EPIGENETIC REGULATORS IN NEUROBLASTOMA: BRG1, A FUTURE THERAPEUTIC TARGET

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Is this the real life?

Is this just fantasy?

Caught in a landslide

No escape from reality;

Open your eyes

Look up to the skies and see

Bohemian Rhapsody, Queen

To my Mom
To my Dad
To Adri

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Desde que escribí la primera palabra en el libro de mi vida mucha gente se ha cruzado en mi camino para dejar su huella en él. Gente a la que no solo debo agradecer su ayuda y apoyo sino que han hecho hoy de mí la persona que fui, soy y seré.

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ABBREVIATIONS

Abbreviations

(v/v)	(volume/volume)
ABC	ATP-binding cassette
Ac-DEVD-afc	N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Trifluoromethylcoumarin
ADAADi	Active DNA-dependent ATPase A Domain inhibitor
AHSCT	autologous hematopoietic stem cell transplantation
ALK	Anaplastic Lymphoma Receptor Tyrosine Kinase
BCL-2	B-Cell CLL/Lymphoma 2
BET	Bromodomains and extraterminal domain
BMP	bone morphogenic proteins
BRD	Bromodomains
BRG1	or SMARCA4, SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4
BRM	or SMARCA 2, SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 2
BSA	Bovine serum albumin
CASP8	Caspase-8
cDNA	complementary DNA
CI	Combination Index
CNV	copy number variations
COJEC	cisplatin, vincristine, carboplatin, etoposide and cyclophosphamide
CRC	chromatin remodeler complexes
CTNNB1	β-Catenin
DFMO	difluoro-methylornithine
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferases
DNMTi	DNMT inhibitors
dNTP	Deoxynucleotides
ECL	Enhanced chemoluminsecence
EDTA	ethylene-diamine-tetraacetic acid
EFS	event-free survival
EMT	Epithelial-Mesenchymal Transition
EZH2	Enhancer of Zeste 2
FDA	Food and Drug administration
FGF	Fibroblast growth factor
GD-2	disialoganglioside
GFP	green fluorescent protein
GWAS	Genome wide association studies
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HDACi	HDAC inhibitor
HDM	Histone demethylases
HMT	Histone methyltransferases

ABBREVIATIONS

HRP	Horseradish Peroxidase
iBET	BET inhibitor
IL-2	Interleukin-2
INPC	international neuroblastoma pathology classification system
INRGSS	International Neuroblastoma Risk Group Staging System
INSS	International Neuroblastoma Staging System
JHDM	Jumonji domain-containing histone demethylase
KDM1 (LSD1)	Lysine-specific histone demethylase 1
lncRNA	long non-codign RNA
LOH	loss of heterozygosity
MDR	multidrug resistance
mIBG	^{123}I-Meta-iodobenzylguanidine
miRNA	micro RNA
miRNA	micro RNA
mRNA	messenger RNA
MYCN	Neuroblastoma MYC Oncogene
NAD+	nicotinamide adenin dinucleotide
NAIP	neural apoptosis inhibitor protein
NB	Neuroblastoma
ncRNA	non-coding RNA
NGF	Nerve growth factor
NSC	Non-silencing control
OD	optycal density
OMS	opsoclonus myoclonus syndorme
PARP	Poly(ADP-Ribose) Polymerase
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PI3KCA	p110α
PKMT	Lysine methyltransferases
PRC	Polycomb Repressive Complex
PRMT	Argininte methyltransferases
PVDF	polyvinylidine diflouride
qPCR	quantitative PCR
RIPA	Radioimmunoprecipitation assay
RT	reverse transcription
shRNA	short-hairpin RNA
SIOPEN	International Society of Paediatric Oncology Europe Neuroblastoma
siRNA	small-interfering RNA
SIRT	Sirtuins
SnAC	Snf2 ATP-coupling
sncRNA	small non-coding RNA
SNP	Single nucleotide polimorfisms
SRC	Nuclear receptor coactivators
SWI/SNF	SWItch/Sucrose Non-Fermentable
TBS-T	Tris buffered saline-Tween

TF	Transcription Factor
TGF	Transformin growth factor
UTR	Untranslated region
VAP	Valproic acid
WNT	Wingless-related integration site

INTRODUCTION

1. INTRODUCTION

1.1. NEUROBLASTOMA

Childhood cancers are considered a rare disease; only 10,270 cases arise every year in the United States of America (USA) and around 15,000 cases in Europe. Despite the low incidence (compared to adulthood cancers), cancer related deaths is the leading cause of children death by disease in the western countries[1].

The most common cancers diagnosed during the childhood are: leukemia, brain and other central nervous system tumors, neuroblastoma, lymphoma, Wilms tumor, rhabdomyosarcoma, osteosarcoma, retinoblastoma, Ewin sarcoma and testicular or ovarian germ cell tumors.

In 1975, the 5-year overall survival of children with cancer was only 50%[1]. In defiance of increasing childhood cancer incidence in the past decades, in 2010 the overall survival after 5 years reached 80%[2]. The improvement in the survival rates is due to the advance in tumor treatments of some cancers such as leukemia, but many others like gliomas[3] remain in very low rates of survival.
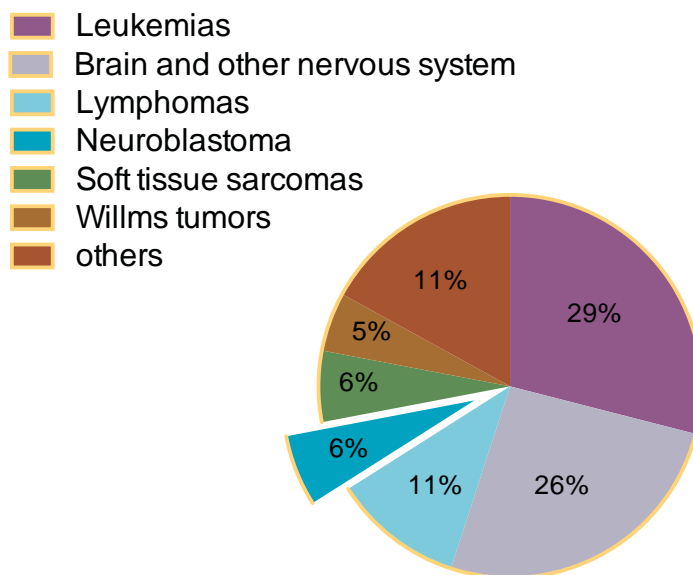
Figure 1: Percentage of childhood cancers. Leukemias are the most common childhood cancer, followed by brain and other nervous system tumors and lymphomas. Neuroblastoma is the fourth most common childhood cancer accounting for 6% of all childhood cancers. Adapted from Siegel R.L. et al[4].

Neuroblastoma (NB) is the fourth most common childhood cancer (Figure 1), accounting for 6-10% of western countries childhood cancers; and is the most common solid tumor of the infancy. The incidence rate in the western countries varies depending on the age of the patient. Approximately 90% of these tumors are diagnosed during the 5 years of life[5,6]. In general 1.1 cases per 100,000 people are diagnosed among 0-14 years old, whereas less than 0.1 cases per 100,000 people are diagnosed among 15-19 years old. The 5-years survival rate in western countries of NB is 80%, but this can vary depending on the stage of the disease[1,4,6].

NB is a very heterogeneous neoplasm, with prognosis raging from spontaneous regression to fatal death[7]. Hence, the study of this tumor has been hard due to the variable behavior. In the following sections, the current knowledge of the origin, diagnosis, treatment and challenges of this disease will be exposed.

1.1.1. Neuroblastoma origin: where all begins?

NB is considered an embryonal tumor, which means that these tumors arise during intrauterine or early postnatal development from an immature organ or tissue. Therefore, embryonic cancer initiation appears to be an aberration of our prenatal self[8]. In fact, it is widely accepted that embryonic tumors are initiated by resting cells. In the model proposed by Marshall G. et al, these cells derive from normal embryogenesis and suffer a first hit that allows them to continuously proliferate. After birth, some of these cells can receive a second hit that confer them a mechanism for surviving in the early postnatal environment, transforming them in a precancerous cell. Finally a third hit accelerates the genome instability leading to a cancer lesion[8] (Figure 2).
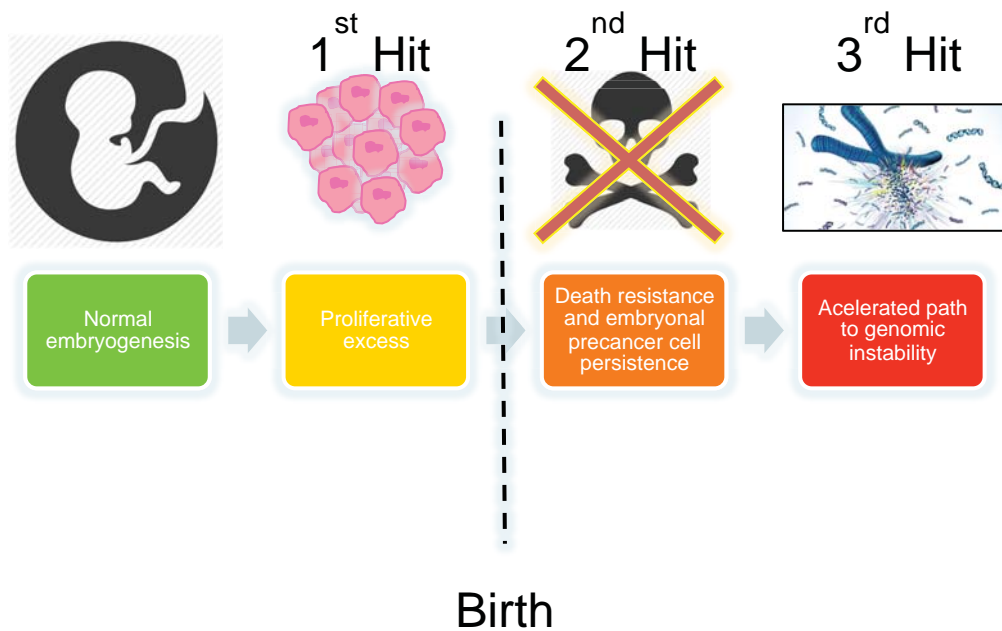
Figure 2: Model of embryonal tumorogenesis. Embryonal cancers need three hits to develop: first they need a prenatal proliferative excess in the tissue of origin, then after birth cells acquires an intrinsic mechanism for surviving the post-natal environment, finally they need an accelerated pathway toward genomic instability (adapted from Marshall G. et al[9]).

Wright J.H. pointed out for the first time that NB tumors derived from neural cells (reviewed in [5]). Nowadays, based on the primary tumor location (usually adrenal gland or sympathetic ganglia[10,11]), cellular and neurochemical features of NB and expression profiling showing that human fetal adrenal neuroblasts have gene signatures that are similar to NB[12], there is enough evidence to

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affirm that NB arises from the sympathoadrenal lineage of the neural crest during development[13].

The neural crest is a transient structure that exists during early embryogenesis[14]. Consist of small number of pluripotent cells with the ability to migrate and differentiate in a great variety of adult tissues that includes peripheral neurons, enteric neurons, glia, melanocytes, Schwann cells, cells of the craniofacial skeleton and adrenal medulla[15]. In order to get to the target sites and differentiate, neural crest cells need to detach and go through an epithelial-mesenchymal transition (EMT)[16]. The induction of EMT and migration is a very intricate process that involves the interaction with ectoderm, mesoderm and neural plate (embryonic structures that are crucial for the development of vertebrates)[17]. In addition, EMT process needs the coordination of several transcription factors, cell signal pathways and cell to cell interaction, cellular adhesion and extracellular matrix changes[18]. All these factors are orchestrated by gradients generated by excreted ligands and extracellular matrix components, such as TGF, WNT, FGF and BMP which are produced by the ectoderm, mesoderm and neural plate[19]. When neural crest cells contact this gradients induce the expression of several transcription factors (Snail1, Snail2, Twist, SoxE, FoxD, ETS, MYCN…)[17,20]. In turn, these transcription factors directly regulate the transcription of cadherins[17,21], metaloproteases, ADAM and integrins, leading to a loss of cellular polarity, cell to cell junctions and extracellular matrix digestion[18].

The processes involved on EMT during embryogenesis is recapitulated in tumor EMT[16]. Moreover, transcription factors that are crucial for the correct development are altered in many cancers[17]. Therefore, defects in the mechanism that control embryonic processes can promote cell transformation making developing cells more sensitive to tumorigenesis[22].

As described before, NB is thought to arise from sympathoadrenal lineage cells from the neural crest. These cells has not received or responded properly to all the signals that determine neuronal or chromaffin cell fate. This raises the question of which signals are altered in NB.

In the case of sympathoadrenal development, the expression of MYCN (driven by WNT gradient[20]) is increased in the early post-migratory neural crest, regulating expansion and ventral migration of neural crest cells[23]. However,

sympathoadrenal maturation requires low or absent MYCN expression[23]; therefore, the levels of this protein are gradually reduced during the differentiation of sympathetic neurons[23–25]. This reduction is necessary for a proper selection of cells (usually by apoptosis) at the final stage of sympathoadrenal maturation, a process catalyzed by NGF deprivation[26]. In line with these results, zebra fish and mouse models have demonstrated that MYCN persistent expression in sympathoadrenal precursors recapitulate neuroblastoma resting disease[27–31]. Moreover, studies with MYCN overexpression brought to light the importance of a robust p53 stress response in the susceptibility to oncogenic stress in these embryonal cells[28,32,33].

Another early regulator in the sympathoadrenal development is ALK. It protects neuroblasts in utero against nutrient deprivation[34,35]. Interestingly, this protein has been found actively mutated in sporadic and familial NB[36–38]. Moreover, the neural crest specific expression of ALK F1174L mutation (the most aggressively activating mutation[39]) is sufficient to generate tumors in transgenic mice[40].

At the fifth week of development, dorsal aorta secretes bone morphogenetic protein and forms a gradient. Neural crest cells that migrates ventrally respond to this gradient and starts a transcriptional program that specifies neuronal and catecholaminergic properties[41–43]. Then, PHOX2A and PHOX2B are expressed and drive catecholamine biosynthesis enzymes expression. Additionally, PHOX2B starts the transcriptional program that controls terminal sympathoadrenal differentiation. PHOX2B mutations is associated with a subset of familial NB[44,45].

Additionally, TRKA and TRKB, two neurotrophin factor receptors, have been found altered in NB. On the one hand, TRKA, is downregulated in MYCN amplified NB cells and thus, neural differentiation is blocked. On the other hand, TRKB is capable to respond to BDNF (a survival and growth promoter in neurons). BDNF and TRKB are expressed in MYCN amplified NB to maintain an autocrine cell survival loop (reviewed in [11]).

Finally, let-7 miRNA family is a relevant regulator of developmental timing and cell growth during neural crest cell lineage commitment. LIN28A and LIN28B RNA-binding proteins regulate the processing of let-7 family members[46], thereby making them important regulators of the neural crest

development. In fact, LIN28B expression is high in poor prognosis NB tumors[47]. Furthermore, ectopic expression of LIN28B in the developing neural crest of transgenic mice is sufficient to induce NB through the downregulation of let-7 family of miRNAs, which in turn, increases MYCN expression and maintain the undifferentiated neuroblast phenotype[46] (Figure 3).
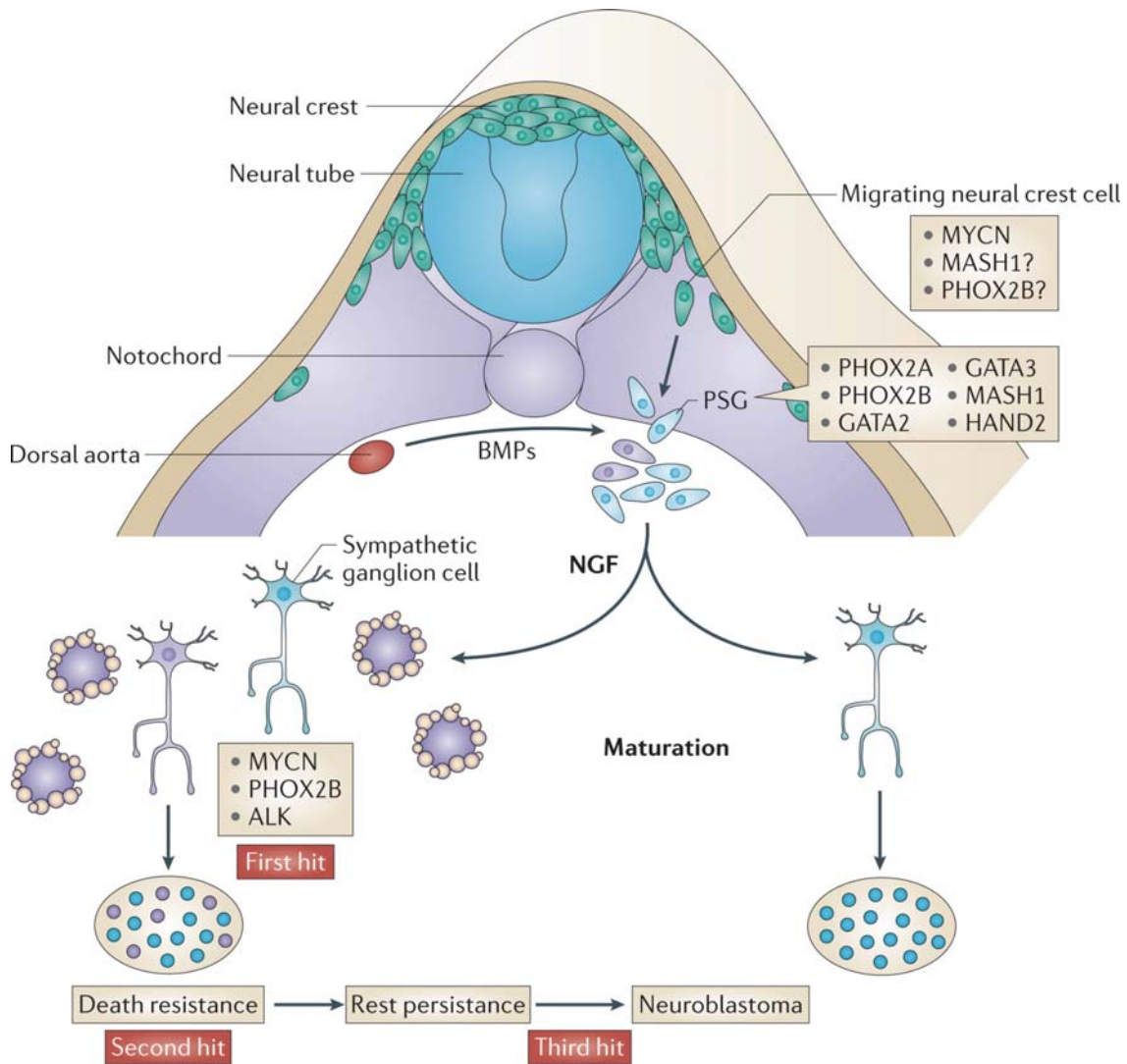
Figure 3: Physiological neural crest development and alterations that leads to neuroblastoma formation. Neuroblast progenitors migrate from the neural crest to a region near the notochord and the dorsal aorta under the regulation of MYCN and BMP. At this site, the cells undergo specification as the primary sympathetic ganglia before divergence into neural cells of the mature sympathetic ganglia or chromaffin cells of the adrenal medulla. MYCN, mutations/alterations in anaplastic lymphoma kinase (*ALK*) and paired-like homeobox 2 (*PHOX2B*) can be first hits of NB.. Local access to nerve growth factor (NGF) determines whether the normal sympathetic ganglia matures into a terminal ganglion cell or undergoes apoptotic cell death. A relatively common pathologic state is postnatal survival of neuroblast resting disease which requires the cell destined to become malignant to be resistant to trophic factor withdrawal before these persistent rest cells undergo a third change to induce transformation, which presents as a clinical malignancy in early childhood (Adapted from Marshall G et al) [9].

1.1.2. Familial and Sporadic Neuroblastoma: Clues to understand the genetic and epigenetic alterations of the disease

FAMILIAL NEUROBLASTOMA

Familial NB accounts for 1-2% of all NB and is inherited in an autosomal dominant pattern with incomplete penetrance[48–50]. Moreover, families show clinical variability in severity of disease, with low- and high-risk cases observed in the same pedigree[51–55]. In contrast to sporadic cancer behavior, familial NB has an earlier age of onset and is usually presented with multiple primary tumor sites[49].

PHOXB2 gene was the first gene found to predispose to neuroblastoma[56,57]. Initially, it was associated with neural crest-derived cells disorder, the Hirschsprung disease and or hypoventilation syndrome. These types of disorders occasionally are coincident with NB[58–62]. Consequently, mutations in PHOX2B were found in 10% of pedigrees with familial NB [63,64]. PHOX2B low mutational frequency indicates that it is not the major pathogenic gene in NB[65]. Nevertheless, the implication of PHOX2B in neural crest development is clear and malfunctions in this gene lead to an aberrant sympathoadrenal neural-crest cell development.

A genome-wide linkage scan at 6000 single nucleotide polymorphisms (SNP) was done in 20 NB families in order to identify more hereditary predisposition genes[36]. The study identified a linkage in 2p23-p24 chromosome bands. This region contained 104 genes, among which MYCN and ALK were included. Moreover, ALK was also identified as a potential oncogene in this malignancy[66,67]. Additional studies confirmed that 80% of familial NB harbor mutations in ALK[36,37,39,68] and mutation type correlates with different penetrance (Table 1).

