.; Schomig-Markiefka, B.; Merkelbach-Bruse, S.; Bartenhagen, C.; Cartolano, M.; Ackermann, S.; Theissen, J.; Blattner-Johnson, M.; et al. Genomic ALK alterations in primary and relapsed neuroblastoma. *Br J Cancer* **2023**, *128*, 1559-1571, doi:10.1038/s41416-023-02208-y.
230. Hasan, M.K.; Nafady, A.; Takatori, A.; Kishida, S.; Ohira, M.; Suenaga, Y.; Hossain, S.; Akter, J.; Ogura, A.; Nakamura, Y.; et al. ALK is a MYCN target gene and regulates cell migration and invasion in neuroblastoma. *Sci Rep* **2013**, *3*, 3450, doi:10.1038/srep03450.
231. Berry, T.; Luther, W.; Bhatnagar, N.; Jamin, Y.; Poon, E.; Sanda, T.; Pei, D.; Sharma, B.; Vetharoy, W.R.; Hallsworth, A.; et al. The ALK(F1174L) mutation potentiates the oncogenic activity of MYCN in neuroblastoma. *Cancer Cell* **2012**, *22*, 117-130, doi:10.1016/j.ccr.2012.06.001.
232. Mosse, Y.P.; Laudenslager, M.; Khazi, D.; Carlisle, A.J.; Winter, C.L.; Rappaport, E.; Maris, J.M. Germline PHOX2B mutation in hereditary neuroblastoma. *Am J Hum Genet* **2004**, *75*, 727-730, doi:10.1086/424530.
233. Dubreuil, V.; Hirsch, M.R.; Pattyn, A.; Brunet, J.F.; Goridis, C. The Phox2b transcription factor coordinately regulates neuronal cell cycle exit and identity. *Development* **2000**, *127*, 5191-5201, doi:10.1242/dev.127.23.5191.
234. Yang, C.; Kim, H.S.; Seo, H.; Kim, C.H.; Brunet, J.F.; Kim, K.S. Paired-like homeodomain proteins, Phox2a and Phox2b, are responsible for noradrenergic cell-specific transcription of the dopamine beta-hydroxylase gene. *J Neurochem* **1998**, *71*, 1813-1826, doi:10.1046/j.1471-4159.1998.71051813.x.
235. Raabe, E.H.; Laudenslager, M.; Winter, C.; Wasserman, N.; Cole, K.; LaQuaglia, M.; Maris, D.J.; Mosse, Y.P.; Maris, J.M. Prevalence and functional consequence of PHOX2B mutations in neuroblastoma. *Oncogene* **2008**, *27*, 469-476, doi:10.1038/sj.onc.1210659.
236. Naftali, O.; Maman, S.; Meshel, T.; Sagi-Assif, O.; Ginat, R.; Witz, I.P. PHOX2B is a suppressor of neuroblastoma metastasis. *Oncotarget* **2016**, *7*, 10627-10637, doi:10.18632/oncotarget.7056.
237. Stutterheim, J.; Gerritsen, A.; Zappeij-Kannegieter, L.; Kleijn, I.; Dee, R.; Hooft, L.; van Noesel, M.M.; Bierings, M.; Berthold, F.; Versteeg, R.; et al. PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. *J Clin Oncol* **2008**, *26*, 5443-5449, doi:10.1200/JCO.2007.13.6531.
238. Fan, H.; Xing, T.; Hong, H.; Duan, C.; Zhao, W.; Zhao, Q.; Wang, X.; Huang, C.; Zhu, S.; Jin, M.; et al. The expression of PHOX2B in bone marrow and peripheral blood predicts adverse clinical outcome in non-high-risk neuroblastoma. *Pediatr Hematol Oncol* **2022**, *39*, 343-356, doi:10.1080/08880018.2021.1995090.
239. Picketts, D.J.; Higgs, D.R.; Bachoo, S.; Blake, D.J.; Quarrell, O.W.; Gibbons, R.J.J.H.m.g. ATRX encodes a novel member of the SNF2 family of proteins: mutations point to a common mechanism underlying the ATR-X syndrome. **1996**, *5*, 1899-1907.
240. Gibbons, R.J.; Suthers, G.K.; Wilkie, A.O.; Buckle, V.J.; Higgs, D.R. X-linked alpha-thalassemia/mental retardation (ATR-X) syndrome: localization to Xq12-q21.31 by X inactivation and linkage analysis. *Am J Hum Genet* **1992**, *51*, 1136-1149.
241. Zeineldin, M.; Federico, S.; Chen, X.; Fan, Y.; Xu, B.; Stewart, E.; Zhou, X.; Jeon, J.; Griffiths, L.; Nguyen, R.; et al. MYCN amplification and ATRX mutations are incompatible in neuroblastoma. *Nat Commun* **2020**, *11*, 913, doi:10.1038/s41467-020-14682-6.
242. Cheung, N.K.; Zhang, J.; Lu, C.; Parker, M.; Bahrami, A.; Tickoo, S.K.; Heguy, A.; Pappo, A.S.; Federico, S.; Dalton, J.; et al. Association of age at diagnosis and genetic mutations in patients with neuroblastoma. *JAMA* **2012**, *307*, 1062-1071, doi:10.1001/jama.2012.228.
243. Peifer, M.; Hertwig, F.; Roels, F.; Dreidax, D.; Gartlgruber, M.; Menon, R.; Kramer, A.; Roncaioli, J.L.; Sand, F.; Heuckmann, J.M.; et al. Telomerase activation by genomic rearrangements in high-risk neuroblastoma. *Nature* **2015**, *526*, 700-704, doi:10.1038/nature14980.
244. Weinrich, S.L.; Pruzan, R.; Ma, L.; Ouellette, M.; Tesmer, V.M.; Holt, S.E.; Bodnar, A.G.; Lichtsteiner, S.; Kim, N.W.; Trager, J.B.; et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nat Genet* **1997**, *17*, 498-502, doi:10.1038/ng1297-498.
245. Valentijn, L.J.; Koster, J.; Zwijnenburg, D.A.; Hasselt, N.E.; van Sluis, P.; Volckmann, R.; van Noesel,

- M.M.; George, R.E.; Tytgat, G.A.; Molenaar, J.J.; et al. TERT rearrangements are frequent in neuroblastoma and identify aggressive tumors. *Nat Genet* **2015**, *47*, 1411-1414, doi:10.1038/ng.3438.
246. Fong, C.T.; Dracopoli, N.C.; White, P.S.; Merrill, P.T.; Griffith, R.C.; Housman, D.E.; Brodeur, G.M. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci U S A* **1989**, *86*, 3753-3757, doi:10.1073/pnas.86.10.3753.
  247. Ohira, M.; Kageyama, H.; Mihara, M.; Furuta, S.; Machida, T.; Shishikura, T.; Takayasu, H.; Islam, A.; Nakamura, Y.; Takahashi, M.; et al. Identification and characterization of a 500-kb homozygously deleted region at 1p36.2-p36.3 in a neuroblastoma cell line. *Oncogene* **2000**, *19*, 4302-4307, doi:10.1038/sj.onc.1203786.
  248. Fujita, T.; Igarashi, J.; Okawa, E.R.; Gotoh, T.; Manne, J.; Kolla, V.; Kim, J.; Zhao, H.; Pawel, B.R.; London, W.B.; et al. CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. *J Natl Cancer Inst* **2008**, *100*, 940-949, doi:10.1093/jnci/djn176.
  249. Mosse, Y.P.; Laudenslager, M.; Longo, L.; Cole, K.A.; Wood, A.; Attiyeh, E.F.; Laquaglia, M.J.; Sennett, R.; Lynch, J.E.; Perri, P.; et al. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* **2008**, *455*, 930-935, doi:10.1038/nature07261.
  250. Schlisio, S.; Kenchappa, R.S.; Vredeveld, L.C.; George, R.E.; Stewart, R.; Greulich, H.; Shahriari, K.; Nguyen, N.V.; Pigny, P.; Dahia, P.L.; et al. The kinesin KIF1Bbeta acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. *Genes Dev* **2008**, *22*, 884-893, doi:10.1101/gad.1648608.
  251. Lastowska, M.; Roberts, P.; Pearson, A.D.; Lewis, I.; Wolstenholme, J.; Bown, N. Promiscuous translocations of chromosome arm 17q in human neuroblastomas. *Genes Chromosomes Cancer* **1997**, *19*, 143-149.
  252. Meddeb, M.; Danglot, G.; Chudoba, I.; Venuat, A.M.; Benard, J.; Avet-Loiseau, H.; Vasseur, B.; Le Paslier, D.; Terrier-Lacombe, M.J.; Hartmann, O.; et al. Additional copies of a 25 Mb chromosomal region originating from 17q23.1-17qter are present in 90% of high-grade neuroblastomas. *Genes Chromosomes Cancer* **1996**, *17*, 156-165, doi:10.1002/(SICI)1098-2264(199611)17:3<156::AID-GCC3>3.0.CO;2-3.
  253. Bown, N.; Cotterill, S.; Lastowska, M.; O'Neill, S.; Pearson, A.D.; Plantaz, D.; Meddeb, M.; Danglot, G.; Brinkschmidt, C.; Christiansen, H.; et al. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med* **1999**, *340*, 1954-1961, doi:10.1056/NEJM199906243402504.
  254. Bown, N.; Lastowska, M.; Cotterill, S.; O'Neill, S.; Ellershaw, C.; Roberts, P.; Lewis, I.; Pearson, A.D.; Group, U.K.C.C.; the, U.K.C.s.C.S.G. 17q gain in neuroblastoma predicts adverse clinical outcome. U.K. Cancer Cytogenetics Group and the U.K. Children's Cancer Study Group. *Med Pediatr Oncol* **2001**, *36*, 14-19, doi:10.1002/1096-911X(20010101)36:1<14::AID-MPO1005>3.0.CO;2-G.
  255. Lastowska, M.; Cotterill, S.; Pearson, A.D.; Roberts, P.; McGuckin, A.; Lewis, I.; Bown, N. Gain of chromosome arm 17q predicts unfavourable outcome in neuroblastoma patients. U.K. Children's Cancer Study Group and the U.K. Cancer Cytogenetics Group. *Eur J Cancer* **1997**, *33*, 1627-1633, doi:10.1016/s0959-8049(97)00282-7.
  256. Stallings, R.L.; Nair, P.; Maris, J.M.; Catchpoole, D.; McDermott, M.; O'Meara, A.; Breatnach, F.J.C.r. High-resolution analysis of chromosomal breakpoints and genomic instability identifies PTPRD as a candidate tumor suppressor gene in neuroblastoma. **2006**, *66*, 3673-3680.
  257. Guo, C.; White, P.S.; Weiss, M.J.; Hogarty, M.D.; Thompson, P.M.; Stram, D.O.; Gerbing, R.; Matthay, K.K.; Seeger, R.C.; Brodeur, G.M.; et al. Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. *Oncogene* **1999**, *18*, 4948-4957, doi:10.1038/sj.onc.1202887.