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Table 1: ALK mutation type and their correlation with NB penetrance and phenotype

ALK mutation	Phenotype	References
R1275Q	Near complete penetrance, highly activating mutation	69,70
G1128A	25% of penetrance, weakly activating mutation	69,70
F1174	Present in NB cursing with neurocognitive defects and brain stem abnormalities, strongly activating mutation	71
F1245	Present in NB cursing with neurocognitive defects and brain stem abnormalities, strongly activating mutation	71

There is still some familial NB that does not present mutations in ALK or PHOX2B. For instance, other less frequent germline mutations have been reported in GALNT14[72], TP53, SDHB, PTPN11, APC and NF1 genes (reviewed in[73]).

Additionally, two complex congenital malformation syndromes have been reported to curse with NB. These are the subtelomeric deletions 1p36.3 and 11q23[73,74].

Some researchers have proposed that screening of germline mutations of ALK and PHOX2B for children with NB family history or two-sided adrenal gland enlargement should be performed (since these type of NB are susceptible to be hereditary)[75]. In fact, genetic testing for both genes are currently available for identifying susceptibility and informing decisions about screening NB family members (http://www.ncbi.nlm.nih.gov/sites/GeneTests/).

SPORADIC NEUROBLASTOMA

Sporadic NB represents 99% of the cases. Thus, many studies attempted to uncover the genetic and epigenetic mechanism underlying the disease. These discoveries can be classified into: chromosomal aberrations, mutations and epigenetic alterations.

Chromosomal aberrations

Comparing to adult tumors, the number of somatic mutations in NB is low, but the frequency of copy number variations (which includes chromosomal aberrations and gene amplifications) alteration is high. This indicates that copy number variations could be used as biomarkers[65].

DNA amplification can cause an overexpression of oncogenes, thereby playing an important role in cancer development. MYCN amplification was discovered in early 80's and is the most relevant gene amplification in NB[76]. It is present in 22% of NB[77]. Moreover, it associates with advanced stages of the disease and highly malignant phenotypes[78] and it has been demonstrated to be sufficient to initiate neuroblastoma formation in mice.

ALK is the second most frequently amplified gene accounting for 4% of NB[79–81]. Other less frequent amplifications has been reported, usually co-occurring with MYCN amplification such as DDX1, ODC1, CDK4, MDM2 and NAG or MYCN-independent such as CDK6 and CCND1 (reviewed in [65]).

Hyperdiploidy can be also a predictive factor of outcome for infants, though the prognostic value is lost for older patients[82]. Younger patients (less than 1-2 years old) with localized and good prognosis tumors usually are hyperdiploid and have very few or even zero structural aberrations[83,84].

Chromosome 1p deletions are quite often in NB; loss of heterozygosity (LOH) of the short arm of chromosome 1 is found in 20-35% of NB tumors[85,86]. It is associated with poor outcome[87] and MYCN amplification[88]. The shortest region of consistent LOH is 1p36.3 (spanning 261kb)[89–91], which contains many potential tumor suppressor genes. Some of the more relevant are: CHD5, a chromodomain helicase the loss of which was correlated with advanced stage and unfavorable histology[92–94], CAMTA1 a transcription factor the low expression of which correlates with poor clinical outcome[95,96], CASZ1 a zinc-finger transcription factor associated with decreased overall survival[97,98], UBE4B and ubiquitination factor[99] and APITD1 and apoptotic related protein[100].

Another frequent structural aberration is LOH of 11q, which occur in 40-45% of NB patients and is associated with poor clinical outcome[77]. In this case, 11q deletion and MYCN amplification are mutually exclusive, though a small sub-set of tumors present both aberrations[101–104]. Given that 11q LOH is important for aggressive NB, many efforts have been done to identify the genes that contribute to this phenotype. One of such example is *H2AFX*, a gene coding for Histone 2A, is located in 11q23.3 region and its loss showed enhanced susceptibility to linfomas, rabdomyosarcomas and central nervous system cancers in mice[105,106]. CADM1, an adhesion molecule, is involved in neural cell development and is associated with poor NB survival[105,106].

Additionally, constitutional rearrangements of 11q were found in NB patients, indicating that 11q genes may be involved in development of NB[105,106].

NB most common aberration is the unbalanced gain of 17q that occurs in approximately 70% of patients[107–109], though its role is controversial in NB. This is usually caused by unbalanced translocations of 17q21-q terminal segment and 1p or 11q distal part[107,110–114], though other chromosomes can be involved[115]. The role of 17q gain as a prognostic factor is controversial[109,116]. Some studies reported that 17q gain correlates with advanced stage, increased patient age, 1p LOH, 11q LOH and MYCN amplification[109,112–115,117]. Only two genes mapping to 17q gain has been associated to aggressive phenotype of neuroblastoma: BIRC5 and NME1[118–120].

Finally, other less frequent chromosomal aberrations can be important for NB development: gains of 1q, 2p, 7q, 9p and 11p or losses of 3p, 4p, 14q, 16p, and 19q[115]. Nevertheless, the role of these genes present in these regions for NB origin or progression remain elusive[121].

Mutations

Mutation frequencies in NB are lower compared to adult tumors; however, these mutations frequently drive initation or progression of NB[122]. Mutations can be classified in to somatic and germ line, understanding the first ones as the mutations that occur only in the tumor and the second ones as the mutations that carry the individual in all cells.

Somatic mutations in NB include ATRX genetic loss-of-function alterations. They are present in 10% of all NB[123,124] and ATRX is found mutated in ~50% of adolescent and young adults with NB[123,124]. ARID1A and ARID1B are two genes involved in chromatin regulation; they are part of the SWI/SNF family members[125]. It was found that mutations in both genes could lead to resistance to early stage treatment for NB patients and low survival rates[126]. Another gene that has been found mutated in NB is NTRK1, which encodes the TRKA receptor[127] and is important for the development as explained before. Its high expression has also been reported to be associated with a favorable prognosis in NB[128,129]. Other chromatin regulators have been found mutated, including EP300, CREBBP, TTF2, KDM5A, CHD9 and IKZF1[122],.

Genome-wide association studies (GWAS) have resulted in the identification of SNP or copy number variations (CNVs), associated with sporadic NB[130–132]. These variations can be classified into three categories. The first one includes SNPs in genes that correlate with high risk NB: CASC15/14, BARD1, LMO1, LIN28B and HACE1[133,134]. The second one includes DNA alleles of the genes: DUSP12, DDX, IL31RA and HSD17B12[135], which were present in low risk NB. The last one includes germline CNV that correlates with NB. So far, only been found in the NBPF23 gene[135]. Further studies revealed mutations of some of these genes at somatic level, such as LMO1 that was found mutated in 12% of NB[136], validating their relevance in NB development.

Finally, germline mutations have been found in BRCA1/2, PALB2, FANCD2 and CHECK2 DNA repair genes[137–142].

Epigenetic alterations

Many types of epigenetic alterations have been described in NB tumors. Among them, changes on DNA methylation, expression of non-coding RNAs and mutations in epigenetic regulators have been characterized.

In 2000 methylation of caspase-8 (CASP8) promoter was described in NB. CASP8 alleles were found completely methylated in 68% of MYCN amplified NB[143], thereby suggesting a potential role in the development of NB tumors[143,144]. Delving on the methylation of apoptotic genes, TRAILR1, 2, 3 and 4, APAF1 and PYCARD were described to bear DNA promoter hypermethylation in some NB cell lines and primary tumors[145–147]. RASSF1A was another gene found frequently methylated in NB, whose low expression levels correlated with stage of the disease and MYCN status[148–150]. Just 7kb upstream of RASF1A, ZMYND10 promoter was also found methylated and correlated with higher risk NB[151].

In the case of ncRNA, it has been described that every genomic subtype of NB presents specific miRNA profile[152] and these profiles correlates with prognosis, differentiation and apoptosis. In fact, miRNA expression profile can be used to classify high- and low-risk patients with a high sensitivity and specificity[153]. Thus, this suggests the importance of miRNA in NB. To date, numerous miRNAs have been identified that regulate different oncogenic

properties in NB. Some miRNA have been described to have oncogenic roles in such as miR-17-5p-92 cluster, the upregulation of which promotes tumorigenesis[154] or miR-380-5p the expression of which was found higher in advance NB stages, this miRNA regulates TP53 expression, therefore injection of anti-miR against miR-380-5p successfully reduce tumor growth in MYCN-dependent tumors by inducing TP53-mediated cell death[155]. On the contrary, let-7 and miR-101 directly regulate MYCN expression and together with miR-542-5p, miR-497, miR-34a, miR-335, miR-138 and miR-145 act as tumor suppressors in NB[47,156–162]. Other miRNA have different functional roles in NB: miR-204 increase drug sensitivity and miR-125b, miR-10a and miR10b promotes NB differentiation (reviewed in [163]). Studies in lncRNA have shown that T-UCR (transcribed ultra-conserved region) expression signatures can be used to classify high-risk NB[164]. Also DEIN (differentially expressed in NB) is a ncRNA that is highly expressed in stage 4S tumors[165].

Regarding epigenetic regulators, many of them have been described to be mutated, correlated with NB outcome or even associated with the development of NB. They will be further discussed in section 1.2.

1.1.3. Neuroblastoma clinical presentation, biological behavior and diagnosis: a disease profile

CLINICAL PRESENTATION

Neuroblastoma median age at diagnosis is 18 months; 90% of patients are younger than 10 years old and 40% are infants at the diagnosis[166]. Age at diagnosis correlates with survival, where patients younger than 18 months have a significant better overall survival than older patients[167,168]. Adolescent and adult NB cases accounts for less than 5% of the cases but it is usually a more indolent disease[169].

Primary tumors can arise from anywhere in the sympathetic nervous system and clinical signs and symptoms are directly linked to this location and metastatic sites if present (Figure 4). More than 50% of the primary tumors appear in the adrenal gland medulla, usually as a unilateral tumor; nevertheless bilateral primary adrenal tumors represent ~1% of all cases. Adrenal gland medulla tumors are associated with lower survival when compared to primary tumors in other locations[170]. Localized tumors in the abdomen are rare, besides large abdominal tumors can cause hypertension, abdominal distension and pain. Tumors that appear in the neck can cause damage on ganglion stellatum (a cervical ganglion), leading to the development of Horner syndrome, which includes ptsosis (dropping of the upper eyelid), miosis (constriction of the pupil), enophthalmos (posterior displacement of the eyeball) and anhidrosis (lack of sweat). Paraspinal sympathetic ganglia primary tumors can expand into the neural foramina, which leads to spinal cord compression[171].

A   B
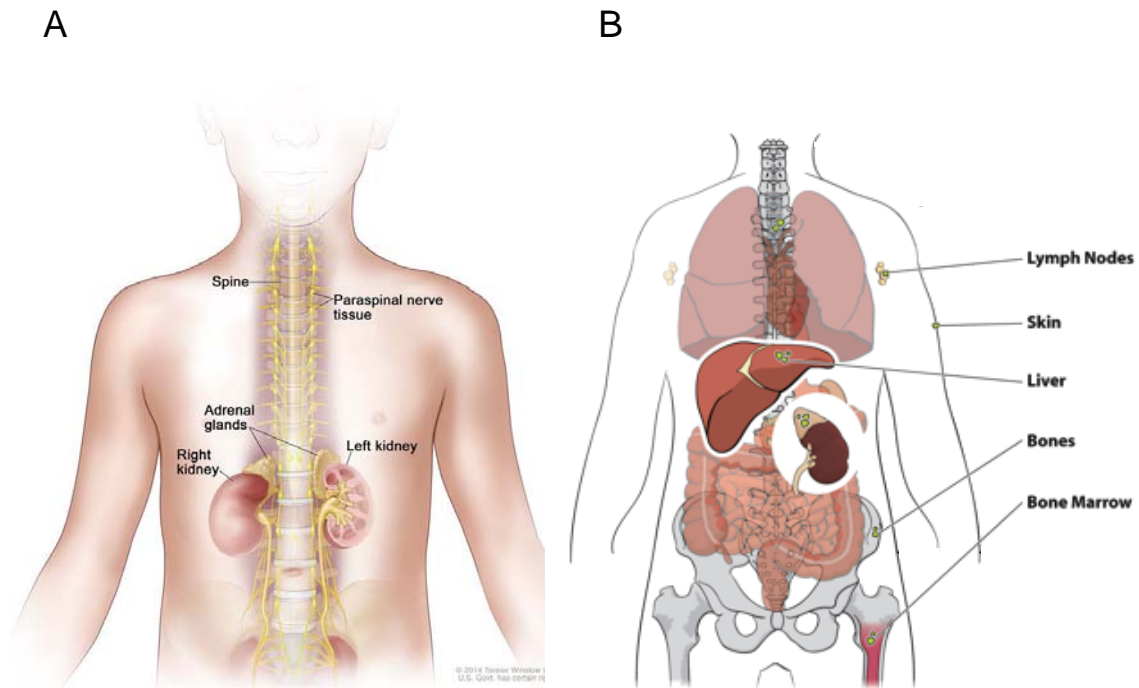
Figure 4: NB primary and metastatic sites. A. Primary sites of NB can be found in the adrenal glands (50% of the cases) and in the paraspinal nerve tissue from the neck to the pelvis (adapted from www.cancer.gov), B. Common metastatic sites in NB (adapted from[172]).

Fifty percent of patients present metastatic disease at diagnosis. Metastatic preferential sites are the regional lymph nodes, liver, skin, bone and bone marrow, whereas metastases in the lungs or the central nervous system are rare[170] (Figure 4). Symptoms related to metastatic disease are: bone pain, fever, weight loss and pallor or bleeding derived from anemia and thrombocytopenia. Other symptoms are specific to the metastatic sites such as localized bone pain and limping for bone metastasis, proptosis (protrusion of the eyeball out of the eye socket) or periorbital bruising for periorbital metastasis. Additionally, respiratory distress, coagulation disorders or even renal impairment can occur derived from massive tumor cell infiltration of the liver, particularly in patients younger than 3 months[173]. Finally 2-3% patients with NB will develop OMS (opsoclonus myoclonus syndrome), a rare paraneoplastic neurological disorder which curse with rapid, involuntary and multidirectional conjugate eye movements (opsoclonus), brief, involuntary twitching of a muscle or a group of muscles (myoclonus), severe irritability and cerebellar ataxia[174].

BIOLOGICAL BEHAVIOR

Neuroblastomas do not follow a common cancer progression pattern; instead, they present heterogeneous progression. A subset of NB tumors can undergo through spontaneous maturation and regression, especially stage 4S tumors (patients younger than 1 year with metastasis limited to the skin, liver or bone marrow)[175,176]. Furthermore, a screening in infants showed a higher detection of NB tumors than the ones that later developed[177], revealing that more NB than were thought undergo spontaneous regression.

The mechanisms underlying the spontaneous regression in NB are not fully understood, perhaps because the great majority of them regress before ever manifesting and only a few ever fully develop. This regression is characterized by a massive death of immature neuroblasts[178–181]. Several hypotheses have tried to explain this phenomenon. One hypothesis proposes that the immunologic system is involved in the regression[182]. In fact, murine models of liver metastasis infused with IL-2 induced natural killer infiltration lead to the regression of the disease, as seen in 4S NB tumors[183]. Others suggest that telomere crisis leads to tumor regression since, at least half of the NB that spontaneously regress, lack telomerase expression[184]. Another hypothesis proposes that the lack of neurotrophic factors in the tumor environment is the cause of tumor cell death[185]. Nerve growth factor (NGF) depletion in developing sympathetic neurons induces the expression of pro-apoptotic genes[186–188]. Finally, other studies reported that programmed cell death, autophagy or lysosomal-mediated cell death have been observed during NB regression[189–191].

Some NB cases undergo spontaneous maturation and differentiation towards benign neuroma or mature NB. Neuroblastic tumors main cell populations are neuroblastic cells and Schawnn cells[192–194]. Schawnn cells are attracted by mitogen agents and chemotactic factors produced by neoplastic neuroblasts. Once in the tumor, Schawnn cells produce and release factors that inhibit proliferation and induce differentiation of the tumoral cells[195] through neurotrophins and their receptors[196].

On the contrary, 50% of NB do not have favorable outcomes and instead, present aggressive behavior, ability to metastasize and become refractory to all current therapies[7].

DIAGNOSIS

Diagnosis of NB requires for a variety of tests that ultimately determines the tumor stage. This will help to stratify patients into risk groups and different treatments will be applied depending on the risk group. Tests used to determine NB include laboratory tests, different types of imaging and pathology evaluation. These test are summarized in Table 2.

Table 2: Diagnostic test to determine NB (Reviewed in Matthay K et al[171])

LABORATORY	Complete blood and platelet count
	Prothrombin time and partial thromboplastin time
	Electrolyte, creatinine and uric acid levels and liver function
	Ferritin and lactate dehydrogenase levels
	Urine catecholamines or catecholamine derivates levels
IMAGING	CT or MRI of the primary site, chest, abdomen and pelvis
	CT or MRI of the head and neck if clinically involved
	123I-metaiodobenzylguanidine (mIBG) scan and then 18F-fluorodeoxyglucose-PET scan if the tumor is not mIBG-avid
PATHOLOGY	Tumor biopsy with immunohistochemistry and the International Neuroblastoma Pathology Committee classification
	Fluorescence in situ hybridization for MYCN
	Array comparative genomic hybridization or other study for segmental chromosomal alterations
	DNA index (ploidy)
	Bilateral bone marrow aspirate and biopsy with immunohistochemistry
	Optional: genomic analysis for ALK and other gene mutations

1.1.4. Neuroblastoma stratification and staging: towards a better patient classification

Since NB has a wide range of clinical and biological behavior, a lot of efforts have been invested in the definition of risk groups. The main goal of these definitions is to identify homogeneous treatment risk groups and facilitate the comparison of clinical trials between international groups[197]. Multiple staging systems have been proposed but the most widely accepted for the past three decades is the International Neuroblastoma Staging System (INSS) [198]. The INSS was completed with the International Neuroblastoma Risk Group Staging System (INRGSS)[197].

INSS CLASSIFICATION

The INSS system was formulated in 1988[199] and revised in 1993[200]. The aim of INSS system was to unify and standardized the classification criteria to facilitate comparison among institutions. The INSS definitions of NB stages are listed in the following table.

Table 3: International NB staging system (adapted from[200])

Stage 1	Localized tumour with complete gross surgical excision, with or without residual disease; no metastasis to the representative ipsilateral lymph nodes that were not attached to tumour.
Stage 2A	Localized tumour with incomplete gross surgical excision and no metastasis to the lymph nodes.
Stage 2B	Localized tumour with or without complete gross surgical excision, with tumour metastasis to the ipsilateral lymph nodes but no tumour metastasis noted in any enlarged contralateral lymph nodes.
Stage 3	Unresectable, unilateral tumour infiltrating across the midline, with or without regional lymph node metastasis, or localized unilateral tumour with contralateral regional lymph node metastasis, or midline tumour with bilateral infiltration or lymph node involvement.
Stage 4	Any primary tumour with metastasis to distant lymph nodes and/or other organs, except as defined for stage 4S.
Stage 4S	Localized primary tumour (stages 1, 2A or 2B) in patients <1 year of age, with metastasis limited to the skin, liver or bone marrow (<10% tumour involvement otherwise it will be considered stage 4).

INRGSS CLASSIFICATION

INRGSS classification was created to solve the problems that INSS had. Surgery differs from one institution to another; therefore, patients stage vary among institutions. In 2005, worldwide experts from pediatric cooperative NB groups met to create the INRGSS. Data from patients around the world between 1990 and 2002 was reviewed to define a pre-surgical staging system that classifies the disease in a uniform manner across institutions[197,201]. These experts tested the prognosis significance of many factors and found that the best ones to define homogeneous cohorts of patients were stage, age, histologic category, tumor differentiation status, MYCN genomic status, 11q aberrations and DNA ploidy.

STAGE

The international NB risk group created a new staging system based on preoperative diagnostic image, which is more robust and reproducible among institutions. They called them image-defined risk factors, which consists of data obtained by imaging technologies[201]. Stages are described in Table 4.

Table 4: International NB risk group staging system (Adapted from[201]).

Stage	Description
L1	Localized tumor confined to one body compartment and not involving vital structures
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease, except those included in stage MS
MS	Metastatic disease in childern <18 months. Metastases confined to skin, liver and/or bone marrow

AGE AT DIAGNOSIS

Age was found to be a strong predictive factor, since NB gradually worsens with increasing age. An 18 months cutoff has shown to be optimal and feasible for patient classification[167,197]. Other studies showed that younger patients usually curse with localized tumors whereas metastases are more frequently found in older children[202].