  258. Spitz, R.; Hero, B.; Simon, T.; Berthold, F. Loss in chromosome 11q identifies tumors with increased risk for metastatic relapses in localized and 4S neuroblastoma. *Clin Cancer Res* **2006**, *12*, 3368-3373, doi:10.1158/1078-0432.CCR-05-2495.
  259. Michels, E.; Hoebeeck, J.; De Preter, K.; Schramm, A.; Brichard, B.; De Paepe, A.; Eggert, A.; Laureys, G.; Vandesompele, J.; Speleman, F.J.B.c. CADM1 is a strong neuroblastoma candidate gene that maps within a 3.72 Mb critical region of loss on 11q23. **2008**, *8*, 1-9.

260. Javanmardi, N.; Fransson, S.; Djos, A.; Umapathy, G.; Ostensson, M.; Milosevic, J.; Borenas, M.; Hallberg, B.; Kogner, P.; Martinsson, T.; et al. Analysis of ALK, MYCN, and the ALK ligand ALKAL2 (FAM150B/AUGalpha) in neuroblastoma patient samples with chromosome arm 2p rearrangements. *Genes Chromosomes Cancer* **2019**, doi:10.1002/gcc.22790.
261. Jeison, M.; Ash, S.; Halevy-Berko, G.; Mardoukh, J.; Luria, D.; Avigad, S.; Feinberg-Gorenshtein, G.; Goshen, Y.; Hertz, G.; Kapelushnik, J.; et al. 2p24 Gain region harboring MYCN gene compared with MYCN amplified and nonamplified neuroblastoma: biological and clinical characteristics. *Am J Pathol* **2010**, *176*, 2616–2625, doi:10.2353/ajpath.2010.090624.
262. Nuchtern, J.G.; London, W.B.; Barnewolt, C.E.; Naranjo, A.; McGrady, P.W.; Geiger, J.D.; Diller, L.; Schmidt, M.L.; Maris, J.M.; Cohn, S.L.; et al. A prospective study of expectant observation as primary therapy for neuroblastoma in young infants: a Children's Oncology Group study. *Ann Surg* **2012**, *256*, 573–580, doi:10.1097/SLA.0b013e31826cbbbd.
263. Smith, V.; Foster, J. High-Risk Neuroblastoma Treatment Review. *Children (Basel)* **2018**, *5*, doi:10.3390/children5090114.
264. Matthay, K.K.; Reynolds, C.P.; Seeger, R.C.; Shimada, H.; Adkins, E.S.; Haas-Kogan, D.; Gerbing, R.B.; London, W.B.; Villablanca, J.G. Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. *J Clin Oncol* **2009**, *27*, 1007–1013, doi:10.1200/JCO.2007.13.8925.
265. Pearson, A.D.; Pinkerton, C.R.; Lewis, I.J.; Imeson, J.; Ellershaw, C.; Machin, D.; European Neuroblastoma Study, G.; Children's, C.; Leukaemia, G. High-dose rapid and standard induction chemotherapy for patients aged over 1 year with stage 4 neuroblastoma: a randomised trial. *Lancet Oncol* **2008**, *9*, 247–256, doi:10.1016/S1470-2045(08)70069-X.
266. Garaventa, A.; Poetschger, U.; Valteau-Couanet, D.; Luksch, R.; Castel, V.; Elliott, M.; Ash, S.; Chan, G.C.F.; Laureys, G.; Beck-Popovic, M.; et al. Randomized Trial of Two Induction Therapy Regimens for High-Risk Neuroblastoma: HR-NBL1.5 International Society of Pediatric Oncology European Neuroblastoma Group Study. *J Clin Oncol* **2021**, *39*, 2552–2563, doi:10.1200/JCO.20.03144.
267. Matthay, K.K.; Villablanca, J.G.; Seeger, R.C.; Stram, D.O.; Harris, R.E.; Ramsay, N.K.; Swift, P.; Shimada, H.; Black, C.T.; Brodeur, G.M.; et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N Engl J Med* **1999**, *341*, 1165–1173, doi:10.1056/NEJM199910143411601.
268. Berthold, F.; Boos, J.; Burdach, S.; Erttmann, R.; Henze, G.; Hermann, J.; Klingebiel, T.; Kremens, B.; Schilling, F.H.; Schrappe, M.; et al. Myeloablative megatherapy with autologous stem-cell rescue versus oral maintenance chemotherapy as consolidation treatment in patients with high-risk neuroblastoma: a randomised controlled trial. *Lancet Oncol* **2005**, *6*, 649–658, doi:10.1016/S1470-2045(05)70291-6.
269. Pritchard, J.; Cotterill, S.J.; Germond, S.M.; Imeson, J.; de Kraker, J.; Jones, D.R. High dose melphalan in the treatment of advanced neuroblastoma: results of a randomised trial (ENSG-1) by the European Neuroblastoma Study Group. *Pediatr Blood Cancer* **2005**, *44*, 348–357, doi:10.1002/pbc.20219.
270. Ladenstein, R.; Potschger, U.; Pearson, A.D.J.; Brock, P.; Luksch, R.; Castel, V.; Yaniv, I.; Papadakis, V.; Laureys, G.; Malis, J.; et al. Busulfan and melphalan versus carboplatin, etoposide, and melphalan as high-dose chemotherapy for high-risk neuroblastoma (HR-NBL1/SIOPEN): an international, randomised, multi-arm, open-label, phase 3 trial. *Lancet Oncol* **2017**, *18*, 500–514, doi:10.1016/S1470-2045(17)30070-0.
271. Park, J.R.; Kreissman, S.G.; London, W.B.; Naranjo, A.; Cohn, S.L.; Hogarty, M.D.; Tenney, S.C.; Haas-Kogan, D.; Shaw, P.J.; Kravka, J.M.; et al. Effect of Tandem Autologous Stem Cell Transplant vs Single Transplant on Event-Free Survival in Patients With High-Risk Neuroblastoma: A Randomized Clinical Trial. *JAMA* **2019**, *322*, 746–755, doi:10.1001/jama.2019.11642.
272. Desai, A.V.; Gilman, A.L.; Ozkaynak, M.F.; Naranjo, A.; London, W.B.; Tenney, S.C.; Diccianni, M.;

- Hank, J.A.; Parisi, M.T.; Shulkin, B.L.; et al. Outcomes Following GD2-Directed Postconsolidation Therapy for Neuroblastoma After Cessation of Random Assignment on ANBL0032: A Report From the Children's Oncology Group. *J Clin Oncol* **2022**, *40*, 4107-4118, doi:10.1200/JCO.21.02478.
273. Peinemann, F.; van Dalen, E.C.; Enk, H.; Berthold, F. Retinoic acid postconsolidation therapy for high-risk neuroblastoma patients treated with autologous haematopoietic stem cell transplantation. *Cochrane Database Syst Rev* **2017**, *8*, CD010685, doi:10.1002/14651858.CD010685.pub3.
  274. Yu, A.L.; Gilman, A.L.; Ozkaynak, M.F.; London, W.B.; Kreissman, S.G.; Chen, H.X.; Smith, M.; Anderson, B.; Villablanca, J.G.; Matthay, K.K.; et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med* **2010**, *363*, 1324-1334, doi:10.1056/NEJMoa0911123.
  275. Berlanga, P.; Canete, A.; Castel, V. Advances in emerging drugs for the treatment of neuroblastoma. *Expert Opin Emerg Drugs* **2017**, *22*, 63-75, doi:10.1080/14728214.2017.1294159.
  276. Mazloom, A.; Louis, C.U.; Nuchtern, J.; Kim, E.; Russell, H.; Allen-Rhoades, W.; Krance, R.; Paulino, A.C. Radiation therapy to the primary and postinduction chemotherapy MIBG-avid sites in high-risk neuroblastoma. *Int J Radiat Oncol Biol Phys* **2014**, *90*, 858-862, doi:10.1016/j.ijrobp.2014.07.019.
  277. Qayed, M.; Chiang, K.Y.; Ricketts, R.; Alazraki, A.; Tahvildari, A.; Haight, A.; George, B.; Esiashvili, N.; Katzenstein, H.M. Tandem stem cell rescue as consolidation therapy for high-risk neuroblastoma. *Pediatr Blood Cancer* **2012**, *58*, 448-452, doi:10.1002/pbc.23155.
  278. Zhou, M.J.; Doral, M.Y.; DuBois, S.G.; Villablanca, J.G.; Yanik, G.A.; Matthay, K.K. Different outcomes for relapsed versus refractory neuroblastoma after therapy with (131I)-metaiodobenzylguanidine ((131I)-MIBG). *Eur J Cancer* **2015**, *51*, 2465-2472, doi:10.1016/j.ejca.2015.07.023.
  279. Park, J.R.; Kreissman, S.G.; London, W.B.; Naranjo, A.; Cohn, S.L.; Hogarty, M.D.; Tenney, S.C.; Haas-Kogan, D.; Shaw, P.J.; Kravaka, J.M.J.J. Effect of tandem autologous stem cell transplant vs single transplant on event-free survival in patients with high-risk neuroblastoma: a randomized clinical trial. **2019**, *322*, 746-755.
  280. De Ioris, M.A.; Crocoli, A.; Contoli, B.; Garganese, M.C.; Natali, G.; Toma, P.; Jenkner, A.; Boldrini, R.; De Pasquale, M.D.; Milano, G.M.; et al. Local control in metastatic neuroblastoma in children over 1 year of age. *BMC Cancer* **2015**, *15*, 79, doi:10.1186/s12885-015-1082-7.
  281. Norman, A.W.; Mizwicki, M.T.; Norman, D.P. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov* **2004**, *3*, 27-41, doi:10.1038/nrd1283.
  282. Laudet, V. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J Mol Endocrinol* **1997**, *19*, 207-226, doi:10.1677/jme.0.0190207.
  283. Zhang, Z.; Burch, P.E.; Cooney, A.J.; Lanz, R.B.; Pereira, F.A.; Wu, J.; Gibbs, R.A.; Weinstock, G.; Wheeler, D.A. Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome. *Genome Res* **2004**, *14*, 580-590, doi:10.1101/gr.2160004.
  284. Olivares, A.M.; Moreno-Ramos, O.A.; Haider, N.B. Role of Nuclear Receptors in Central Nervous System Development and Associated Diseases. *J Exp Neurosci* **2015**, *9*, 93-121, doi:10.4137/JEN.S25480.
  285. Stergiopoulos, A.; Politis, P.K. The role of nuclear receptors in controlling the fine balance between proliferation and differentiation of neural stem cells. *Arch Biochem Biophys* **2013**, *534*, 27-37, doi:10.1016/j.abb.2012.09.009.
  286. Bonkhoff, H.; Berges, R. The evolving role of oestrogens and their receptors in the development and progression of prostate cancer. *Eur Urol* **2009**, *55*, 533-542, doi:10.1016/j.eururo.2008.10.035.