HISTOLOGY AND GRADE OF DIFFERENTIATION

NB are composed by small, round cells with dense hyperchromatic nuclei and scant cytoplasm. Tumors are classified accordingly to the international neuroblastoma pathology classification system (INPC)[203] which consists of four morphologic categories: neuroblastoma, ganglioneuroblastoma intermixed, ganglioneuroblastoma nodular and ganglioneuroma. More details can be found in Figure 5.
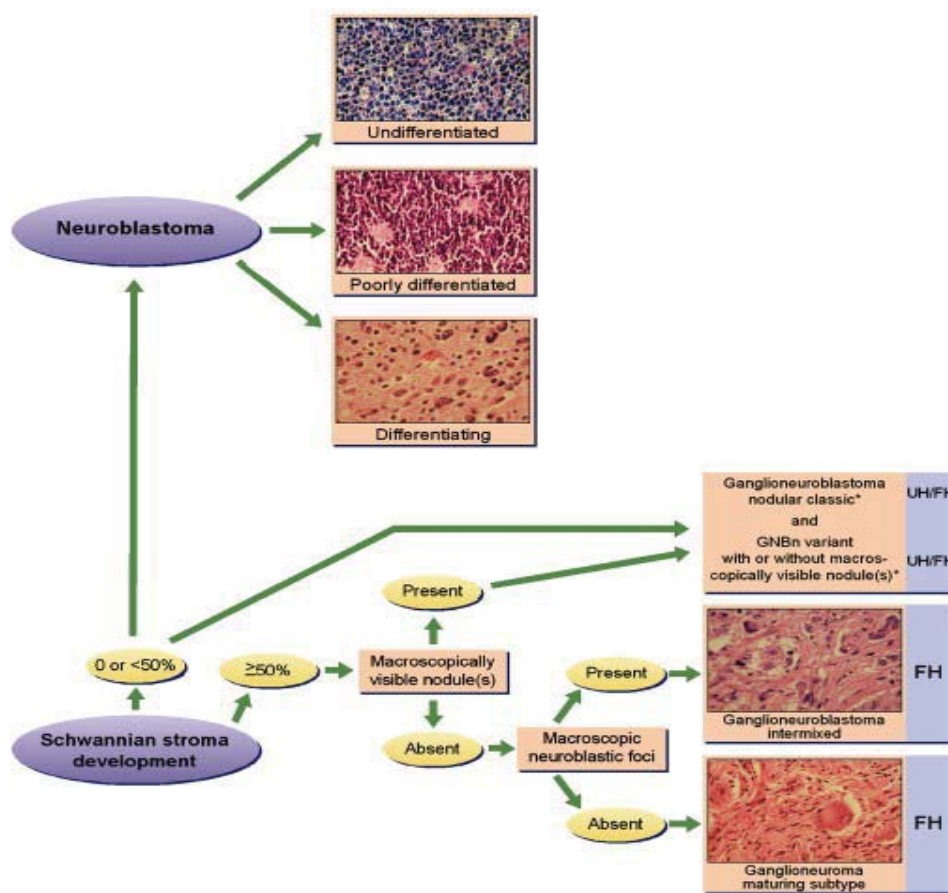
Figure 5: International neuroblastoma pathology classification. Neuroblastoma tumors consists of small, round cells called neuroblasts and have low presence of Schwann cells. In turn, neuroblastoma tumors can be classified in three categories depending on the differentiation status. The rest of the tumors show partial histological differentiation and are rich in Schwann cells. MKC: mitosis-karyorrhexis index, which indicates the percentage of cells that undergo division or apoptosis. UH: unfavorable histology. FH: Favorable histology (Adapted from[577]).

DNA AND CHROMOSOMAL ALTERATIONS

MYCN amplification, 11q deletion and DNA hyperdiploidy are events that strongly correlate with patient outcome.

Combination of these prognostic factors led to the pre-treatment and treatment groups summarized on Table 5.

Table 5: International neuroblastoma Risk group consesus pre-tretment Classification Schema.
GN: ganglioneuroma; GNB: ganglioneuroblastoma; NA: non-amplified; Amp: amplified (adapted from [574]).

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	MYCN	11q Aberration	Ploidy		Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A	Very low
L1		Any, except GN maturing or GNB intermixed		NA			B	Very low
				Amp			K	High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D	Low
					Yes		G	Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E	Low
					Yes		H	Intermediate
			Poorly differentiated or undifferentiated	NA				
				Amp			N	High
M	< 18			NA		Hyperdiploid	F	Low
	< 12			NA		Diploid	I	Intermediate
	12 to < 18			NA		Diploid	J	Intermediate
	< 18			Amp			O	High
	≥ 18						P	High
MS	< 18			NA	No		C	Very low
					Yes		Q	High
				Amp			R	High

Based on the 5-year event-free survival (EFS), these risk categories have the following proportions (Figure 6): very low risk (85% EFS), low risk (75-85% EFS), intermediate risk (50-75% EFS) and high risk (<50% EFS).
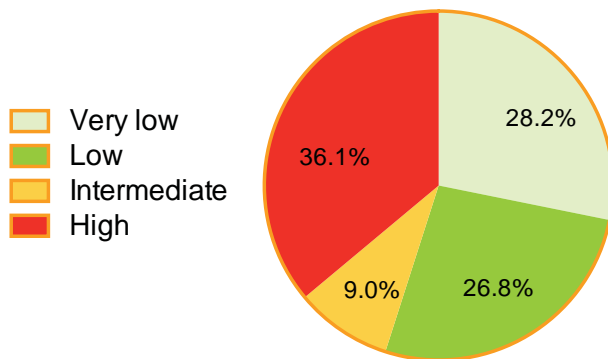
Figure 6: Proportion of Neuroblastoma patients in risk groups. (adapted from Cohn L. S. et al[197])

1.1.5. Neuroblastoma treatment: what is used and needed

TREATMENT OVERVIEW

Current therapeutic strategies are applied based on the INRGSS. These treatments vary depending on the risk group where the patient is included. Figure 7 summarizes the different treatments given to each of the risk groups.
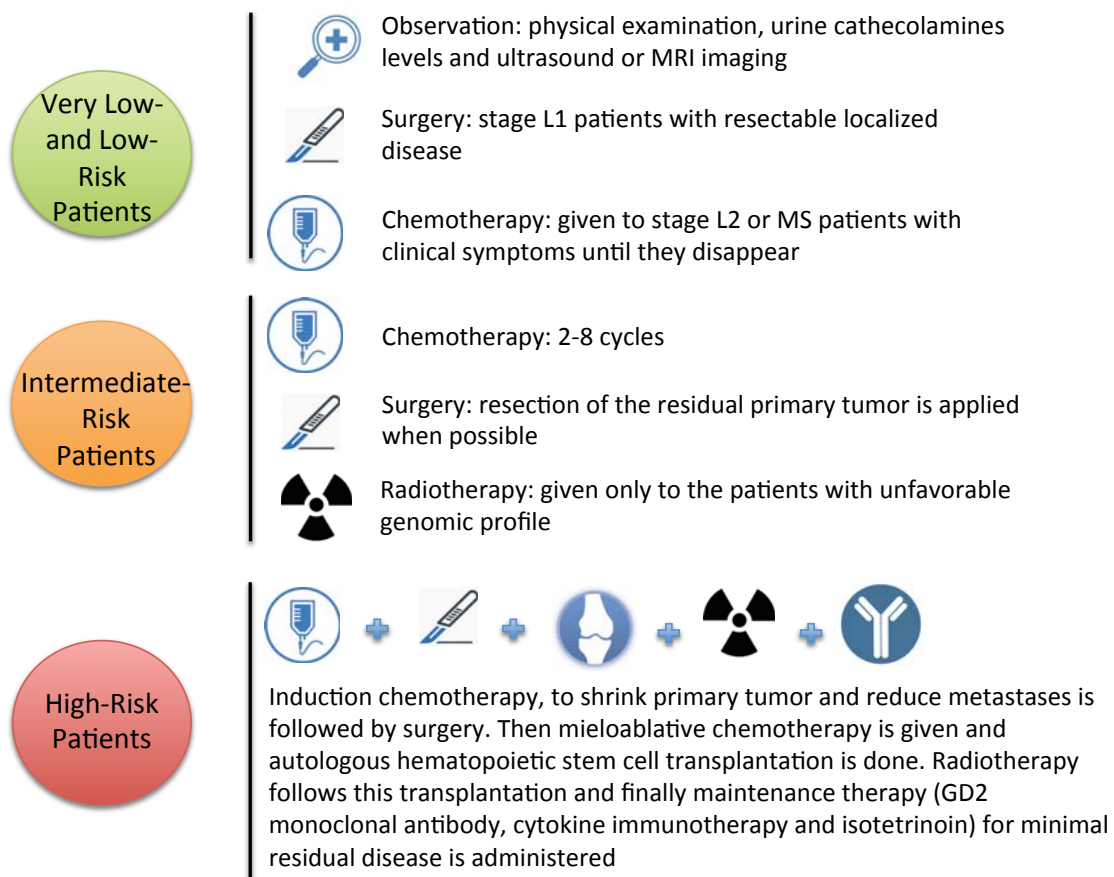
Figure 7: Neuroblastoma treatment overview. Low-risk treatments aim to deliver the minimum therapy wile maintaining good patient survival. High-risk patients survival has increased from 29 to 50% since the introduction of the myeloablative chemotherapy, but still major advances in the treatment of these patients are imperative (reviewed in [171]).

In very low-, low- and intermediate-risk patients usually surgery and non-aggressive chemotherapy is applied. Around 36% of NB patients are classified as high-risk[197]. Standard treatment of this group includes at least four phases and is very aggressive. A detailed explanation of the therapies is outlined below.

INDUCTION CHEMOTHERAPY

The objective of the induction therapy is to reduce the tumor size both, at the primary site and metastases. This reduction facilitates later surgical resection. Along the years, this induction therapy has changed since it was shown that higher dose intensity significantly improved the event free survival[204–206]. Therefore, a combination of chemotherapeutic agents that include anthracyclines, alkylators, platinum compounds and topoisomerase II inhibitors has been developed and demonstrated to be the most effective[207]. This combination is called COJEC: cisplatin, vincristine, carboplatin, etoposide and cyclophosphamide[207].   At the Vall d'Hebron Hospital, COJEC is administered during three months at 10-day intervals following SIOPEN (International Society of Paediatric Oncology Europe Neuroblastoma) instructions[208].

LOCAL CONTROL

Complete resection of the primary tumor is difficult in high-risk NB patients, even after chemo-reduction. The complexity is usually associated to tumor encasement of renal and abdominal vessels or infiltration of the neural foramina. Nevertheless, it has been difficult to determine whether total resection improves patient outcome, mainly because of the difficulties of conducting a randomized surgery trial and the frequent failure of surgery to eradicate metastases on bone and bone marrow[209]. Also, radiotherapy to primary tumor bed after the myeloablative therapy can obscure surgical resection effects[210]. Despite that, in L2 high-risk tumors, gross total resection has shown to improve outcome[211–213], but in M high-risk patients showed no difference whether resection was partial or total[214].

CONSOLIDATION THERAPY

The aim of consolidation therapy is to remove any residual tumor cells after induction chemotherapy and surgery. It consists of myeloablative chemotherapy followed by autologous hematopoietic stem cell transplantation (AHSCT) and

completed with radiotherapy. Initially, a combination of body irradiation and myeloablative chemotherapy was used, but total body irradiation was associated with infertility, impaired growth and secondary malignances. Therefore, whole body radiation was replaced with higher doses of chemotherapy[171].

The introduction of myeloablative chemotherapy supposed an improvement of overall survival to 60% of the patients[212,215]. Trials to assess the best regimen are currently ongoing. One trial showed that a combination of busulfan and melphalan showed same survival results compared to other myeloablative chemotherapy regimen (carboplatin, etoposide and melphalan) but with less severe adverse effects[216]. In addition, trials to evaluate the use of tandem AHSCT are still on going[217–219]. One trial, comparing tandem transplantation versus single transplantation showed a significant improvement in event free survival with tandem regimen[217,219]. Some studies are being conducted to test the use of mIBG to eliminate residual metastatic disease before AHSCT[220,221].

The addition of radiotherapy to the preoperative tumor bed after the AHSCT has shown decreased local recurrence and is currently accepted as standard care for high-risk NB patients[210,222].

MAINTENANCE THERAPY

Despite the myeloablative chemotherapy survival improvement, 50% of children relapse after the transplantation[204]. Therefore, maintenance therapy was included to eliminate persistent minimal residual disease. The addition of isotretinoin (retinoic acid), anti-GD2 monoclonal antibodies conjugated to IL-2 factor or a combination of both after consolidation therapy showed a significant improvement in event free survival[204,223]. Currently, a combination of isotretinoin and anti-GD2 plus IL-2 is the standard therapy at Vall d'Hebron Hospital.

Targeted therapies

Despite the aggressive therapy for high-risk NB, half of the children relapse. Survival for relapsed patients is never more than 1-3 years without recurrence of disease or death. Until now, the most effective treatments for these children

are highly aggressive chemotherapy combinations (either topotecan with cyclophosphamide, irinotecan with temozolomide or topotecan with temozolomide)[224–227], which have achieved temporary complete or partial remission of the tumors.

Nevertheless, patients who survived NB are at risk of substantial disease-related and treatment-induced toxicity. One study showed an increased risk of neurological and musculoskeletal conditions compared with a sibling cohort[228]. Risk of a secondary malignancy after 25 years is 3.6%[229], and many other studies have shown an increase of secondary tumors due to the aggressive treatments[228–230]. In addition, high-risk patients survivors present higher prevalence of endocrinopathy, renal dysfunction, hearing loss[230–232], impaired growth and short stature, poor weigh gain, chronic diarrhea[233], diabetes mellitus and metabolic syndrome[234,235], and reproductive system abnormalities[230].

Given the failure of the treatment in some cases and the aggressive secondary effects for the survivors there is an urgent need to develop new therapies. Some early clinical trials are focused on the study of targeted therapies that directly target cell signaling pathways implicated in NB or antigens frequently expressed in these tumors, thereby reducing secondary effects and inmproving patient outcome.

Anti-GD2 antibodies

GD2 (Disialoganglioside) is a surface glycolipid expressed on more than 98% of NB. GD2 expression in normal tissue is weak and exclusively restricted to peripheral sympathetic pain fibers, melanocytes and neurons[236]. Thus GD2, is a good candidate for NB targeted immunotherapy. Murine, chimeric and humanized monoclonal GD2-directed antibodies have been developed and investigated in clinical trials. GD2 immunotherapy is given as maintenance therapy since GD2 antibody treatment as monotherapy only achieved partial response in clinical trials[237–239]. Some clinical trials are testing whether IL-2 is crucial for the efficacy and if increasing the length of the antibody infusion can reduce adverse effects[240,241]. Humanized anti-GD2 monoclonal antibody with a single point mutation (K322A), which reduces complement-dependent cell lysis, is tested in clinical trials to see if efficacy is retained but treatment toxicity is

reduced[242]. Early-phase trials are evaluating anti-GD2 immunoconjugate Hu14.18-IL2[243], vaccines containing GD2 and GD3 NB antigens[244], anti-GD2 antibody with NK cells[245] and T cells engineered to express chimeric antigen receptors targeting GD2[246].

mIBG therapy

[131]I-metaiodobenzylguanidine (mIBG) is an aralkylguanidine norepinephrine analogue developed to visualize sympathetic neural origin tissue[247]. This molecule targets the norepinephrine transporter, which is present in 90% of NB. It has been recently used as salvage treatment on high-risk patients since it destroys the cells by directed radiation[248] and showed 30-40% of response rate in refractory and relapsed tumors[249–251]. mIBG is also tested in induction chemotherapy and in combination with radio sensitizers[252,253]. The principal acute toxicity of mIBG is myelosupression, which can by bypassed by AHSCT[254,255].

ALK inhibitors

ALK targeted therapies are very promising considering that ALK is expressed on the surface of most NB cells, acting as a tumor-associated antigen[256] and its inhibition leads to cell death[257]. Inhibitors of this protein were previously developed for ALK translocation adult malignancies (anaplastic large cell lymphoma, non-small cell lung cancer, etc). Crizotinib (PF2341066; Pfizer) was the first drug approved by the FDA for cancer treatment and showed remarkable effects in adult tumors[258–260]. In NB, clinical trials with crizotinib alone [261] or in combination with topotecan and cyclophosphamide are being tested in NB with ALK mutations[262]. Also improved ALK inhibitors are being tested in pediatric clinical trials[263,264].

Other targeted therapies

A variety of targeted therapies are currently being developed such as Aurora Kinase inhibitors, which have been shown to destabilize MYCN and induce cell

cycle arrest. In combination with chemotherapy showed favorable response rates in phase I trials[265]. Another examples are polyamine antagonists[266] such as difluoro-methylornithine (DFMO), or inhibitors of ornithine decarboxylase (a MYCN target gene)[267].

RESISTANCE TO THERAPY

Primary NB tumors may present resistance to chemotherapeutic drugs (intrinsic resistance), which leads to relapse. The relapsed tumors may also show multidrug resistance (MDR), which is acquired during the course of treatment (acquired resistance), thereby indicating that molecular changes and mutations are acquired and selected during the chemotherapy regimens. In fact, NB relapsed tumors have shown activating mutations in RAS-MAPK pathways in 78% of the cases[268] and inactivating mutations on the p53 pathway in 49% of the tumors[269]. All these alterations contribute to the resistance of the tumor, permitting the survival of the tumor cells.

MDR mechanisms can be classified into seven categories (reviewed in [166]) (Figure 8):

Drug inactivation

Some anticancer drugs must suffer a metabolic activation in order to be effective, therefore drugs interact with different proteins that can modify, partially degrade or complex the drug with other molecules, leading to its activation. Cancer cells can develop resistance to this type of drugs through the downregulation or mutation of the proteins that activates the drug, such as cytochromes, glutathione-S-transferase superfamily or uridine diphospho-glucuronsoyltransferase superfamily. In NB the lack of GSTM1, a drug-metabolizing enzyme, increases the risk of relapse. Also *NAT1*3*, *NAT1*4*, or *NAT1*10* alleles increase the risk of relapse. On the contrary, *NAT1*11* allele is related to less patients relapse[270].

Drug target alteration

Drug effectiveness depends on the molecular alterations of its target such as mutations or expression levels (either of the target or in one of the downstream components of the target pathway). Targeted therapies against one important protein for cancer are more prone to acquire this type of drug resistance. Many targeted therapies are arising to treat NB, such as Crizotinib, an ALK inhibitor, which has shown effectiveness in NB patients[271]. Nevertheless, Crizotinib is ineffective when ALK has F1174L and D1091N activating mutations[272].

Drug efflux

Enhance efflux leads to a reduction of drug accumulation inside the cells. These mechanisms involves the members of the ATP-binding cassette (ABC) transporter family, which are a family of transmembrane proteins in charge of regulate efflux of many substances, including some anticancer drugs. MDR1,3 and 4 members of the ABC transporter family have been associated with drug resistance and NB outcome. Additionally, its expression is regulated by MYCN[270,273,274].

DNA damage repair

Most of the chemotherapeutic drugs affect DNA integrity; therefore DNA damage repair plays a crucial role in the resistance to these therapies by reversing DNA drug-induced damage. In NB, platinum based chemotherapy can be bypassed through alteration of DNA damage response pathway[275]. Additionally, p53 is infrequently mutated in NB, nevertheless p53 loss confers multidrug resistance in NB[276,277].

Cell death inhibition

Cancer cell are often resistant to therapy trough an impaired cell death (whether if it is apoptotic or autophagic). This resistance can be bypassed by

using a drug that renders cell susceptible to death. For example, BCL2 inhibitors restores chemosensitivity in NB[278]. Also NAIP (neural apoptosis inhibitor protein), has been found overexpressed in NB chemoresistant cells, and exerts this resistance through apoptosis inhibition[279].

Epithelial-mesenchymal transitions

Several factors that are induced during EMT process play important roles in the development of drug resistance. Several integrins and other autocrine factors have been related to drug resistance in cancer. In the case of neuroblastoma, it has been found that during the process of cisplatin-resistance acquisition the EMT process is activated[280]. Also resistance to ALK inhibitor, ceritinib, is mediated through the activation and induction of EMT process[281].

Epigenetics

Epigenetic changes play an important role on the development of drug resistance through the modulation of expression of cellular components that regulates the above-explained mechanisms. More importantly, one epigenetic modulator can control several of the above-mentioned drug resistance mechanisms. Many epigenetic modulators has been found to play an important role in NB chemoresistance, such as JARID1B, the expression of which has been found increased in chemoresistant cells[282], or DNMT3A and B, the overexpression of which has been correlated with cisplatin ressitance[283]. Also miRNA are important players in this process[284].
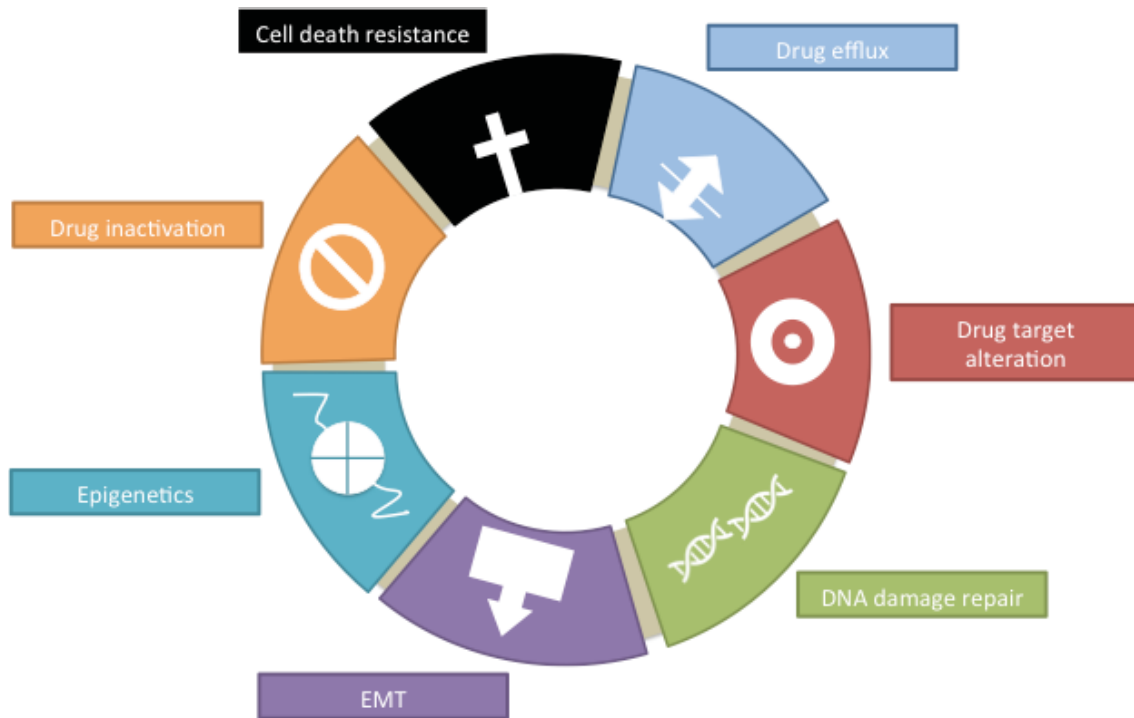
Figure 6: Hallmarks of cell drug resistance. Cancer cells can develop drug resistance through a great variety of mechanisms, ending in resistant tumors. The image shows schematically the different mechanisms that help cancerous cells to become resistant.