  287. Horvath, L.G.; Henshall, S.M.; Lee, C.S.; Head, D.R.; Quinn, D.I.; Makela, S.; Delprado, W.; Golovsky, D.; Brenner, P.C.; O'Neill, G.; et al. Frequent loss of estrogen receptor-beta expression in prostate

- cancer. *Cancer Res* **2001**, *61*, 5331-5335.
288. Dey, P.; Jonsson, P.; Hartman, J.; Williams, C.; Strom, A.; Gustafsson, J.A. Estrogen receptors beta1 and beta2 have opposing roles in regulating proliferation and bone metastasis genes in the prostate cancer cell line PC3. *Mol Endocrinol* **2012**, *26*, 1991-2003, doi:10.1210/me.2012.1227.
  289. Ma, Z.Q.; Spreafico, E.; Pollio, G.; Santagati, S.; Conti, E.; Cattaneo, E.; Maggi, A. Activated estrogen receptor mediates growth arrest and differentiation of a neuroblastoma cell line. *Proc Natl Acad Sci U S A* **1993**, *90*, 3740-3744, doi:10.1073/pnas.90.8.3740.
  290. Teppola, H.; Sarkanen, J.-R.; Jalonen, T.O.; Linne, M.-L.J.N.r. Morphological differentiation towards neuronal phenotype of SH-SY5Y neuroblastoma cells by estradiol, retinoic acid and cholesterol. **2016**, *41*, 731-747.
  291. Loven, J.; Zinin, N.; Wahlstrom, T.; Muller, I.; Brodin, P.; Fredlund, E.; Ribacke, U.; Pivarcsi, A.; Pahlman, S.; Henriksson, M. MYCN-regulated microRNAs repress estrogen receptor-alpha (ESR1) expression and neuronal differentiation in human neuroblastoma. *Proc Natl Acad Sci U S A* **2010**, *107*, 1553-1558, doi:10.1073/pnas.0913517107.
  292. Dzieran, J.; Rodriguez Garcia, A.; Westermarck, U.K.; Henley, A.B.; Eyre Sanchez, E.; Trager, C.; Johansson, H.J.; Lehtio, J.; Arsenian-Henriksson, M. MYCN-amplified neuroblastoma maintains an aggressive and undifferentiated phenotype by deregulation of estrogen and NGF signaling. *Proc Natl Acad Sci U S A* **2018**, *115*, E1229-E1238, doi:10.1073/pnas.1710901115.
  293. Ribeiro, D.; Klargvist, M.D.R.; Westermarck, U.K.; Oliynyk, G.; Dzieran, J.; Kock, A.; Savatier Banares, C.; Hertwig, F.; Johnsen, J.I.; Fischer, M.; et al. Regulation of Nuclear Hormone Receptors by MYCN-Driven miRNAs Impacts Neural Differentiation and Survival in Neuroblastoma Patients. *Cell Rep* **2016**, *16*, 979-993, doi:10.1016/j.celrep.2016.06.052.
  294. Han, G.; Chang, B.; Connor, M.J.; Sidell, N. Enhanced potency of 9-cis versus all-trans-retinoic acid to induce the differentiation of human neuroblastoma cells. *Differentiation* **1995**, *59*, 61-69, doi:10.1046/j.1432-0436.1995.5910061.x.
  295. Masetti, R.; Biagi, C.; Zama, D.; Vendemini, F.; Martoni, A.; Morello, W.; Gasperini, P.; Pession, A.J.A.i.t. Retinoids in pediatric onco-hematology: the model of acute promyelocytic leukemia and neuroblastoma. **2012**, *29*, 747-762.
  296. Kam, R.K.; Deng, Y.; Chen, Y.; Zhao, H. Retinoic acid synthesis and functions in early embryonic development. *Cell Biosci* **2012**, *2*, 11, doi:10.1186/2045-3701-2-11.
  297. Sidell, N.; Altman, A.; Haussler, M.R.; Seeger, R.C. Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. *Exp Cell Res* **1983**, *148*, 21-30, doi:10.1016/0014-4827(83)90184-2.
  298. Sidell, N. Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells in vitro. *J Natl Cancer Inst* **1982**, *68*, 589-596.
  299. Kaplan, D.R.; Matsumoto, K.; Lucarelli, E.; Thiele, C.J. Induction of TrkB by retinoic acid mediates biologic responsiveness to BDNF and differentiation of human neuroblastoma cells. Eukaryotic Signal Transduction Group. *Neuron* **1993**, *11*, 321-331, doi:10.1016/0896-6273(93)90187-v.
  300. Reynolds, C.P.; Matthay, K.K.; Villablanca, J.G.; Maurer, B.J. Retinoid therapy of high-risk neuroblastoma. *Cancer Lett* **2003**, *197*, 185-192, doi:10.1016/s0304-3835(03)00108-3.
  301. Pavlova, N.N.; Zhu, J.; Thompson, C.B. The hallmarks of cancer metabolism: Still emerging. *Cell Metab* **2022**, *34*, 355-377, doi:10.1016/j.cmet.2022.01.007.
  302. Warburg, O.; Minami, S. Versuche an überlebendem carcinom-gewebe. *Klinische Wochenschrift* **1923**, *2*, 776-777.
  303. Tao, L.; Mohammad, M.A.; Milazzo, G.; Moreno-Smith, M.; Patel, T.D.; Zorman, B.; Badachhpe, A.; Hernandez, B.E.; Wolf, A.B.; Zeng, Z.; et al. MYCN-driven fatty acid uptake is a metabolic vulnerability in neuroblastoma. *Nat Commun* **2022**, *13*, 3728, doi:10.1038/s41467-022-31331-2.