Neuroblastoma poses a major challenge in the development of new therapies due to the therapy resistance that some patients present. Thereby, developing Epigenetic therapies to treat these children are the best option for future clinical strategies. Epigenetic treatments are capable to bypass therapy resistance at the same time that they can affect drug-sensitive NB tumors. Additionally, these treatments are reversible, reducing the chances of having severe secondary effects at long term.

1.2. EPIGENETICS

Epigenetics play a major and important role in NB. They are necessary not only for the development of the disease but also for the progression and acquisition of therapy resistance. Thereby, a better understanding of the epigenetic mechanisms in NB is needed. But, first of all: What are epigenetics?

Conrad Waddington first introduced the term Epigenetics in 1942. Though the literal meaning comes from the greek prefix epi- (over, outside of, around) and the word genetics. Specifically, Waddington used "epigenetic landscape" to refer to the molecular mechanisms that converts genetic information into observable traits or phenotypes[285]. In fact, he used the term as a metaphor for how gene regulation modulates development in drosophila melanogaster; the cell can go through a series of mountain ranges and valleys in order to differentiate towards a final tissue type.

In the 1990's, epigenetics term was redefined as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences". For many years the term was used to give an explanation for all the phenomena that could not be explained by genetics[286].

Finally, in 2008, the scientific community reached a consensus definition of epigenetics at a Cold Spring Harbor meeting: "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"[287]. This new definition considers that to initiate a new epigenetic state, transient mechanisms are different from the ones in charge to maintain it. There are three main levels of epigenetic regulation: DNA methylation, histone modifications (e.g. methylation, acetylation, ubiquitination, etc) and regulation of gene expression by non-coding RNA (ncRNA)[288] (Figure 9).
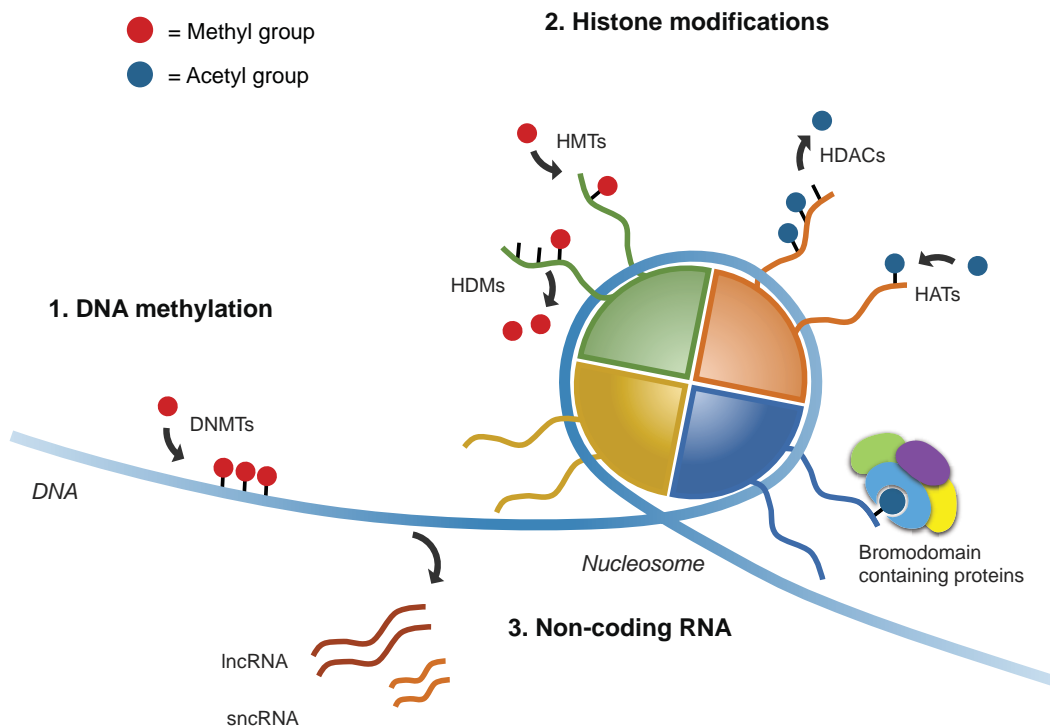
Figure 9: Epigenetic regulation levels. There are three main epigenetic levels. 1. DNA methylation is level, consists of the addition of methyl groups to DNA. 2. Histone modifications is the second level, in this level are encompassed proteins that place, remove and recognize histone covalent modifications. 3. Non-coding RNA is the last level and involves RNA that don't codify for proteins, the non-coding RNA.

These processes may happen simultaneously, maintain a balance and may have additive roles in the control of genome stability and gene regulation. In fact, deregulation of the balance of the epigenetic regulators may lead to different diseases such as cancer[289]. Moreover, epigenetic changes play a major role in the development of cancer resistance to therapies. Therefore, epigenetic therapies are an emerging option for overcoming this problem. Epigenetic regulators usually control the expression of multiple genes and cell signaling pathways; thus inhibition of one of them could have the same effect on several cell processes as if all these pathways were individually targeted with a specific drug. A further advantage of epigenetic therapies is that they act at transcriptional level, which enables the repression of certain genes or the transcriptional reactivation of genes epigenetically silenced in cancer[290,291]. In the past two decades, interest in developing and validating drugs targeting epigenetic regulators has continued to increase. Some compounds have already been approved for the treatment of certain tumors and many other compounds are currently at a pre-clinical stage or already under clinical trials[292–297]. All these advances render epigenetic therapies a promising

alternative for cancers which survival rates are still poor due to resistance to current treatments.

In this section the most important classes of epigenetic regulators that regulate the different epigenetic levels will be defined.

1.2.1.   DNA Methylation

The bases of the epigenetic field were founded on the study of DNA methylation in the 1960's. DNA methylation leads to stable long-term transcription repression, whereas unmethylated DNA tends to remain in a more relaxed structure, thereby facilitating entry of the replicative and transcription machinery[298]. For this methylation to occur, four DNA methyltransferases (DNMT1, DNMT3A DNMT3B and DNMT3L) exist in mammals, the activity of which consists of transferring a methyl group from S-adenosyl-$_L$-methionine to the C5 position of cytosine residues. DNMT are capable of performing *de novo* methylation when the initial pattern is made during embryogenesis and perpetuating this methylation throughout the individual's life. DNMT3A and B are considered the *de novo* DNMT. On the other hand, DNMT1 is responsible for maintaining methylation in the daughter DNA strand during replication. Finally, DNMT3L is a related protein lacking catalytic activity that stimulates *de novo* methylation by DNMT3A and is required for the establishment of maternal genomic imprints (Reviewed in[299]) (Figure 11A).

The association between DNA methylation and cancer was established soon after being discovered. In 1965, Craddock and Magee analyzed DNA methylation in the liver during carcinogenesis[300]; one year later, Silber *et al.* described methylation in normal and leukemic leukocytes[301]. Currently, aberrant DNA methylation patterns have been observed in many different cancers.

The expression of DNMT have been shown to be altered in neuroblastoma. Particularly, DNMT3A/B expression was observed to be higher in high-risk NB tumours and overexpressed in cisplatin-resistant NB cells[302]. Recently, a truncated form of DNMT3B, DNMT3B7, was identified in primary NB tumors. Interestingly, while DNMT3B expression correlates with NB with poor outcome, DNMT3B7 expression was associated with better clinical behavior. In fact, ectopic expression of DNMT3B7 in NB cells inhibited tumor growth *in vivo*

by reducing cell proliferation and increasing apoptosis. Furthermore, reduced tumor vascularity was also observed. Genomic and transcriptomic analyses revealed that DNMT3B7-overexpressing cells had higher levels of genomic methylation and increased expression of genes related to the retinoic acid pathway. Consistent with these findings, treatment of DNMT3B7-overexpressing cells with all-trans retinoic acid enhanced NB differentiation[303]. Why DNMT3A/B and DNMT3B7 have opposite roles and whether these DNMT target different genomic regions remain to be elucidated.

1.2.2.   Histone modifications

Histones are the evolutionary solution to compact large amounts of DNA in the nucleus of eukaryotic cells. Approximately 147 bp of DNA are wrapped on histone octamers (formed by H2A, H2B, H3 and H4) forming a nucleosome. Nucleosomes are assembled in successively higher-order structures to eventually form a chromosome. Nucleosomes build chromatin, which can exist as euchromatin (decondensed and transcriptionally active) or heterochromatin (condensed and transcriptionally inactive). The compaction of chromatin is regulated by modifications on the histone tails. N- and C-terminal domains protrude from the nucleosome and are subjected to different covalent post-transcriptional modifications such as methylation, acetylation, phosphorylation and sumoylation. The enzymes responsible for these covalent modifications are called "writers", whereas the enzymes that remove these marks are called "erasers". Finally enzymes capable of recognizing histone marks are called "readers" (reviewed in [304–306]).

HISTONE METHYLTRANSFERASES

Histone methyltransferases (HMT) are a class of histone writers that transfer methyl groups from S-adenosyl methionine to histone-specific lysine or arginine residues on histones[307,308]. Histone methylation is involved in different processes such as chromatin compaction, X-chromosome inactivation, genomic imprinting and repression or activation of transcription, among other tasks. These functions are influenced by the site and degree of methylation on specific residues (reviewed in[308,309]). Histone methylation usually occurs on H3 and H4 histone tails.

To date, approximately 60 HMTs have been identified. HMT are classified depending on the histone amino acid that is methylated: lysine methyltransferases (PKMT) modify lysine residues by mono-, di- or tri-methylation and are classified in a SET domain containing or a non-SET domain containing PKMT [307,308]. Arginine residues are mono- and symmetrically or asymmetrically di-methylated by arginine methyltransferases (PRMT)[310] (Figure 11B).

In the last decade, some SET-PKMT have been shown to participate in the development and maintenance of NB. On the one hand, low expression levels of NSD1 (a PKMT) were found to be indicative of poor outcome in high-risk NB. NSD1 expression restoration caused cell growth inhibition *in vitro*[311]. On the other hand, high expression levels of other PKMT such as WHSC1 have been shown to correlate with poor survival, negative prognostic factors and metastatic disease[312]. Despite their association with prognostic factors, the functional significance of these HMT remain to be determined. Regarding PRMT, only PRMT5 to date has been studied in NB. PRMT5 expression is elevated in NB with MYCN amplification. PRMT5 silencing reduced cell growth and induced apoptosis, only in MYCN-amplified NB cell lines but not in non-MYCN amplified or non-transformed cell lines. Furthermore, PRMT5 interacts with MYCN and regulates its stability[313].

HISTONE DEMETHYLASES

Histone methylation is a balance resulting from the opposed activity of HMT and histone demethylases (HDM). KDM1 (also known as LSD1) was the first enzyme found to be capable of removing the methyl group from mono- and di-methylated Lys 4 in histone 3 (H3K4me1/2)[314]. HDM can be classified into two lysine HDM families: i) the KDM1 family and ii) the JHDM family. While proteins from the KDM1 family demethylate mono- or di-methylated lysines, those of the JHDM family demethylate tri-methylated lysines. Of note, JMJD6 (a member of the JHDM family) is also an arginine-specific HDM that demethylates H3R2me1/2 and H4R3me1/2[315] (Figure 11C).

KDM1A was the first HDM the expression of which was found to correlate with adverse outcome and undifferentiated tumors. Loss of function experiments showed that KDM1A silencing resulted in a reduction in cell proliferation, colony formation, migration and invasion of NB cell lines[316,317]. Mechanistically, KDM1A can interact with MYCN and repress the expression of some tumor-suppressors (e.g. CDKN1A/p21) and differentiation-associated genes[318,319].

The JHMD family is larger than the KDM1A family and, therefore, there are more members of the family associated with NB. One of these members is

JMJD1A (also known as KDM3A), found in a search for MYCN direct mediators on cell migration, invasion and metastasis. Genetic and pharmacologic inhibition of JMJD1A suppressed NB cell migration and invasion[320].

Similarly to JMJD1A, KDM4B knockdown reduced NB cell proliferation and induced differentiation, *in vitro* and *in vivo*[321]. Mechanistically, KDM4B physically interacts with MYCN, removes histone methylation marks at MYCN binding sites, and blocks the transcription of MYCN direct targets such as miR-17–92 cluster, CDC25A, TRIP13, and VCAN. [321].

Recently, JARID1B was found overexpressed in NB cell lines, and high expression levels were observed to negatively correlate with survival rates in human tumor samples. Moreover, JARID1B was also found highly expressed in tumor spheres; thereby conferring resistance on chemotherapeutics. Silencing JARID1B resulted in a decrease in tumor invasion, sphere formation and increased sensitivity to cisplatin[322]. The effects of JARID1B inhibition *in vivo* remain to be determined.

HISTONE ACETYLASES

A further histone modification is the addition of acetyl groups from acetyl-CoA to specific histone lysine residues. This process can be carried out by histone acetyltransferases (HAT)[323]. HAT are capable of modulating gene transcription by altering histone acetylation patterns [324,325] or by acetylating non-histone substrates such as transcription factors (Reviewed in [326]). HAT are usually classified based on sequence similarity and structure which define five families: GNAT, p300/CBP, MYST, SRC (nuclear receptors coactivators) and others (Reviewed in[327]) (Figure 11D).

To date, no HAT expression or functional studies have been conducted either in clinical or preclinical NB models. Nevertheless, our *in silico* analyses did show that several HAT are differentially expressed in advanced stages of NB. In most cases, HAT levels are found to be lower expressed in patients with poor prognosis (i.e. stage 4 with MYCN amplified), thereby indicating that strong criteria for selection of patients who could benefit from HAT inhibition therapies must be taken into account (Figure 10).
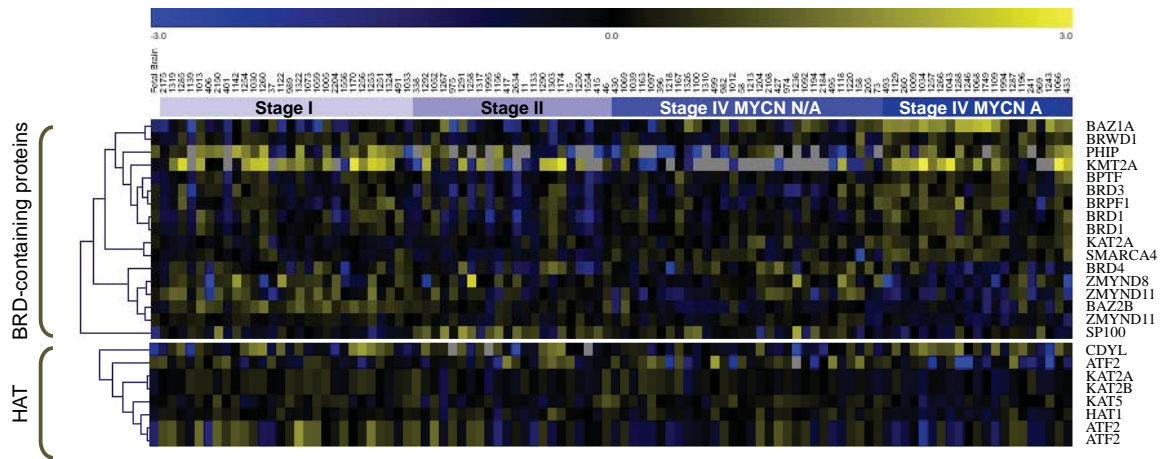
Figure 10: *In silico* **analysis of HAT and BRD-containing proteins expression in NB.** mRNA expression levels from 39 different BRD-containing proteins and 16 histone acetyltransferases were analyzed in neuroblastoma samples from the E-GEOD-3960 (n = 101) dataset. Samples were sorted according to disease stage and MYCN status. Heatmap represents the differentially expressed genes (p<0.05) between the groups of patients with poor prognosis (stage 4, MYCN amplification) versus the rest of patients (i.e. stage 1, 3 and 4 MYCN non-amplified).

HISTONE DEACETYLASES

Histone acetylation and deacetylation have a dynamic balance that controls gene transcription. While histone acetylation is associated with active transcription, histone deacetylation is associated with transcriptional repression. Hypoacetylated nucleosomes usually result in tightly compacted chromatin, thereby restricting access of transcription factors to their target DNA and leading to transcription repression (reviewed in [328]). An alteration in this acetylation balance may result in the development of diseases such as cancer. The 18 Histone deacetylases (HDAC) encoded in our genome can be classified based on their homology with yeast HDAC[329] as: class I, which includes HDAC1,2,3 and 8, class II HDAC4,5,6,7, 9 and 10, class III sirtuins (SIRT1-7) and class IV only HDAC11[330].

All HDAC share a conserved histone deacetylase domain; however, they vary in location, structure and expression patterns[331]. Classes I, II and IV share homology in structure and sequence and require a zinc ion for their catalytic activity. Class III HDAC share no similarities with the other classes and require nicotinamide adenin dinucleotide (NAD$^+$) for their activity[332] (Figure 11E).

Only two HDAC have been reported to be associated with NB prognosis. Particularly, HDAC8 and HDAC10 was found to be overexpressed in high-risk

NB and their inhibition resulted in reduced NB cell proliferation *in vitro*[333,334]and *in vivo*[335]. Moreover, the inhibition of HDCA8 and 10 was found to increase doxorubicin sensitivity[333,336].

One of the most important genetic factors associated with NB outcome is the genomic amplification of the transcription factor MYCN, a driver oncogene in NB, which in turn regulates the expression of a myriad of genes associated with cell proliferation, survival and metastasis, among others. Some HDAC have been shown to participate in a positive feedback loop with MYCN. One of these examples is HDAC2, which is necessary for the expression of MYCN[337] and, in turn, MYCN drives the expression of HDAC2[338]. Other studies suggested that HDAC2 cooperates with MYCN to repress the expression of the tumor suppressor gene TP53INP1 by direct binding to its promoter, resulting in increased cell proliferation and survival[339]. HDAC5 has also been found to be upregulated by MYCN in NB and *vice versa*. Similar to HDAC2, MYCN and HDAC5 interact and form a protein complex. Overexpression of HDAC5 was able to block NB differentiation and induced proliferation[340].

A similar positive feedback loop was also described for the Class III HDACs such as SIRT1 and SIRT2. SIRT1 studies revealed that MYCN induced the transcription of SIRT1 directly and increased the stability of this oncogenic protein. Furthermore, the pharmacologic inhibition of SIRT1 (cambinol) reduced tumorigenesis in a MYCN-driven neuroblastoma transgenic mouse model[30]. SIRT2 was also proven to regulate and be regulated by MYCN. SIRT2 aids to the stabilization of MYCN. SIRT2 inhibitors (i.e. AC-93253) suppressed NB cancer cell proliferation[31]; however, there is no evidence of the effects of SIRT2 inhibition *in vivo*.

The therapeutic potential of inhibiting other HDAC regardless of their association with clinical parameters in NB has been studied. For instance, silencing of HDAC11[341] or HDAC6[342] resulted in apoptotic cell death. Similarly, HDAC1 downregulation through siRNA led to increased sensitivity of NB cell lines to chemotherapeutic agents such as etoposide[343].

CHROMATIN READERS

To translate the pattern of histone modifications into a functional phenotype, these modifications must be recognized by proteins called "readers". These readers are bromodomain (BRD), chromodomain and tudor-domain containing proteins, which recognize histone marks and recruit other proteins needed to start or inhibit transcription. Bromodomain-containing proteins are capable of recognizing the acetylation of histones whereas chromodomains and tudor-domains recognize methylated histones[344,345]. BRD-containing proteins are highly conserved throughout evolution, and can perform various functions such as histone acetylation, chromatin remodeling and transcriptional activation[346]. The human homolog of the drosophila gene Brahma (Brm)[347] was the first of 61 human BRD to be described, part of 46 BRD-containing proteins [348]. All known BRD have a central hydrophobic pocket with a highly conserved asparagine residue responsible for the binding to the acetylated lysines of histones[349] (Figure 11F).

Only one study to date has demonstrated the correlation of a BRD-containing protein and NB outcome. BPTF was found amplified in 55% of NB cases due to gain of the 17q24.3 locus[350]. *In silico* analysis of mRNA expression data sets in NB (EGEOD-3960, Figure 10) shows that, at least, 15 BRD-containing proteins are differentially expressed in patients with advanced disease and poor prognosis (10 found upregulated, 5 downregulated), thereby suggesting that they could be new potential therapeutic targets for NB.
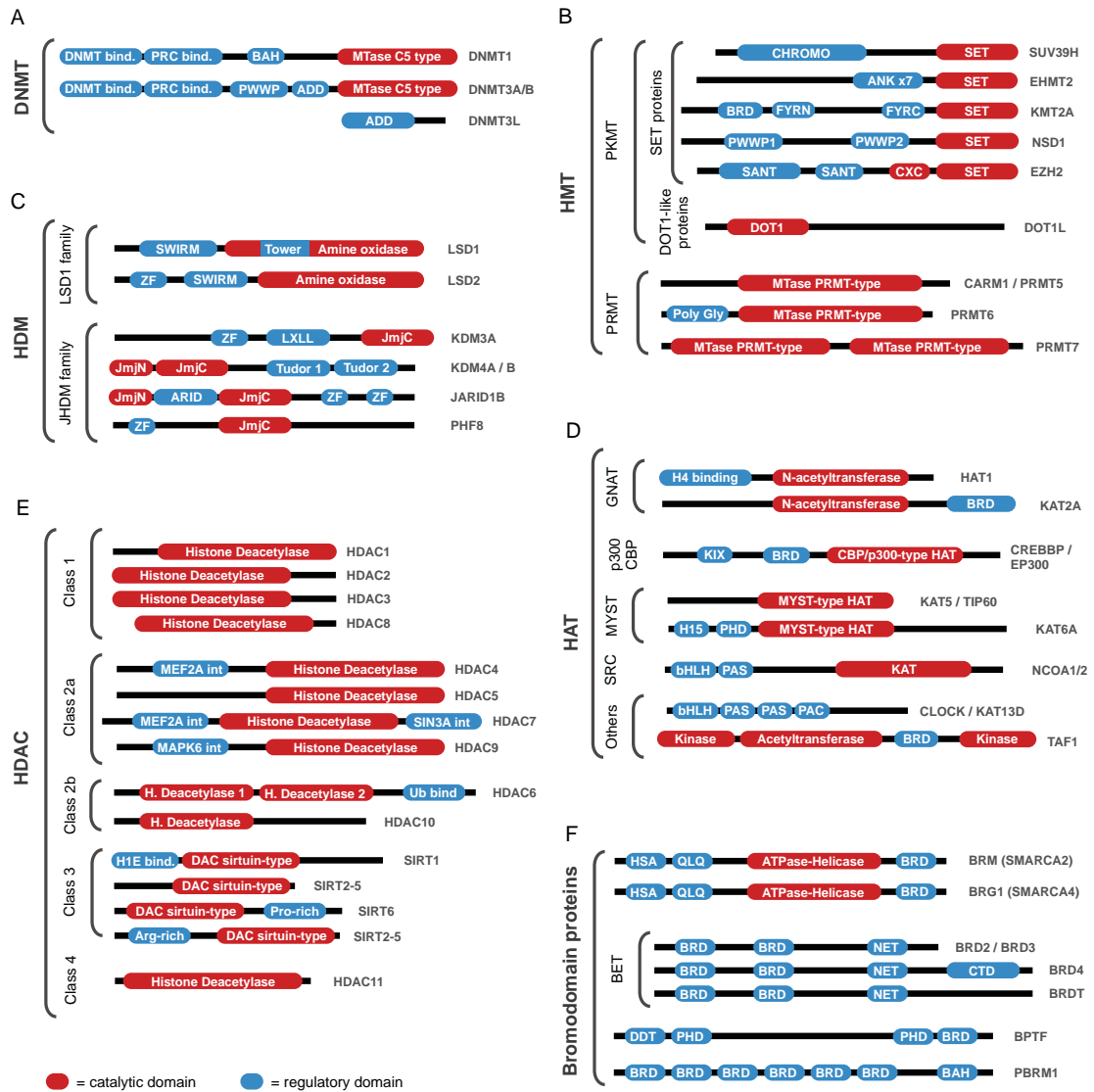
Figure 11: Schematic representation of the members of the main histone modifiers families and subfamilies. Representative members of (A) DNMT, (B) HMT, (C) HDM, (D) HAT, (E) HDAC and (F) BRD-containing proteins are included showing their domain configurations and indicating the catalytic region, the main target of epigenetic drugs. Sources: UniProt[326], InterPro[327].

1.2.3.   Chromatin remodeler complexes

Chromatin is a dynamic structure that requires many proteins working in perfect synchronization to result in correct gene transcription and regulation. Chromatin remodeler complexes (CRC) are sets of proteins responsible to maintain this synchronization and regulate the access to nucleosomal DNA. There are two major classes of CRC: histones or DNA covalent-modifiers complexes and ATP-dependent chromatin remodeling complexes (Figure 12). Regarding the first major class of CRC, PRC2 complex is the most well-known and studied complex. It consist of at least 5 subunits: EZH1 or EZH2 (enzymatic subunits) and EDD, SUZ12, RBBP4/7 and AEBP2[351]. Depending on the cellular context many other associated factors (proteins and RNA entities) can regulate the activity, targeting and specificity of PRC2[352]. EZH1 or EZH2 are responsible of the enzymatic activity; they catalyze the addition of methyl groups to lysine residues of histones. On the contrary, other PRC2 proteins are responsible of diverse non-catalytic functions, such EDD or SUZ12 that are described to recognize histone marks (reviewed in [353]).

All ATP-dependent chromatin remodeling complexes contain an ATPase subunit belonging to the SNF2 family of helicases. They can be classified into four families based on their sequence similarity among their ATPase domains: SWI/SNF (switch/sucrose non-fermentable), ISWI (imitation SWI), CHD (chromodomain helicase DNA-binding) and INO80 (Inositol requiring 80 or SWI2/SNF2 related (SWR)). Each of these CRC can contains up to 16 subunits (reviewed in [354,355]).

CRC exert a gatekeeper function, thus many cancer present mutated complexes since it confers them an advantage to proliferate and survive. In fact, mutations and alterations in CRC expression ultimately lead to aberrant chromatin organization thus, triggering or helping to develop cancer[356,357].

Covalent Modifiers

ATP-dependent Chromatin Remodelers

Covalent modifiers CRCs (i.e. PRC2)

ATP-dependentC RCs (i.e. SWI/ SNF)

Placement of covalent histone modifications

DNA translocation and loosening of chromatin

Covalent histone modifications

Nucleosome sliding

Nucleosome ejection
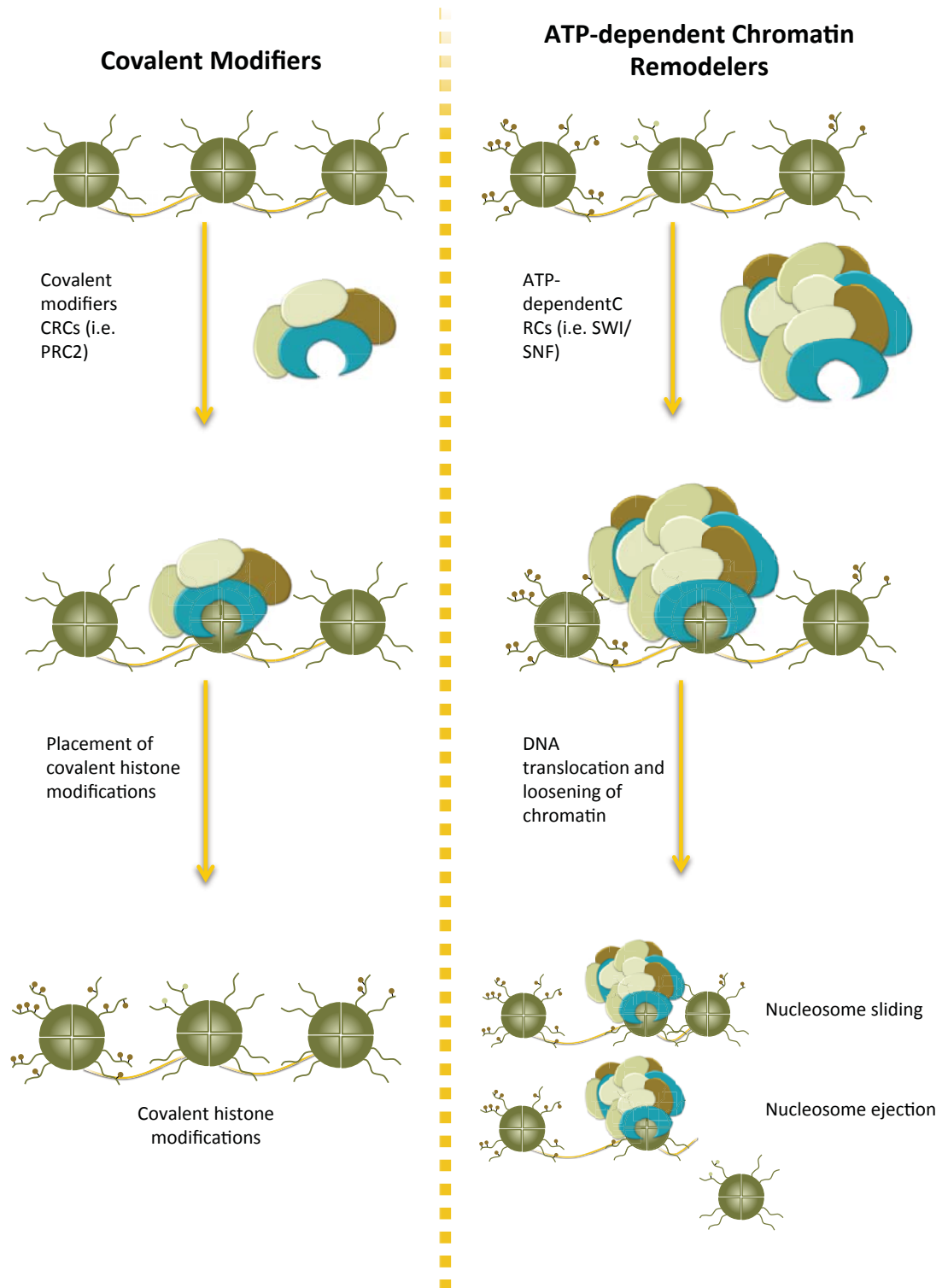
Figure 12: Chromatin Remodeler Complexes. Chromatin remodeler complexes are classified into: Covalent modifiers, which are capable of placing histone covalent modifications, and ATP-dependent chromatin remodelers, which recognize and bind to chromatin covalent modifications and opens or close the chromatin.

1.2.4.   Non-coding RNA

The last level of epigenetic regulation is through non-coding RNAs (ncRNA). These RNA are never transcribed into proteins, yet they exert regulatory functions. They can be classified as small ncRNA (sncRNA), when their length is less than 200 nucleotides, or long ncRNA (lncRNA), when their length is above 200 nucleotides. Among the functions they can perform are chromatin modifications, polymerase III activity regulation, transcriptional interference, splicing, editing, mRNA stability and translation initiation[357] (Figure 13).
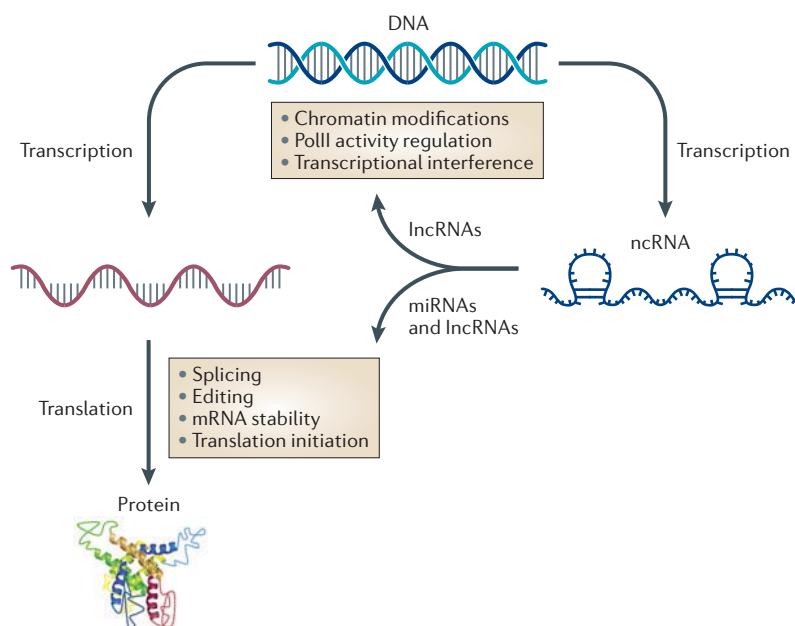
Figure 13: Changing of the Central dogma for non-coding RNA. Francis Crick in 1958 proposed the central dogma of biology, but this dogma was changed due to the discovery of ncRNA, which have the capacity to regulate gene expression at different levels (Adapted from Wahlestedt C. et al 2013[358])

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

Despite all advances in standard and targeted therapies, 5-year survival rates of high-risk NB are still below 50% and up two thirds of patients who survive carry throughout their lives serious health problems derived from the aggressive treatments. The low survival rate of high-risk NB is mainly due to the development of resistance to current therapeutic agents and progression of the disease. Therefore, new treatment strategies must be developed to improve the efficacy and safety of high-risk NB therapies.

Owing to the multiple mechanisms that are involved in resistance to therapy, our hypothesis is that epigenetic regulators could be a good therapeutic strategy to overcome resistance in cancer treatment. Additionally, targeting epigenetic regulators, by definition, do not cause DNA damage, thereby making these treatments reversible and reducing potential long-term secondary effects.

Our hypothesis will be validated through the following objectives:

o **Objective 1:** Analyze the expression levels of epigenetic regulators in primary NB tumor samples and correlate their expression with clinical variables such as disease stage and/or survival.

o **Objective 2:** Study the functional role of the epigenetic regulator that best correlates with clinical data and characterize its implication in NB, both *in vivo* and *in vitro*.

o **Objective 3:** Characterize the mechanisms of action of the selected epigenetic regulator in NB and develop strategies to modulate its function.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Reagents

Reagents used in this thesis are listed on Table 6:

Table 6: Reagents

Reagent	Description	Supplier
Lipofectamine	transfection reagent	ThermoFisher
Crystal Violet	staining reagent	Sigma
Hoechst 33258	fluorescent reagent	Sigma
Ac-DEVD-afc	Caspase substrate	Millipore
PI	Propidium Iodied	Sigma
FGF2	Fibroblast growth factor	ProSpec-Tany Technogene Ltd
EGF	Epidermal growth factor	ProSpec-Tany Technogene Ltd
B27	growth factor	Invitrogen
L-Glutamine	Amino acid supplement	Invitrogen

Drugs used in this work are listed in the following table:

Table 7: Drugs

Reagent	Description	Working concentration	Supplier
PI_103	multitargeted PI3K inhibitor (p110α/β/δ/γ)	1 μM	Sigma-Aldrich
ABT-283 (Navitoclax)	Bcl-2, Bcl-xL and Bcl-w inhibitor	3_12 μM	APExBio
iCRT14	inhibits interaction between β-catenin and TCF4	25 μM	Sigma-Aldrich
XAV939	Inhibits tankyrase1/2	0_100μM	Selleckchem
DMSO (Dimethyl sulfoxide)	A polar and non-polar dissolvent	_	Sigma-Aldrich

3.2. *In silico* analysis of mRNA neuroblastoma datasets

Gene expression data of NB patients (Table 7) were used to analyze the most relevant epigenetic regulator genes (see Annex Table1) with the R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi).

Table 8: Neuroblastoma expression and prognosis data sets employed in this work.

Dataset	Number of samples	Parameter	Reference
GSE3960	101	mRNA expression levels	[359]
GSE16237	51	mRNA expression levels	[360]
GSE16476	88	event-free and overall survival	[361]
GSE45547	649	event-free and overall survivial	[362]

Two different analyses were made to evaluate the potential role of epigenetic regulators in advanced NB. The first approach encompassed the use of two different primary NB datasets to find mRNA level deregulation among tumor stages. The expression of each epigenetic regulator was analyzed comparing Stage 4 MYCN-amplified tumors (the most aggressive NB tumors) *versus* other stages present in the study (i.e. Stage 1, 2, 3, and 4 MYCN non-amplified).

The second approach was to generate Kaplan-Meier survival curves. These curves are used in medical research to measure the fraction of patients that are alive for a certain period of time In this analysis, patients were divided into two groups that expressed high or low levels (above or below median) for each gene.

3.3.   Human samples

Primary tumor tissue samples from 19 NB patients referred to the Spanish Reference Centre for NB Biological and Pathological studies (Department of Pathology, University of Valencia) were obtained immediately after surgery and snap frozen in liquid nitrogen and stored at –80 °C until processing. Tumors were examined by the pathologist to confirm NB diagnosis and the presence of at least 60% of tumor tissue sample and histopathologic classification. Approval to collect neuroblastoma specimens was granted by Hospital Clínico of Valencia, Clinical Research Ethics Committee (protocol number B.0000339, 2014/399). All patients gave their written informed consent.

3.4.   Immunohistochemistry

Sections were deparaffinized in xylene, rehydrated through graded alcohols and rinsed in distilled water. Heat-induced epitope retrieval was performed in a

1200-Watt microwave oven at 100% power in 10 mM citrate buffer pH 6.0 for 20 min. Primary antibody incubation and detection were carried out at 37 °C on a NEXes instrument (Ventana Medical Systems) using Ventana's reagent buffer and detection kits. Appropriate secondary antibodies conjugated with streptavidin-horseradish-peroxidase were used. The complex was visualized with 3,3-diaminobenzidene and nuclei were counterstained with hematoxylin, dehydrated and mounted with permanent media. Immunoreactivity of BRG1 was scored by intensity (0–4) and percentage of positive cells (0–4). Relative expression was obtained by multiplying intensity by percentage scored by an attending pathologist (SN).

3.5.   Gene expression analysis by qPCR

3.5.1.   RNA extraction and quantification

Before extraction, cells were harvested, washed with PBS 1x, and pelleted at 800g in a tabletop centrifuge. At this point, samples can be kept at -80ºC until further use.

RNA was extracted from cell lysates using RNeasy Mini Kit (Qiagen) following manufacturer's instructions. It consists on a guanidine-isothiocyanate sample lysis and homogenization, followed by the addition of ethanol to the lysate to create the ideal binding conditions of RNA to the silica membrane used for purification, which allows to wash contaminants out. Finally, RNA is eluted with RNase free water.

Quantification of the RNA was done using a Nanodrop (Thermo Fisher Scientific) spectrophotometer.

3.5.2.   RNA reverse transcription

Before assessing mRNA expression levels, RNA samples must be converted to complementary DNA (cDNA) through reverse transcription (RT).

To this end, between 0.3 and 1 µg of RNA were subjected to DNase treatment and retrotranscription using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturer's instructions. This kit contains the necessary components for mRNA-cDNA conversion, i.e. Reverse transcriptase, RNase inhibitor protein, dNTPs, random hexamers, and oligo dT. These last two are oligonucleotiedes that anneal at random regions or the poly A-tail of mRNA, respectively. The mix was loaded into a thermal cycler under the following protocol: 25ºC during 10 min and 42ºC during 60 min to retro-transcribe RNA; 95ºC during 5 min to stop the reaction; 4ºC, ∞, to maintain samples in optimal conditions. The resulting cDNA was stored at -20ºC until further use.

3.5.3. Quantitative PCR

To analyze mRNA expression levels, cDNA samples were submitted to quantitative PCR using a SYBR-based protocol. Briefly, primers against the corresponding genes were designed using primer designing tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with an optimal Tm of 60ºC, a PCR product ranging from 80-200 nucleotides and adding exon-exon spam conditions which allow that both primers will anneal in the same exon; thereby, allowing the detection of genomic DNA. In the case that the primer binds at an exon junction, genomic DNA detection is impeded. In order to ensure that no genomic DNA is contaminating our samples, DNase is added. Primers were synthetized through Sigma-Aldrich. Before performing the qPCR, primers were tested in a conventional PCR. The primer sequences used in this project are listed in Table 9.

Table 9: qPCR primers

Name	Sequence		Amplicon size (bp)
BCL2	Fw: 5'GGTGGGGTCATGTGTGTGG3'		89
	Rv: 5'CGGTTCAGGTACTCAGTCATCC3'		
BRG1	Fw: 5'TACAAGGACAGCAGCAGTGG3'		80
	Rv: 5'TAGTACTCGGGCAGCTCCTT3'		
CDK6	Fw: 5'GGTGGGGTCATGTGTGTGG3'		89
	Rv: 5'CGGTTCAGGTACTCAGTCATCC3'		
CXCR4	Fw: 5'ACGCCACCAACAGTCAGAG3'		96
	Rv: 5'AGTCGGGAATAGTCAGCAGGA3'		
DKK1	Fw: 5'CCTTGAACTCGGTTCTCAATTCC3'		138
	Rv: 5'CAATGGTCTGGTACTTATTCCCG3'		
FAM69	Fw: 5'GCAGCGTAACGAGTTCCTG3'		91
	Rv: 5'GGTGAGGTAGAGGTCCCCAC3'		
FN1	Fw: 5'AGGAAGCCGAGGTTTTAACTG3'		106
	Rv: 5'AGGACGCTCATAAGTGTCACC3'		
GAPDH	Fw: 5'CGCTCTCTGCTCCTCCTGTT3'		100
	Rv: 5'CCATGGTGTCTGAGCGATGT3'		
L27	Fw: 5'AGCTGTCATCGTGAAGAA3'		127
	Rv: 5'CTTGGCGATCTTCTTCTTGCC3'		
MAP2K6	Fw: 5'AAACGGCTACTGATGGATTTGG3'		78
	Rv: 5'CAGTGCGCCATAAAAGGTGAC3'		
SEMA3A	Fw: 5'CTATCTTCCGAACTCTTGGGCA3'		77
	Rv: 5'CTTTGGATCATTGAGCCACCT3'		

Then, SYBR Green master mix (Thermo Fisher Scientifics) was mixed at 1x concentration with the primers (0.4µM) and 1:10 diluted cDNA. qPCR reaction was performed in a 94- or 384-well plate and all the reactions were done in triplicate. To normalize, GAPDH or L27 ribosomal gene were used as housekeeping genes. Detection was performed using the 7900HT Real-Time PCR system using standard settings (40 cycles, 15 sec at 95ºC followed by 1min at 60ºC).