  304. Zirath, H.; Frenzel, A.; Oliynyk, G.; Segerstrom, L.; Westermarck, U.K.; Larsson, K.; Munksgaard Persson, M.; Hultenby, K.; Lehtio, J.; Einvik, C.; et al. MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells. *Proc Natl Acad Sci U S A* **2013**, *110*, 10258-10263, doi:10.1073/pnas.1222404110.
  305. Oliynyk, G.; Ruiz-Perez, M.V.; Sainero-Alcolado, L.; Dzieran, J.; Zirath, H.; Gallart-Ayala, H.;

- Wheelock, C.E.; Johansson, H.J.; Nilsson, R.; Lehtio, J.; et al. MYCN-enhanced Oxidative and Glycolytic Metabolism Reveals Vulnerabilities for Targeting Neuroblastoma. *iScience* **2019**, *21*, 188–204, doi:10.1016/j.isci.2019.10.020.
306. Ruiz-Perez, M.V.; Sainero-Alcolado, L.; Oliynyk, G.; Matuschek, I.; Balboni, N.; Ubhayasekera, S.; Snaebjornsson, M.T.; Makowski, K.; Aaltonen, K.; Bexell, D.; et al. Inhibition of fatty acid synthesis induces differentiation and reduces tumor burden in childhood neuroblastoma. *iScience* **2021**, *24*, 102128, doi:10.1016/j.isci.2021.102128.
307. Hayyan, M.; Hashim, M.A.; AlNashef, I.M. Superoxide Ion: Generation and Chemical Implications. *Chem Rev* **2016**, *116*, 3029–3085, doi:10.1021/acs.chemrev.5b00407.
308. Lu, J.M.; Lin, P.H.; Yao, Q.; Chen, C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *J Cell Mol Med* **2010**, *14*, 840–860, doi:10.1111/j.1582-4934.2009.00897.x.
309. Jan, A.T.; Azam, M.; Siddiqui, K.; Ali, A.; Choi, I.; Haq, Q.M. Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants. *Int J Mol Sci* **2015**, *16*, 29592–29630, doi:10.3390/ijms161226183.
310. Wanders, R.J.; Waterham, H.R.; Ferdinandusse, S. Metabolic Interplay between Peroxisomes and Other Subcellular Organelles Including Mitochondria and the Endoplasmic Reticulum. *Front Cell Dev Biol* **2015**, *3*, 83, doi:10.3389/fcell.2015.00083.
311. Chang, W.T.; Bow, Y.D.; Chen, Y.C.; Li, C.Y.; Chu, Y.C.; Teng, Y.N.; Li, R.N.; Chiu, C.C. The Phenoxypheanol Compound diTFPP Mediates Exogenous C(2)-Ceramide Metabolism, Inducing Cell Apoptosis Accompanied by ROS Formation and Autophagy in Hepatocellular Carcinoma Cells. *Antioxidants (Basel)* **2021**, *10*, doi:10.3390/antiox10030394.
312. Rademaker, G.; Costanza, B.; Bellier, J.; Herfs, M.; Peiffer, R.; Agirman, F.; Maloujahmoum, N.; Habraken, Y.; Delvenne, P.; Bellahcene, A.; et al. Human colon cancer cells highly express myoferlin to maintain a fit mitochondrial network and escape p53-driven apoptosis. *Oncogenesis* **2019**, *8*, 21, doi:10.1038/s41389-019-0130-6.
313. Lloberas, J.; Munoz, J.P.; Hernandez-Alvarez, M.I.; Cardona, P.J.; Zorzano, A.; Celada, A. Macrophage mitochondrial MFN2 (mitofusin 2) links immune stress and immune response through reactive oxygen species (ROS) production. *Autophagy* **2020**, *16*, 2307–2309, doi:10.1080/15548627.2020.1839191.
314. Juan, C.A.; Perez de la Lastra, J.M.; Plou, F.J.; Perez-Lebena, E. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22094642.
315. He, L.; He, T.; Farrar, S.; Ji, L.; Liu, T.; Ma, X. Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cell Physiol Biochem* **2017**, *44*, 532–553, doi:10.1159/000485089.
316. Ronca, R.; Benkheil, M.; Mitola, S.; Struyf, S.; Liekens, S. Tumor angiogenesis revisited: Regulators and clinical implications. *Med Res Rev* **2017**, *37*, 1231–1274, doi:10.1002/med.21452.
317. Huertas-Castano, C.; Gomez-Munoz, M.A.; Pardal, R.; Vega, F.M. Hypoxia in the Initiation and Progression of Neuroblastoma Tumours. *Int J Mol Sci* **2019**, *21*, doi:10.3390/ijms21010039.
318. Calvani, M.; Comito, G.; Giannoni, E.; Chiarugi, P. Time-dependent stabilization of hypoxia inducible factor-1alpha by different intracellular sources of reactive oxygen species. *PLoS One* **2012**, *7*, e38388, doi:10.1371/journal.pone.0038388.
319. Hu, C.J.; Wang, L.Y.; Chodosh, L.A.; Keith, B.; Simon, M.C. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* **2003**, *23*, 9361–9374, doi:10.1128/MCB.23.24.9361-9374.2003.
320. Loboda, A.; Jozkowicz, A.; Dulak, J. HIF-1 and HIF-2 transcription factors--similar but not identical. *Mol Cells* **2010**, *29*, 435–442, doi:10.1007/s10059-010-0067-2.
321. Semenza, G.L. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med* **2001**, *7*, 345–350, doi:10.1016/s1471-4914(01)02090-1.