After PCR reaction, data was analyzed using the 7900HT Sequence Detection System 2.3 software (Thermo Fisher scientific) and applying the relative quantification method. The mRNA levels were quantified using Ct

values, which are the number of cycles at where the fluorescence signal reached a predetermined threshold. Higher cDNA copy numbers correlate with higher mRNA expression levels and those are indicated by lower Ct values, which correlate with fluorescent signal detection at earlier PCR cycles. To eliminate loading errors and normalize, Ct values of the housekeeping genes are subtracted from Ct values of the gene of interest. Relative quantification methodology was described by Livak and Schmittgen[363] and uses the comparative $2^{(-\Delta\Delta Ct)}$ method.

3.6.   Protein extraction and detection

3.6.1.   Protein extraction

Protein extraction must preserve de integrity of the extracted proteins during the process; therefore, the extraction must be efficient and ensure that there is no degradation in order to get a close representation of their physiological state in the living cell.

To achieve a good integrity of the proteins we first rinsed the cells with PBS pH 7.2 before lysis. Then we used RIPA buffer 1x (ThermoFisher Scientific) supplemented with 1x EDTA-free complete protease inhibitor cocktail (Roche) and the phosphatase inhibitors sodium fluoride and sodium orthovanadate. Supplemented RIPA was added to cells keeping a proportion of 1:20 between cell pellet and lysis buffer. Cells were incubated on ice for 20 min prior centrifugation at 16,000 xg, 4ºC for 5 min. Under these conditions, DNA and membranes remains in the pellet while supernatant is enriched in nuclear and cytosolic proteins. Harvested supernatants were kept at -20ºC.

3.6.2.   Protein quantification

Lowry DC protein assay (Biorad) was the chosen method to quantify protein concentration. This method measures peptide bonds and the radical groups of tyrosine and tryptophan, and to a lesser extent cysteine, cysteine and histidine, under alkaline conditions. In fact, divalent cooper ions form complexes with peptide bonds, which reduces them into monovalent copper ions. The

monovalent copper ions and the radical groups induce the Folin reagent (yellow color) reduction, thereby destabilizing the Folin reagent and inducing its reduction to molybdenum/tungsten blue (blue color).

One μL of cell lysate was loaded in triplicates on a transparent 96 well plate. Then, 25μL of Reagent A/Reagent S mix to the wells (100/2 ratio of Reagent A/Reagent S). After that, 200μL of Reagent B was added to the wells and incubated at room temperature for 20 min. Protein concentrations were measured in a spectrometer using absorbance between 650-700nm. Finally, sample protein concentrations were calculated comparing OD values from calibrating curve (from reference BSA protein concentrations) to sample OD.

3.6.3.   Western blot

Western blot is a commonly used technique to analyze protein expression in cell lysates. It consists of separating the proteins by size through gel electrophoresis. This allows to correctly determining the molecular weight of the proteins and facilitates their identification. Then, the proteins are transferred onto a membrane for their identification through specific antibodies.

SAMPLE PREPARATION AND LOADING

After Lowry quantification, 25-30μg of protein per sample were prepared in loading buffer (NuPAGE LSD sample buffer 4x, Invitrogen), which gives density to the sample so facilitates the loading and adds negative charge to facilitate the protein migration during electrophoresis. Before loading the samples in the gel, they were heated at 70⁰ during 10 min for protein denaturation.

Later, protein samples and Precision Plus Protein™ Dual Color Standards protein ladder (Bio-Rad) were loaded into 4-12% tris-glycine sodium dodecyl sulfate polyacrylamide electrophoresis gels (Invitrogen). A 200V current was applied through the gels using MES-running buffer (Thermo Fisher Scientific) for 45 min.

PROTEIN TRANSFER TO MEMBRANES

Two types of membranes are commonly used for immobilizing the proteins: polyvinylidine diflouride (PVDF) and nitrocellulose membranes. Each of them has properties that make them suitable for different proteins. PVDF membrane has a protein binding capacity of 170-200µg/cm^2, whereas nitrocellulose membrane binding capacity is 80-100 µg/cm^2. Thus, PVDF offers a higher sensitivity and is suitable for lowly expressed proteins, but has also more background noise during the antibody detection. Proteins bind to nitrocellulose membranes through hydrophobic interactions while in PVDF membranes they do through hydrophobic and dipole interactions. Furthermore, PVDF membranes have higher chemical resistance making them ideal for reprobing or sequencing applications. Reprobing on nitrocellulose membranes is usually more difficult and is easier to lose signal.

There are different methods to transfer proteins to the above-mentioned membranes. These methods can be classified into wet, semi-dry and dry methods. For this thesis, I used wet and dry methods. Wet method consists of preparing a membrane sandwich and submerging it on transfer buffer inside a transfer tank (Figure 14). This method is the most traditional method and is useful for all types of proteins. The main inconvenience of the method is the time consumed in the preparation of the buffers, the sandwich assembly, the discarding of the hazardous buffers and components and the transferring time usually requires more than an hour.

Prepare transfer buffer

Prepare transfer buffer sufficient for the transfer tank and for equilibration of gels and membranes.

Equilibrate gels and membranes

Equilibrate gels and membranes in transfer buffer.

Assemble the gel and membrane sandwich

Place the membrane and gel between buffer-soaked filter papers.

Set up the transfer cell

Place the gel, membrane and filter paper sandwich in the transfer tank. Fill the tank with transfer buffer. Connect the tank to the power supply and set the power supply for optimal power and time.

Start the transfer

Support Grid
Pads
Filter Paper
Transfer Membrane
Gel

Gel/Membrane/Filter Paper Sandwich

Buffer Tank

Anode (+)
Electrodes
Cathode (-)

Direction of Transfer

Figure 7: Workflow of wet transfer of proteins for western blot. Before assembling the sandwich components are equilibrated in transfer buffer. Then the sandwich is submerged inside a transfer tank, which is filled with transfer buffer until sandwich is completely covered and wet. Finally a current is applied to the tank (Adapted from[364]).

On the other hand, dry method has a higher cost, but significantly reduce the time consumed in the process. For this thesis iBlot Dry Blotting system (ThermoFisher) was used. This method has the advantage that sandwich is already prepared and you only have to add the membrane. This increases the reproducibility among western blots. Additionally, transfer time ranges from 5-9 minutes depending on the size of the proteins to be detected. The major inconvenient of dry-system is that transferring high-molecular weight proteins is less efficient than with wet systems.

PROTEIN DETECTION

Once the proteins are immobilized on the membranes, immunoblotting is used to asses protein levels. Before incubation with the pertinent antibodies,

membrane must be blocked with milk, BSA or a mix of them to reduce the unspecific binding of the antibodies during at least half an hour at room temperature. The protein of interest is detected by incubating primary antibodies overnight at 4ºC. Subsequently, primary antibody is detected by using host-corresponding secondary antibody against the primary antibody during one hour at room temperature. The secondary antibody is coupled to the Horseradish Peroxidase (HRP) enzyme, which transforms HRP substrates (such as luminol) into light that can be detected. Enhanced chemoluminsecence (ECL) is a luminol-based method commonly used for the detection of proteins through the use of autoradiography film or digital imaging system. BSA and non-fat dry milk solutions for dissolving the antibodies or blocking the membrane was prepared in TBS-T (Table 12).

Primary and secondary antibodies used in this work are listed on the following tables:

Table 10: Primary antibodies

Antibodies	Weight (kDa)	Origin	Dilution	Dilution media	Supplier	reference
BRG1	205	Rabbit	1:1000	5% BSA	Santa Cruz	sc_10768
BRM	210	Goat	1:500	5% Milk	Santa Cruz	sc_6450
pan-ERK	42	Mouse	1:20,000	5% Milk	BD	612641
Actine-HRP	42	Rabbit	1:40000	5% BSA	Santa Cruz	sc_1616
Bcl_2	26	Mouse	1:2000	TBS-T	Dako	M0887
Caspasa 3	17, 19,35	Rabbit	1:20000	TBS-T	Cell Signaling	#9665
C3 Cleavaged	17, 19,35	Rabbit	1:750	5% BSA	Cell Signaling	#9664
PARP	17, 19,35	Rabbit	1:5000	5% BSA	Cell Signaling	#9542
p27	27	Rabbit	1:4000	5% BSA	Cell Signaling	#3686
PI3K	110	Rabbit	1:1000	5% BSA	Cell Signaling	#4255
Phospho_S6$_{Ser\ 235/236}$	32	Rabbit	1:1000	5% BSA	Cell Signaling	#2211
Phospho-Akt$_{Ser\ 473}$	60	Rabbit	1:1000	5% BSA	Cell Signaling	#9271
AKT1	62	Goat	1:1000	5% BSA	Santa Cruz	sc_1618
β-Catenin	92	Mouse	1:5000	5% Milk	BD	610154

Table 10: Secondary antibodies

Antibodies	Origin	Dilution	Supplier	Reference
Anti-Mouse IgG-Peroxidase	Rabbit	1:10,000	Sigma	A9044
Anti-Rabbit IgG-Peroxidase	Goat	1:10,000	Sigma	A0545
Anti-Goat IgG-Peroxidase	Rabbit	1:10,000	Sigma	A5420

Table 12: TBS-Tween recipe

TBS-T	
Reagent	**Concentration**
Tris	20mM
NaCl	150mM
Tween-20	$0.1\%_{(v/v)}$
pH 8.0	

MEMBRANE REPROBING

Frequently, membranes can be re-probed. When the second protein that we want to detect has a different molecular weight and are recognized by primaries from different host species, inactivation of HRP of the secondary antibodies bound to the membrane will be enough to eliminate or reduce the first signal. Sodium azide (NaN_3) is commonly used to inactivate HRP, which irreversibly bind to HRP and inactivates the enzyme.

In the case of detection of similar molecular weight proteins, is recommended to strip the membrane before immunoblotting with the next primary antibody. Stripping the membrane releases bound proteins, thereby reducing background signals when reprobing. Release of membrane-bound proteins is accomplished by incubation in denaturing buffers, containing detergents, reducing agents and low pH. The incubation time of the membrane in reprobing buffer must be controlled; otherwise it might result in loss of immobilized proteins. For this thesis Re-Blot Mild stripping Solution (Millipore) was used.

3.7.   Cell culture

3.7.1.   Cell lines

Cell lines and their corresponding medium are listed below. Cell cultures were maintained at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

Table 11: Cell lines and media

Cell Line	Type	Supplier
IMDM 20% FBS (v/v) + 1% Insuline-Transferrin-Selenium Supplement (v/v) + 100U/ml penicillin + 100µg/ml streptomycin + 5µg/ml plasmocin		
CHLA₋90	Neuroblastoma	COG
SH-SY5Y	Neuroblastoma	ATCC
IMR₋32	Neuroblastoma	ATCC
LAI-5S	Neuroblastoma	PHECC
SK₋N₋BE₍2₎	Neuroblastoma	PHECC
SK₋N₋BE₍2₎C	Neuroblastoma	ATCC
DMEM 10% FBS (v/v) + 100U/ml penicillin + 100µg/ml streptomycin + 5µg/ml plasmocin		
HEK293T	Renal epithelial cells	ATCC
HEK293T-L1	Renal epithelial cells	Dr. Hector Palmer
UW 228-3	Medulloblastoma	Dr. Beth Coyle
MEM 10% FBS (v/v) + 100U/ml penicillin + 100µg/ml streptomycin + 5µg/ml plasmocin		
HTB₋82	Rabdomyosarcoma	ATCC
CW9019	Rabdomyosarcoma	ATCC
Suppliers	**Description**	
ATCC	American Type Tissue Collection	
PHECC	Public Health England Culture Collections	
COG	Children's Oncology Group Cell Culture Repository	
Dr. Bethy Coyle	University of Nottingham Children's Brain Tumour Research Centre, Nottingham, UK	
Reagent	**Description**	
IMDM	Iscove's Modified Dulbecco's Medium	
DMEM	Dulbecco's Modified Eagle Medium	
MEM	Minimum Essential Media	

3.7.2.   Thawing and cryopreservation of cell lines

To maintain a continuous stock of cell lines they must be cryopreserved and stored. It is important to maintain the stock of cells without introducing selections or modifications that alter the characteristics of the original population. Then, it is recommended to cryopreserve early passages. To ensure cell integrity during cryopreservation, cells are frozen in a mixture of cell media and 10% of DMSO using a propanol filled container that allows cells to freeze gradually (-1ºC/min). Cryopreserved cell lines must be stored in liquid nitrogen to abrogate cellular processes.

Days, months or even years later we can thaw cryopreserved cells to start cell cultures from them. In this process is important to ensure their recovery and keep the best cell viability. DMSO at concentrations higher than 0.01% (v/v) is toxic for the cell; therefore, cells must be rapidly thawed and resuspended in 10 times media volume to dilute the DMSO. Then cells must be centrifuged and the supernatant is replaced for fresh media to seed them in appropriate culture dishes.

3.8. Cell transfection

Cell membranes and nucleic acid molecules are negatively charged, making improbable the spontaneous internalization of alien DNA or RNA into cells. This inconvenient can be overcomed with a great variety of transfection reagents such as Lipofectamine 2000 (ThermoFisher), a cationic liposoluble reagent. The method is based in the spontaneous formation of positively charged liposomes in an aqueous environment that entrap nucleic acids inside, thereby shielding their negative charge. Then the liposomes are internalized by endocytosis into the cell and the DNA/RNA is liberated inside to be expressed or induce gene silencing.

In this study, Lipofectamine 2000 was used for the production of lentiviral particles in HEK293T cells. The protocol used was adapted from manufacturer's recommendations. One day before transfection, 4 millions of HEK293T cells were seeded on a 10cm cell culture plate coated with 0.1% gelatin. On the transfection day, Lipofectamine 2000 and DNA were diluted separately in Opti-MEM I (ThermoFisher) without supplements. After 5 min of incubation, DNA and Lipofectamine 2000 were combined and incubated for 20 min to allow complex formation. Then complexes were added to the medium (Opti-MEM supplemented with 5% FBS). Culture media with the complexes was changed after 4-6h of transfection in order to avoid Lipofectamine 2000 toxicity. The plasmids used in the study are listed below.

Table 12: Vectors

Vector	Function	Supplier
pLKO-NSC	express a non-silencing sh control	Sigma
pLKO-shBRG1 #1	express an sh against BRG1	Sigma
pLKO-shBRG1 #4	express an sh against BRG1	Sigma
pcDNA3-hBCL2	overexpress BCL-2 protein	Dr. Joan Comella
pLenti-TRE-flag-beta Catenin S33Y IRES GFP	overexpress β-Catenin gene	Dr. Hector G. Palmer
pTA-Luc-TCF/LEF	wnt pathway luciferase reporter	AddGene (Vector #12456)

3.9. Lentiviral production

Lentiviral transduction is a very effective method to induce expression or silencing of proteins of interest. They are capable of integrating a fragment of the transduced DNA transduced into the genomic DNA of the host cell. In addition, lentivirus can infect proliferating and differentiated cells. They derive from HIV-1 lentivirus and currently three plasmid generations are available. Each of them increased the biosafety by dividing the necessary elements for the virus formation in more plasmids. In this work 2nd generation lentiviral plasmids (designed by Didier Trono Laboratory) were used.

This method consists of three different plasmids transfected simultaneously: (detailed components of the plasmids are listed on Table 14)

1. Gene expression lentiviral vector: this vector carries the genetic material to be transferred to target cells (DNA or short hairpin RNAs (shRNA)). The desired transgene is flanked by *cis* elements used for encapsidation, reverse transcription and integration in the host genome. We take advantage of the particularity of reverse transcription in order to obtain self-inactivating vectors derived from HIV-1, which loose the transcriptional capacity of the Long Terminal Repeats (LTR) when they get in to the target cell, thereby minimizing the risk of the appearance of recombinant particles competent for replication.

2. psPAX2, the packaging lentiviral vector: this vector contains a robust CAG promoter for efficient expression of packaging proteins, including TAT protein, DNA polymerase and reverse transcriptase.

3. pMD2G, the envelope lentiviral vector: it encodes a G glycoprotein of vesicular stomatitis virus (VSV) that allows a wider tropism, enables pseudotyping of the viral particle and confers enough stability to allow lentiviral concentration by centrifugation.

In order to produce lentiviral particles, an adapted protocol from the one described by Naldini et al[365] was used. HEK293T cells were transfected as described above. In this case, the proportion of plasmids was:

- Vector of interest: 12μg
- psPAX2: 8μg
- pMD2G: 4μg

After 36h of transfection, viral particles are floating in cell media, which is harvested and centrifuged at 1500 rpm for 5 min to eliminate cells or debris in suspension. Then, supernatant is filtered through a 0.45µm filter and can directly be used to infect cells. The infection can be produced in fresh (right after collecting media) or later if it is keept at -80ºC. To ensure virus integrity, thawing-freeze cycles must be avoided and is recommended to aliquot the virus-containing media.

The elements of lentiviral constructs used in this work are listed on Table 14.

Table 14: Lentiviral elements and their role in lentiviral plasmids from the 2nd generation

Vector of interest (such as pGIPZ or pLKO) common elements		
Viral element	**Description**	**Role**
5' LTR	5' Long terminal repeat	Required for viral RNA (vRNA) transcription, integration and gene expression steps
3'LTR	3' Long terminal repeat	Terminate transcription, required for reverse transcription and integration steps
Ψ	Packagign signal	Targets vRNA for packaging into the viral nucleocapsid
RRE	Rev response element	Rev binding sequence. vRNA export from the nucleus to cytoplasm for viral packaging
pCMV	Cytomegalovirus promoter	Initiation of GFP and Transgene mRNA transcription
tGFP	Turbo Green fuorescent protein	Transfection/Transduction efficacy tracking
IRES	Internal ribosome entry site	Allows the expression of 2 genes from the same transcript
Transgene	Gene of interest	Induces transgene overexpression
sh	Short hairpin of interest	Induces sh expression against the interest protein or control
WPRE	Woodchuck Hepatitis Virus (WHP) posttranscriptional regulatory elemen	Enhances transgene expression through increasing nuclear export
U6	Human U6 promoter	Drives RNA Polymerase III transcription for generation of shRNA transcripts
cPPT	Central polypurine tract	Improves transduction efficiency by facilitating nuclear import of the vector's preintegration complex in the transduced cells
hPGK	Human phosphoglycerate kinase promoter	Drives expression of puromycin
Puro R	Puromycin resistance	Gene for selection in mammalian cells.
psPAX2 Lentiviral elements		
Viral element	**Description**	**Role**
Gag	Polyprotein for matrix, caspid and nucleocapsid components	Lentiviral packaging components
Pol	Precursor protein for reverse transcriptase and integrase	Mediate reverse transcription and integration of the DNA into the genome
Rev	Rev protein	Binds RRE within vRNA and mediates nuclear export
Tat	Trans-activator of transcription	Trans-activator that activates transcription form the 3'LTR promoter
pM2G Lentiviral elements		
Viral element	**Description**	**Role**
VSV-G	Vesicular Somatitits Virus G Glycoprotein	Envelope glycoprotein with broad tropism necessary for plasma membrane penetration

3.10. Cell proliferation assay (Crystal Violet)

Cells were seeded in 96-well plates at low density left in standard culture conditions for different time-points. At the indicated times, cells were fixed with 4% paraformaldehyde and kept at 4ºC until all time points were collected. Then, quantification of amount of cells was performed staining the cells with crystal violet (sigma) for 20 min. Plates were then rinsed with distilled water to remove traces of dye and let dry. Finally crystals were dissolved in 10% of acetic acid and absorbance was measured at 590nm using Epoch plate reader (Biotek).

3.10.1.　　Gene silencing proliferation assays

For loss of BRG1 function proliferation experiments, BRG1-depleted cells alone or previously transfected with β-Catenin or BCL-2 overexpression vectors, were seeded at 5×10^5–1×10^6 cells per p60 plate and infected with pLKO-NSC, pLKO-shBRG1#1 or pLKO-shBRG1#4 lentiviral supernatants. At 2 days post- infection, cells were detached and seeded at 5–10×10^3 cells per well on 96-well plates (n=6/condition). Between 6 and 8 hours post seeding, the first plate was fixed as time zero. At the indicated times, plates were fixed and at the end of the experiments, all plates were stained with crystal violet. Normalization against time zero gave us the rate of proliferation.