322. Barriga, E.H.; Maxwell, P.H.; Reyes, A.E.; Mayor, R. The hypoxia factor Hif-1alpha controls neural crest chemotaxis and epithelial to mesenchymal transition. *J Cell Biol* **2013**, *201*, 759–776, doi:10.1083/jcb.201212100.

323. Zhang, B.; Yin, C.P.; Zhao, Q.; Yue, S.W. Upregulation of HIF-1 $\alpha$  by hypoxia protect neuroblastoma cells from apoptosis by promoting survivin expression. *Asian Pac J Cancer Prev* **2014**, *15*, 8251–8257, doi:10.7314/apjcp.2014.15.19.8251.
324. Chirathivat, S.; Post, M.J. CT demonstration of dural metastases in neuroblastoma. *J Comput Assist Tomogr* **1980**, *4*, 316–319, doi:10.1097/00004728-198006000-00005.
325. Lu, X.; Kang, Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. *Clin Cancer Res* **2010**, *16*, 5928–5935, doi:10.1158/1078-0432.CCR-10-1360.
326. Chen, C.C.; Hsia, C.W.; Ho, C.W.; Liang, C.M.; Chen, C.M.; Huang, K.L.; Kang, B.H.; Chen, Y.H. Hypoxia and hyperoxia differentially control proliferation of rat neural crest stem cells via distinct regulatory pathways of the HIF1 $\alpha$ -CXCR4 and TP53-TPM1 proteins. *Dev Dyn* **2017**, *246*, 162–185, doi:10.1002/dvdy.24481.
327. Mescola, A.; Vella, S.; Scotto, M.; Gavazzo, P.; Canale, C.; Diaspro, A.; Pagano, A.; Vassalli, M. Probing cytoskeleton organisation of neuroblastoma cells with single-cell force spectroscopy. *J Mol Recognit* **2012**, *25*, 270–277, doi:10.1002/jmr.2173.
328. Cabado, A.G.; Leira, F.; Vieytes, M.R.; Vieites, J.M.; Botana, L.M. Cytoskeletal disruption is the key factor that triggers apoptosis in okadaic acid-treated neuroblastoma cells. *Arch Toxicol* **2004**, *78*, 74–85, doi:10.1007/s00204-003-0505-4.
329. Rozzo, C.; Chiesa, V.; Ponzoni, M. Integrin up-regulation as marker of neuroblastoma cell differentiation: correlation with neurite extension. *Cell Death Differ* **1997**, *4*, 713–724, doi:10.1038/sj.cdd.4400304.
330. Portier, M.M.; Croizat, B.; Gros, F. A sequence of changes in cytoskeletal components during neuroblastoma differentiation. *FEBS Lett* **1982**, *146*, 283–288, doi:10.1016/0014-5793(82)80935-6.
331. Shea, T.B.; Fischer, I.; Sapirstein, V.S. Effect of retinoic acid on growth and morphological differentiation of mouse NB2a neuroblastoma cells in culture. *Brain Res* **1985**, *353*, 307–314, doi:10.1016/0165-3806(85)90220-2.
332. Middelbeek, J.; Vrenken, K.; Visser, D.; Lasonder, E.; Koster, J.; Jalink, K.; Clark, K.; van Leeuwen, F.N. The TRPM7 interactome defines a cytoskeletal complex linked to neuroblastoma progression. *Eur J Cell Biol* **2016**, *95*, 465–474, doi:10.1016/j.ejcb.2016.06.008.
333. Goel, M.K.; Khanna, P.; Kishore, J. Understanding survival analysis: Kaplan-Meier estimate. *Int J Ayurveda Res* **2010**, *1*, 274–278, doi:10.4103/0974-7788.76794.
334. Lee, J.; Yoshizawa, C.; Wilkens, L.; Lee, H.P. Covariance adjustment of survival curves based on Cox's proportional hazards regression model. *Comput Appl Biosci* **1992**, *8*, 23–27, doi:10.1093/bioinformatics/8.1.23.
335. Tibshirani, R. The lasso method for variable selection in the Cox model. *Stat Med* **1997**, *16*, 385–395, doi:10.1002/(sici)1097-0258(19970228)16:4<385::aid-sim380>3.0.co;2-3.
336. Spooner, A.; Chen, E.; Sowmya, A.; Sachdev, P.; Kochan, N.A.; Trollor, J.; Brodaty, H. A comparison of machine learning methods for survival analysis of high-dimensional clinical data for dementia prediction. *Sci Rep* **2020**, *10*, 20410, doi:10.1038/s41598-020-77220-w.
337. Arashi, M.; Roozbeh, M.; Hamzah, N.A.; Gasparini, M. Ridge regression and its applications in genetic studies. *PLoS One* **2021**, *16*, e0245376, doi:10.1371/journal.pone.0245376.
338. Cho, S.; Kim, H.; Oh, S.; Kim, K.; Park, T. Elastic-net regularization approaches for genome-wide association studies of rheumatoid arthritis. *BMC Proc* **2009**, *3 Suppl 7*, S25, doi:10.1186/1753-6561-3-s7-s25.
339. Valentijn, L.J.; Koster, J.; Haneveld, F.; Aissa, R.A.; van Sluis, P.; Broekmans, M.E.; Molenaar, J.J.; van Nes, J.; Versteeg, R. Functional MYCN signature predicts outcome of neuroblastoma irrespective of MYCN amplification. *Proc Natl Acad Sci U S A* **2012**, *109*, 19190–19195, doi:10.1073/pnas.1208215109.