3.10.2.　　Cytotoxic assays

For drug toxicity assays, cells were seeded at 8×10^3–10×10^3 cells per well on a 96-well plate (n = 6/condition). The day after, cells were treated with ABT-263 (APExBio), iCRT-14 (Sigma-Aldrich) or PI-103 inhibitors (Sigma-Aldrich). At the indicated time points, cells were fixed and stained with crystal violet. In this case, we compared cell proliferation versus vehicle-treated cells..

3.10.3.　　Combination index analysis

IMR-32 and SK-N-BE(2) cells were incubated with PI-103, ABT-263, or the combination of both and PI-103 and iCRT14, or the combination of both. After 48h cells were fixed and stained with crystal violet. Synergism, additivity or antagonism of the combination was determined by the Chou–Talalay method using the Compusyn Software (ComboSyn Inc.).

Drugs were combined at two different constant ratios, 1:25 and 1:100.

3.11.　　Colony formation assay

This method consists of seeding cells in a very low concentration to measure the ability of single cells to grow independently of paracrine signals form neighbouring cells. Cells were seeded at 5×10^3–10×10^3 cells per well on a six-well plate (n = 3/condition). After 10–11 days, plates where fixed and stained with crystal violet. Then plates where scanned and colonies were

counted using ImageJ software. Differences in colony formation where assessed by comparing the number of colonies against control.

3.12. Cell death assays

Cell death assays measure different types of cell death through death-induced changes in DNA, membrane integrity, protein activity/cleavage, protein release and cellular morphology. In this thesis apoptotic cell death was characterized through chromatin staining with the Hoechst dye and by caspase-3/7 activity assay.

3.12.1. Hoechst staining

This method consists of the staining of DNA with a fluorescent dye, thereby allowing the characterization of apoptosis through the observation of nuclear condensation (pyknosis) and/or fragmentation. In this thesis, Hoechst 33258 was used. It is excited by UV light (350nm is the optimal excitation) and emit blue fluorescent light (461nm as the maximal emission), and does not affect cell viability. Hoechst dye becomes highly fluorescent when binds to adenosine-thymidine rich regions of genomic DNA (Figure 15).
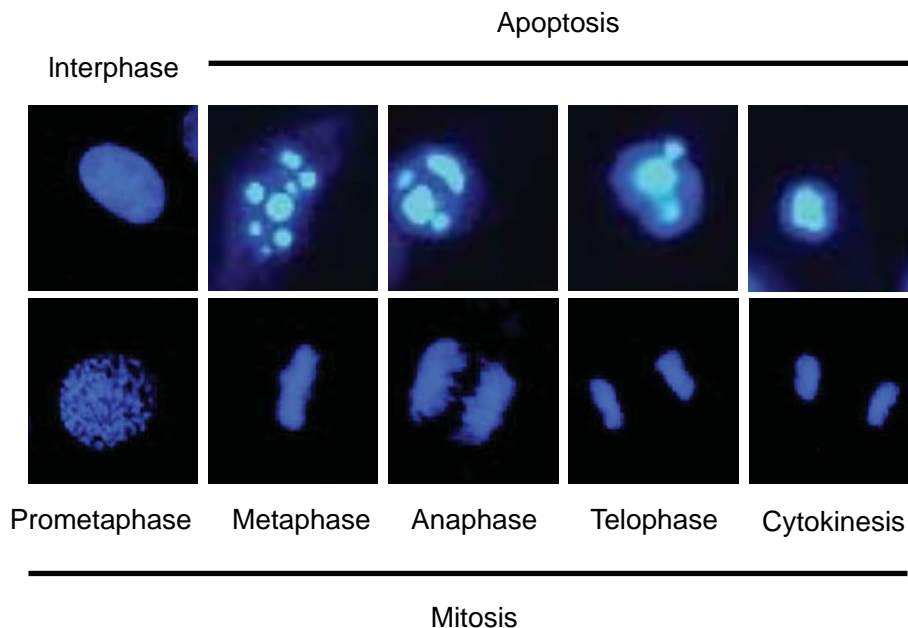
Figure 15: Nuclear morphology. Hoechst staining of CHO cells. Different nuclear morphologies and mitosis stages, such as the ones indicated in the figure, can be detected using this technique. Adapted from[575,576].

For apoptosis detection with Hoeschs staining, cells were plated in 24-well plates (80×10^3 cells/well) 3 days post- infection. Twenty-four hours later, cells were stained with 0.05 mg/ml Hoechst 33258 for 30 min at room temperature. Condensed or fragmented nuclei were counted as dead cells. Five hundred cells were counted for each data point, and the count was repeated three times in independent experiments.

3.12.2. DVDase assay

Caspases are executors of the apoptotic singaling pathways. To measure caspase activity, the method involves a fluorescent substrate susceptible to be cleaved by the caspase, thus emitting light only when the substrate is cleaved. In this work, we used the specific substrate of effector caspases 3 and 7, Ac-DEVD-afc. To measure caspase activity, cells were seeded three days post-infection at 400×10^3 cells/p35. One day later, cells were rinsed once with phosphate-buffered saline and resuspended in lysis buffer composed of 20 mM HEPES/NaOH pH 7.2, 10% sucrose, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% CHAPS and 1 × EDTA-free complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 16,000g for 5 min, and

protein from the supernatants was quantified by the Lowry method (Bio-Rad). Assays were performed in triplicate using 25 µg of protein in the same lysis buffer supplemented with 10 mM dithiothreitol and 25 µM of the fluorogenic substrate Ac-DEVD-afc (Millipore). Plates were read in a fluorimeter using a 405 nm excitation filter and a 535 nm emission filter.

3.13. Multicellular tumor spheroids

Multicellular tumor spheroids (MCT) consists of the formation of spheres from tumoral cells, thereby allowing cells to grow in a more physiological environment. Attached cells lack the 3D structure of a tissue; therefore, MCT mimic physiological conformation better than standard 2D cultures. The major resemblance with tissue conditions leads to results that mimics with more fidelity what happens in an organism, thereby reducing the number of experiments involving animals.For tumor sphere formation, SK-N-BE(2) and IMR-32 cells were grown in serum-free neurobasal medium (Invitrogen), supplemented with B27 (Invitrogen), 2mML-glutamine (Invitrogen), 20ng/ml EGF (ProSpec-Tany Technogene Ltd), 20 ng/ml FGF2 (ProSpec-Tany Techno-gene Ltd), 20U/ml penicillin and 20mg/ml streptomycin. For viability assays, 15 × 10^3 cells were seeded in non-adherent 24-well plates. Forty-eight hours later, the number of spheres was scored (spheres ≥ 40 µm in diameter) and then, incubated with the indicated treatments. Twenty-four and forty-eight hours later, viable spheres were scored the final number of spheres versus the initial number was represented.

3.14. Mouse Xenograft

Mouse xenografts consists of injecting human (or other species) tumoral cells in the flank of the mice. This method resembles betther the human diseases because tumor cells are able to grow in contact with other cell types and tissues. For this study, all mice procedures were approved by the ethical committee of Vall d'Hebron Research Institute (protocol number 52.13).

SK-N-BE(2)C cells were infected with pLKO-NSC or pLKO-shBRG1#4 lentivirus for 3 days and then 5×10^6 cells per mouse were injected () in the flank of 8-week-old female NMRI-nude mice (Janvier, n = 10/group) in 300µl of phosphate-buffered saline and Matrigel (1:1). Tumor volume was measured every 2–3 days for 2 weeks using an electronic caliper. When tumors reached the 1600mm^3 growth limit, mice were sacrificed and tumors removed and weighed (Figure 16). Tumors were then fixed in 10% formalin, paraffin-embedded, and 5 µm sections were stained with hematoxylin and eosin.
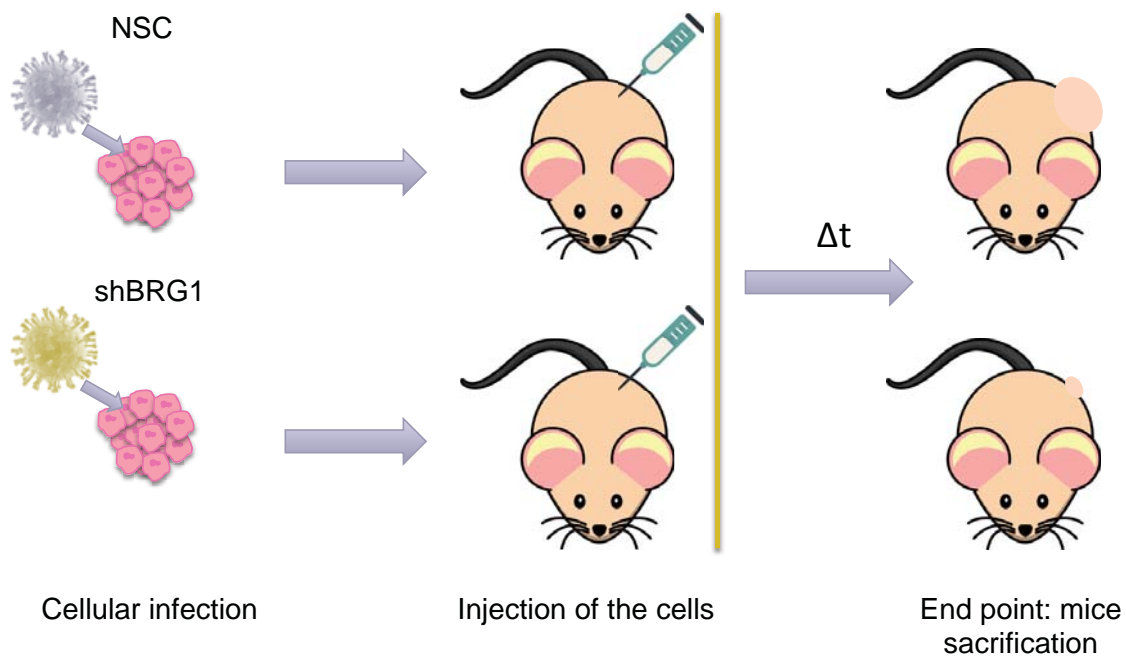
Figure 8: Xenograft mouse model. Cells were infected with NSC or shBRG1 virus. Then 5x10^6 cells were injected in the flank of the mice (10 per group). At the end of the experiment, mice are sacrificed, tumors were extracted and analyzed.

3.15. mRNA microarray analysis

Expression profiling of triplicate experimental sample groups (shNSC versus shBRG1) was performed using the Affymetrix microarray platform and the Genechip Human Gene 1.0 ST Array. Two hundred nanograms of total RNA were hybridized to the arrays with the GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix, Santa Clara, CA, USA). Chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000 and

normalization of the raw data (CEL files) was made with the Robust Multichip Average algorithm (Figure 17).
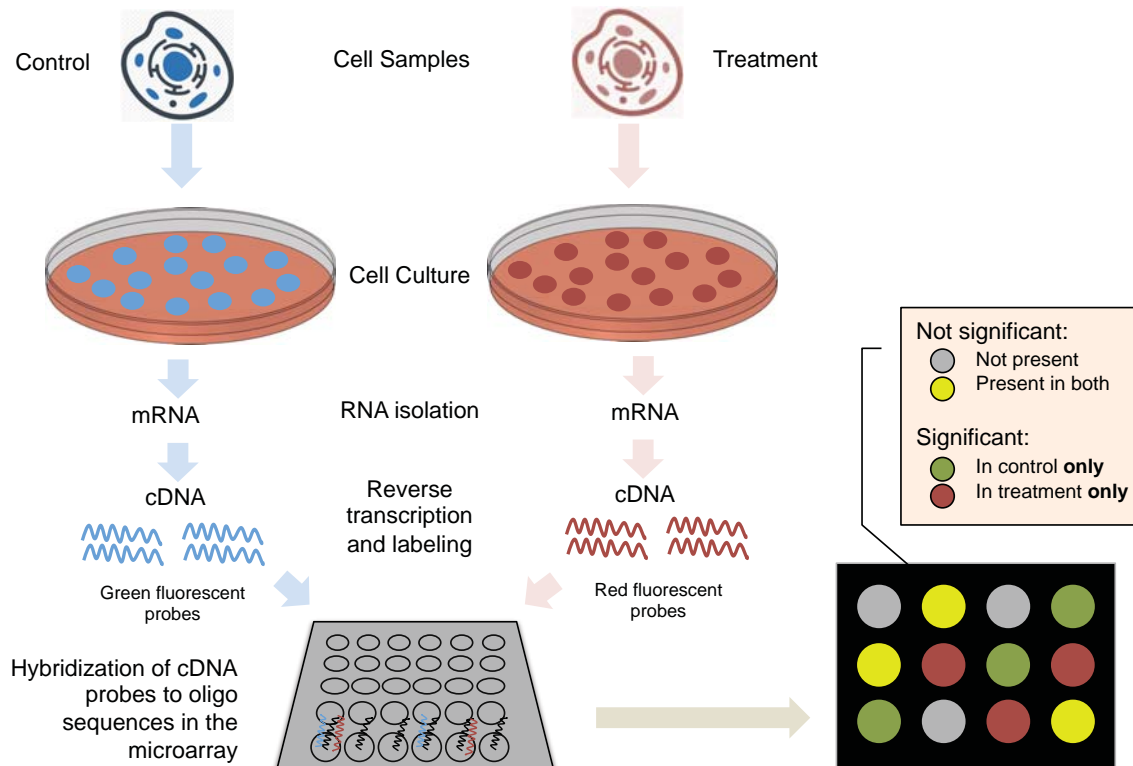
Figure 17: MicroArray overview. Control and treated cells are grown, then mRNA is extracted. Next step converts the mRNA in to cDNA, which is labeled with fluorescent probes and hybridized against the probes in the microarray. The chip is read and different colors and their intensity measures the quantity of each mRNA hybridized on the respective probes.

To filter and perform differential expression analyses, a moderated t-test (P o 0.05 α-level) and fold-change thresholding (433% reproducible change) were considered. Principal component analysis was generated by the Partek Genomics Suite 6.6 software (Partek Inc.). The functional annotations of resulting gene lists were performed using the Ingenuity pathway, the KEGG52, and Gene Set Enrichment Analyses databases. Heatmaps were generated by normalization of the array expression values to the median and log2 transformed, and color converted using the MultipleExperiment- Viewer software (TM455). The publically available gene sets for BENPOR-ATH_PROLIFERATION, ANTI_APOPTOSIS and REACTOME_CELL_CYCLE (Molecular Signatures Database v5.0; http://www.broadinstitute.org/gsea) were used to analyze the impact of BRG1 depletion in NB cells. The accession number for microarray analysis reported in this work is GSE74622.

3.16. WNT GFP-reporter activity assay

To determine the activation of WNT pathway in NB, we used a lentiviral construct, (Figure 18) that contains two reporter genes: first, the mCherry protein (which determines de efficiency of cell infection) and second, the GFP protein which expression is regulated by a β-catenin dependent promoter (7xTcf)[366]. In this setting, GFP is only expressed when the WNT pathway is activated and β-catenin is translocated into the nucleus to exert its function.
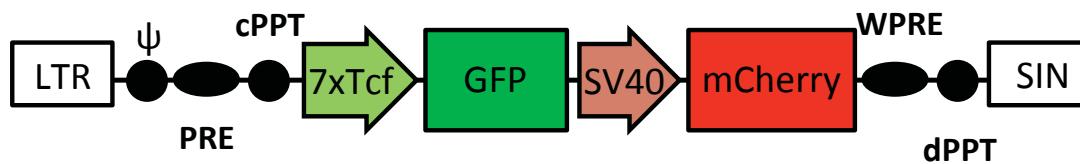
Figure 9: WNT activity reporter. The lentiviral vector contains 7 Tcf (an specific β-catenin binding motif), which regulates de expression of GFP. Additionally mCherry fluorescent protein is expressed constitutively and is used to determine the efficiency of cell transduction.

For β-catenin activity assays, cells were stably transduced with the WNT activity reporter vector and subsequently infected with shNSC and shBRG1 #4. Two days later, cells were treated with mock or WNT3A ligand for 48h. At the end of the experiment, cells were harvested and GFP and mCherry fluorescence was analysed in a Fortessa cytometer.

WNT3A ligand and mock were produced in HEK293T-L1 expressing cells. Cell supernatant was collected during four days and kept at 4ºC. Then supernatant was centrifuged, filtered on a 45μm filter and warmed at 37ºC before applying to the target cells.

3.17. Statistical analysis

Experimental sample size for *in vitro* and *in vivo* experiments was chosen following the criteria of the VHIR Statistics and Bioinformatics Unit. Unless otherwise indicated, mean ± S.E.M. values are representative of one of three independent experiments. Statistical significance was determined by two-sided unpaired t-test or ANOVA Tukey's test (GraphPad Prism Software). In all the experiments variance was similar between groups. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

RESULTS

4. RESULTS

4.1. BRG1 is one of the most consistently deregulated epigenetic gene in Neuroblastoma

In order to characterize the implication of epigenetic regulators and determine the elements that promotes and facilitates the development in advanced NB tumors, we examined the expression of 127 epigenetic regulators in 3 different publicly available NB gene expression datasets: 39 HMT, 37 bromodomains, 16 HAT, 14 HDAC, 8 HDM, 3 DNMT and 12 from other categories, (Annex Table 1). First, GSE960 and GSE16237 human NB tumor sample datasets were used to compare the mRNA expression of these genes between the poorest prognosis group (i.e. stage 4 MYCN amplified) and the rest of stages. Second, GSE16476 human NB tumor samples database was used to determine the correlation of these 127 gene expression with event-free or with overall survival or survival. The top 10 deregulated epigenetic genes are listed in Table 15.

Table 15: Most differentially deregulated epigenetic regulators in NB

Gene Symbol	GSE960		GSE16237		GSE16476	
	Fold Change	P Value	Fold Change	P Value	Worse outcome when expression is	P Value
PRMT5	N/A	N/A	2.81	9.10E-02	High	2.30E-11
WHSC1	1.42	1.00E-04	1.86	2.99E-01	High	2.50E-05
EHMT2	1.44	3.14E-05	1.61	4.59E-02	High	1.10E-04
KAT5 (HTATIP)	-1.47	1.51E-02	1.60	1.72E-02	High	2.80E-03
SMARCA4	1.47	8.07E-05	1.53	2.60E-02	High	1.70E-03
EZH1	-1.25	3.00E-04	-1.93	6.03E-02	Low	6.30E-06
SIRT2	N/A	N/A	-2.27	4.00E-03	Low	2.90E-02
MLL3	N/A	N/A	-2.63	1.76E-04	Low	3.30E-02
BAZ2B	-1.38	1.57E-03	-3.57	6.09E-03	Low	7.10E-15
PRDM2 (RIZ)	-2.94	1.35E-11	-3.73	1.22E-05	Low	6.40E-10

N/A: gene not included in the database.

Among these genes, SMARCA4 (also known as BRG1) was one of the consistently upregulated genes in all databases and its higher expression significantly correlated with worse outcome. Additionally, BRG1 alterations were never described before in NB, despite being one of the most mutated enzymatic subunit of the SWI/SNF chromatin remodeling complex in cancer[367], thereby suggesting an important role in the disease.