340. Cook, A.M.; McDonnell, A.M.; Lake, R.A.; Nowak, A.K. Dexamethasone co-medication in cancer patients undergoing chemotherapy causes substantial immunomodulatory effects with implications for chemo-immunotherapy strategies. *Oncoimmunology* **2016**, *5*, e1066062, doi:10.1080/2162402X.2015.1066062.
341. Kunath, F.; Jensen, K.; Pinart, M.; Kahlmeyer, A.; Schmidt, S.; Price, C.L.; Lieb, V.; Dahm, P. Early

- versus deferred standard androgen suppression therapy for advanced hormone-sensitive prostate cancer. *Cochrane Database Syst Rev* **2019**, *6*, CD003506, doi:10.1002/14651858.CD003506.pub2.
342. Geisler, J.P.; Wiemann, M.C.; Miller, G.A.; Zhou, Z.; Geisler, H.E. Estrogen and progesterone receptors in malignant mixed mesodermal tumors of the ovary. *J Surg Oncol* **1995**, *59*, 45-47, doi:10.1002/jso.2930590112.
  343. Martin, J.D.; Hahnel, R.; McCartney, A.J.; Woodings, T.L. The effect of estrogen receptor status on survival in patients with endometrial cancer. *Am J Obstet Gynecol* **1983**, *147*, 322-324, doi:10.1016/0002-9378(83)91119-5.
  344. Cheung, B.B. Combination therapies improve the anticancer activities of retinoids in neuroblastoma. *World J Clin Oncol* **2015**, *6*, 212-215, doi:10.5306/wjco.v6.i6.212.
  345. Cheung, B.B.; Tan, O.; Koach, J.; Liu, B.; Shum, M.S.; Carter, D.R.; Sutton, S.; Po'uha, S.T.; Chesler, L.; Haber, M.; et al. Thymosin-beta4 is a determinant of drug sensitivity for Fenretinide and Vorinostat combination therapy in neuroblastoma. *Mol Oncol* **2015**, *9*, 1484-1500, doi:10.1016/j.molonc.2015.04.005.
  346. Ali, S.H.; O'Donnell, A.L.; Mohamed, S.; Mousa, S.; Dandona, P. Overexpression of estrogen receptor-alpha in the endometrial carcinoma cell line Ishikawa: inhibition of growth and angiogenic factors. *Gynecol Oncol* **2004**, *95*, 637-645, doi:10.1016/j.ygyno.2004.08.034.
  347. Ali, S.H.; O'Donnell, A.L.; Balu, D.; Pohl, M.B.; Seyler, M.J.; Mohamed, S.; Mousa, S.; Dandona, P. Estrogen receptor-alpha in the inhibition of cancer growth and angiogenesis. *Cancer Res* **2000**, *60*, 7094-7098.
  348. Westermann, F.; Muth, D.; Benner, A.; Bauer, T.; Henrich, K.O.; Oberthuer, A.; Brors, B.; Beissbarth, T.; Vandesompele, J.; Pattyn, F.; et al. Distinct transcriptional MYCN/c-MYC activities are associated with spontaneous regression or malignant progression in neuroblastomas. *Genome Biol* **2008**, *9*, R150, doi:10.1186/gb-2008-9-10-r150.
  349. Gorlick, R.; Kolb, E.A.; Keir, S.T.; Maris, J.M.; Reynolds, C.P.; Kang, M.H.; Carol, H.; Lock, R.; Billups, C.A.; Kurmasheva, R.T.; et al. Initial testing (stage 1) of the Polo-like kinase inhibitor volasertib (BI 6727), by the Pediatric Preclinical Testing Program. *Pediatr Blood Cancer* **2014**, *61*, 158-164, doi:10.1002/pbc.24616.
  350. Bassiri, H.; Benavides, A.; Haber, M.; Gilmour, S.K.; Norris, M.D.; Hogarty, M.D. Translational development of difluoromethylornithine (DFMO) for the treatment of neuroblastoma. *Transl Pediatr* **2015**, *4*, 226-238, doi:10.3978/j.issn.2224-4336.2015.04.06.
  351. Martinez-Monleon, A.; Kryh Oberg, H.; Gaarder, J.; Berbegall, A.P.; Javanmardi, N.; Djos, A.; Ussowicz, M.; Taschner-Mandl, S.; Ambros, I.M.; Ora, I.; et al. Amplification of CDK4 and MDM2: a detailed study of a high-risk neuroblastoma subgroup. *Sci Rep* **2022**, *12*, 12420, doi:10.1038/s41598-022-16455-1.
  352. McBeath, R.; Pirone, D.M.; Nelson, C.M.; Bhadriraju, K.; Chen, C.S. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* **2004**, *6*, 483-495, doi:10.1016/s1534-5807(04)00075-9.
  353. Yourek, G.; Hussain, M.A.; Mao, J.J. Cytoskeletal changes of mesenchymal stem cells during differentiation. *ASAIO J* **2007**, *53*, 219-228, doi:10.1097/MAT.0b013e31802deb2d.
  354. Nakaseko, Y.; Yanagida, M. Cell biology. Cytoskeleton in the cell cycle. *Nature* **2001**, *412*, 291-292, doi:10.1038/35085684.
  355. Lau, E.O.; Damiani, D.; Chehade, G.; Ruiz-Reig, N.; Saade, R.; Jossin, Y.; Aittaleb, M.; Schakman, O.; Tajeddine, N.; Gailly, P.; et al. DIAPH3 deficiency links microtubules to mitotic errors, defective neurogenesis, and brain dysfunction. *Elife* **2021**, *10*, doi:10.7554/eLife.61974.