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4.2. BRG1 expression is associated with neuroblastoma prognosis

To delve in the characterization of BRG1 in NB and validate the results found in the screening, we examined the expression of *BRG1* and *BRM* (the other mutually exclusive enzymatic subunit of SWI/SNF complex) in an independent human NB dataset with 649 patients (GSE45547). *BRG1* expression was found to be higher in stage 4 patients (Stage 4 *vs* Stage 1, p<0.001; stage 4 vs stage 2, p<0.05; stage 4 vs 4S, p<0.05) confirming previous observations, whereas no significant differences were found in *BRM* levels (Figure 19A). Moreover, high levels of *BRG1* were correlated with worse event-free (Figure 19B, p=$1.9 \cdot 10^{-7}$) and overall survival (Figure 19B, p=$2.3 \cdot 10^{-7}$) in the same NB dataset (Figure 19B, n=476), but no correlation was observed with *BRM* (Figure 19C).
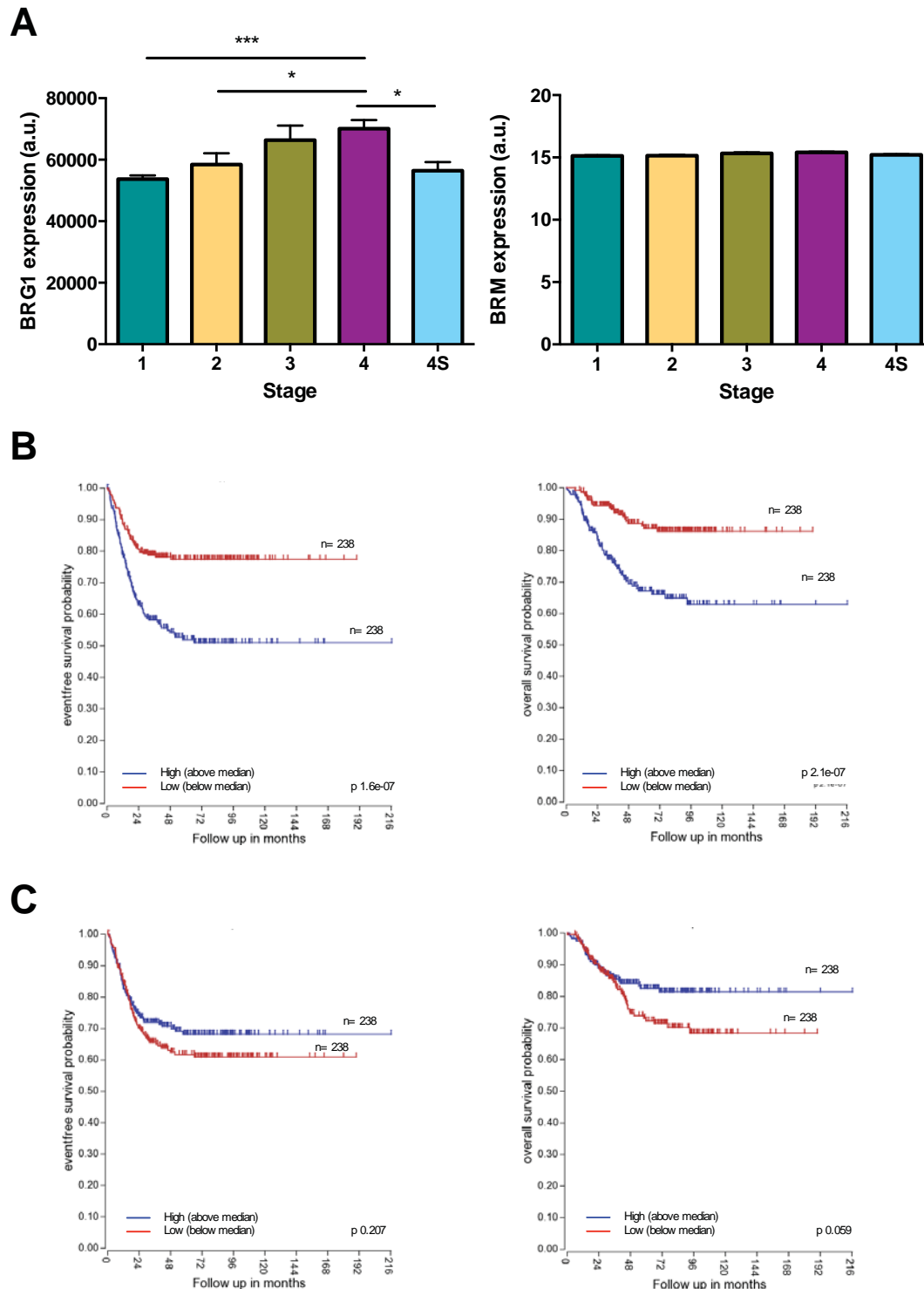
Figure 19: BRG1 mRNA expression correlates with worse oucome in NB. A, RG1 and BRM mRNA expression levels in 649 NBs (stage 1, n = 153; stage 2, n=113; stage 3, n=91; stage 4, n=214; stage 4s, n=78) measured by Agilent 44K oligonucleotide expression microarrays. ***P<0.001; *P<0.05. **B**, BRG1 upregulation is a marker of poor prognosis in NB. Kaplan–Meier event-free survival and overall survival curves in 476 tumors based on high (above median) or low (below median) BRG1 expression. **C**, BRM expression cannot predict outcome in NB. Kaplan–Meier event-free survival and overall survival curves in 476 tumors based on high (above median) or low (below median) BRM expression.

In order to determine whether BRG1 may act as an independent prognostic factor in NB, we analyzed the same data excluding stage 4, which is the only subset of patients with the higher BRG1 levels. Subsequently, we analyzed only the stage 4 patients. Finally, we excluded MYCN amplified patients, which defines the subset of patients with the worst outcome. In all cases, higher BRG1 levels (above median) were correlated with worse event-free (p<0.05) and overall survival (p<0.05) (Figure 20), thereby indicating that BRG1 may be an independent prognostic factor.
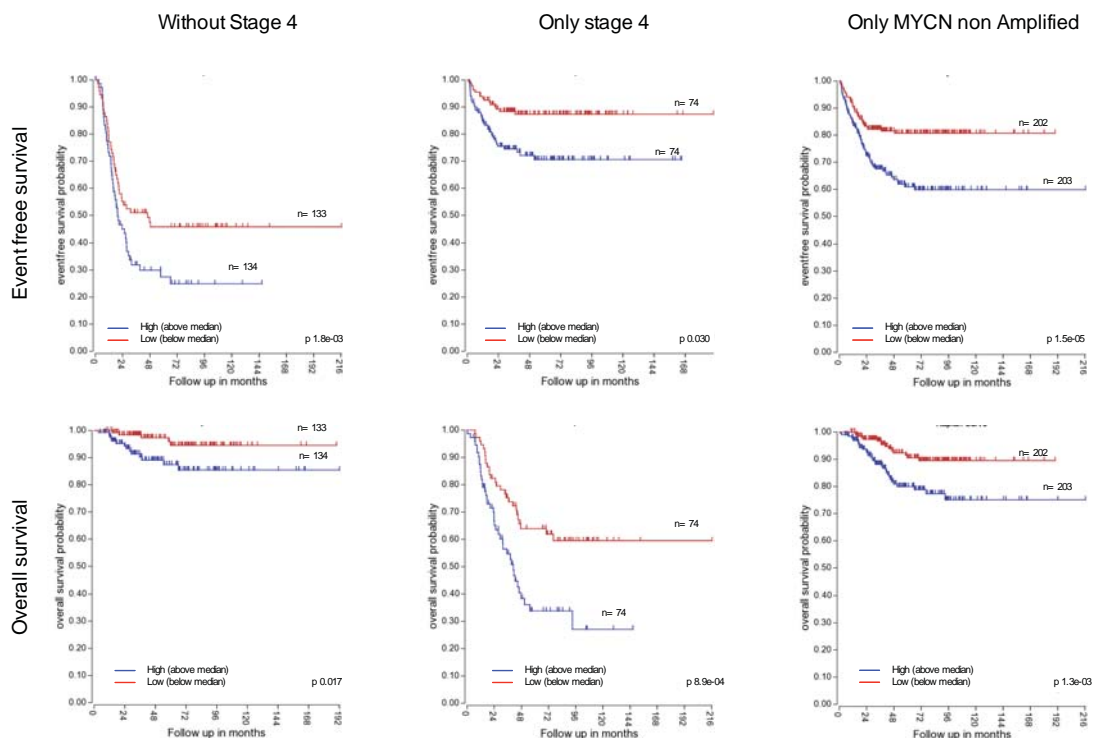
Figure 20: BRG1 may be an independent prognostic factor in NB. Kaplan-Meier event-free survival (above) or overall (below) curves in the three indicated independent sets of patients.

We further analyzed the protein expression of BRG1 in a panel of NB cell lines with clinical features representative of the most aggressive NB such as MYCN amplification, ALK mutations and different status of p53 function (Table 16).

Table 16: NB cell lines and their characteristics

Cell Line	Stage	Age	MYCN Status	P53 Status	ALK Status
IMR–32	4	2 years	Amplified	Functional	PA, wt
SH-SY5Y	4	4 years	Not Amplified	Functional	F1174
SK-N-AS	4	6 years	Not Amplified	Not Functional	NA, wt
LAI-5S	4	3 years	Amplified	Functional	F1174L Mut
CHLA–90	4	8 years	Not Amplified	Not Functional	F1245V Mut
SK–N–BE(2)	4	26 months	Amplified	Not Functional	NA, wt
SK–N–BE(2)C	4	26months	Amplified	Not Functional	NA, wt

BRG1 expression was found to be relatively higher in NB cell lines when compared with other embryonic tumor cell lines such as rhabdomyosarcoma (CW9019, HTB-82), medulloblastoma (UW 228-3) or other embryonic cell lines (HEK293T) (Figure 21A). BRM expression, however, did not show any differential pattern in these samples (Figure 21A). We then proceed to analyze the BRG1 protein levels in human tissues by immunohistochemistry. Mild or not detectable BRG1 were found in the majority of stage 1-2 patients, whereas strong nuclear immunoreactivity was detected in stage 3-4 cases (Figure 21B). In summary, BRG1 is abundantly expressed in advanced stages of NB tumors and is associated with worse patient outcome.
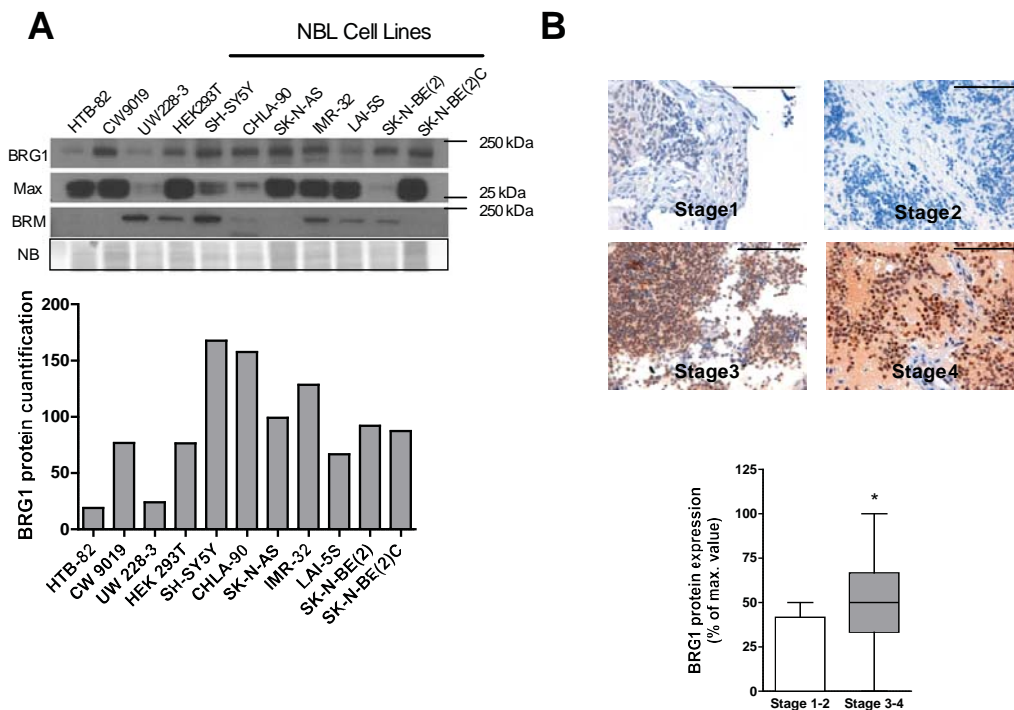
Figure 21: BRG1 protein is upregulated in advanced stages of NB. A, Immunoblot of BRG1, MAX and BRM in embryonic cancer cell lines including NB. Naphtol Blue staining was used to control equal protein loading. Graph shows band intensity quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). **B**, Representative images of BRG1 staining of NB tumor sections (n = 19) of the indicated stages followed by BRG1 quantification (below) *P<0.05. Bar, 100 µm.

4.3. BRG1 is essential for Neuroblastoma growth and viability

4.3.1. BRG1 is essential for neuroblastoma growth

With a view to ascertaining the role of BRG1 in NB oncogenic properties, we proceeded to silence it using two different lentiviral-based short hairpin RNA (shRNA) in multiple NB cell lines. Two independent shRNAs efficiently reduced BRG1 protein levels (Figure 22A) and consistently impaired the growth of multiple NB cell lines with distinct molecular features such as MYCN amplification or p53 functionality (see Table 15 and Figure 22B,C).
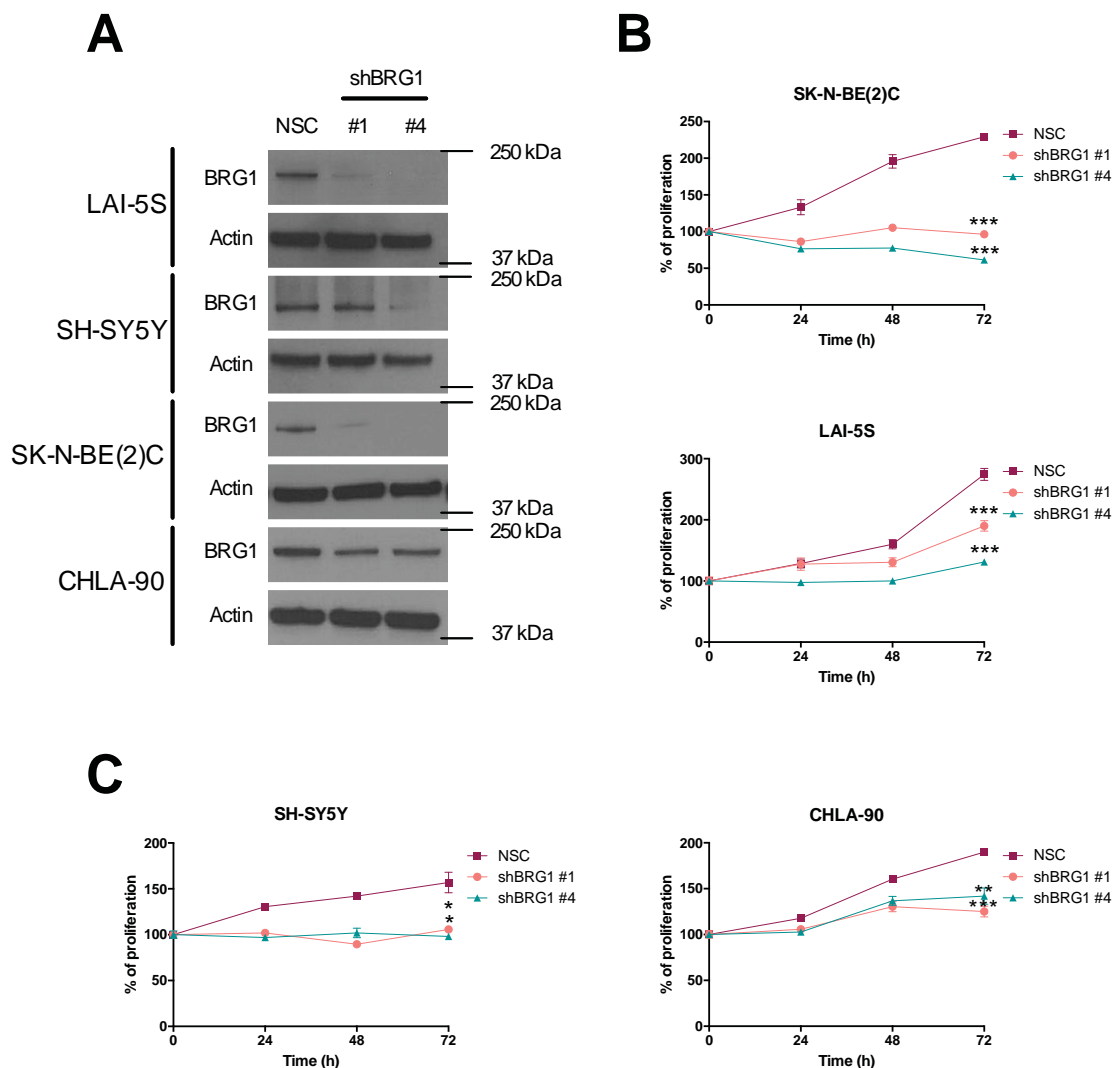
Figure 22: BRG1 inhibition reduces cell proliferation *in vitro*. A, BRG1 levels detected by western blot in the indicated cell lines infected with non-silencing control (NSC) or two different shRNA against BRG1. **B**, Normalized proliferation curves of two MYCN amplified NB cell lines, one p53 non-functional (SK-N-BE(2)C) and one functional (LAI-5S), infected with shNSC, shBRG1#1 and shBRG1#4, measured by crystal violet staining (n=6/condition) and **C**, Normalized proliferation curves of two MYCN non-amplified NB cell lines, one p53 non-functional (SH-SY5Y) and one functional (CHLA-90), infected with shNSC, shBRG1#1 and shBRG1#4, measured by crystal violet staining (n=6/condition). *P<0.05, **P<0.01, ***P<0.001.

These effects were not observed when the same experiments were performed in other embryonic cell lines with low BRG1 levels, such as HTB-82 and UW 228-3 (Figure 23).

A
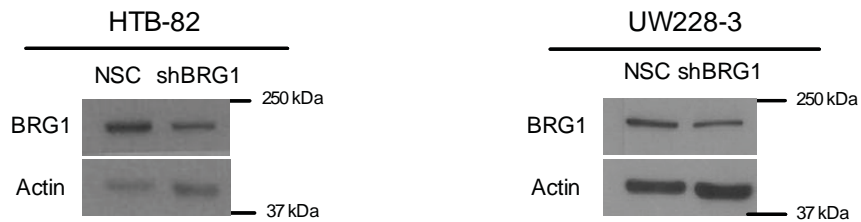
B
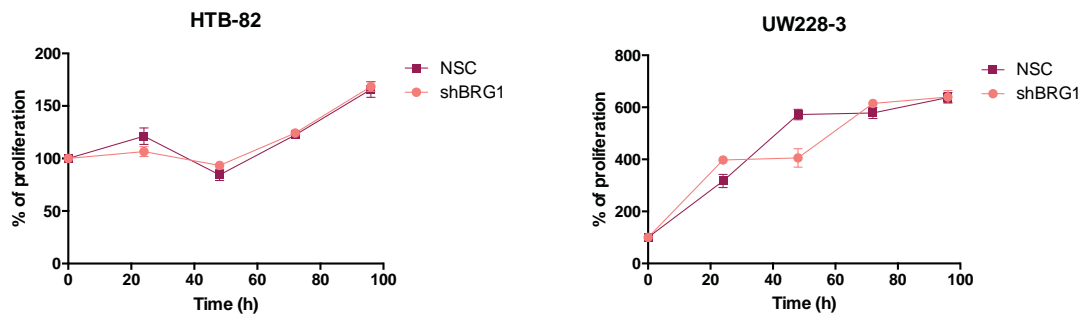
Figure 23: Depletion of BRG1 in other embrioanic cell lines do not affect proliferation. A, BRG1 levels detected by western blot in the indicated cell lines infected with non- silencing control (NSC) or one shRNA against BRG1. **B,** Normalized proliferation curves of two non-NB embryonal cell lines, one rhabdomyosarcoma (HTB-82) and one medulloblastoma (UW228-3) cell line, infected with shNSC, shBRG1#1 and shBRG1#4, measured by crystal violet staining (n=6/condition).

Furthermore, loss of BRG1 expression dramatically reduced the number of colonies in a colony formation assay using two independent NB cell lines (Figure 24).

A

B
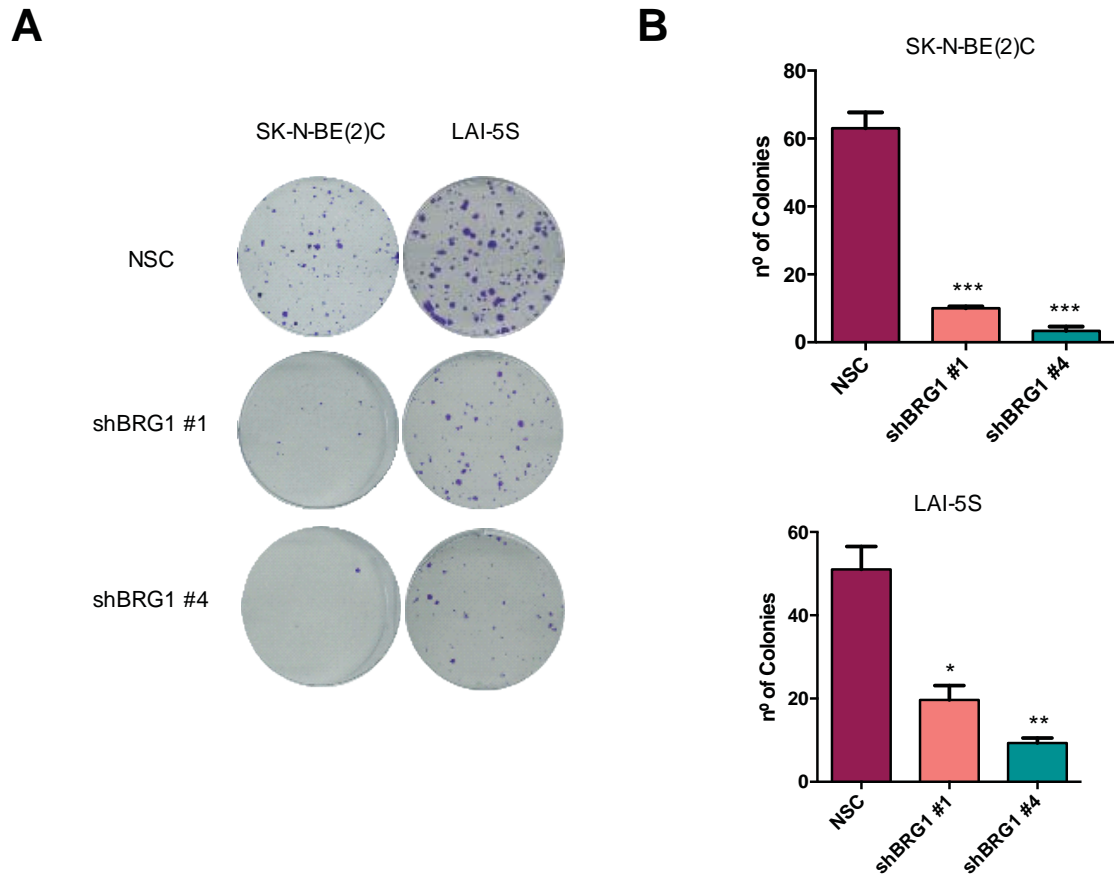
Figure 24: BRG1 knockdown reduces colony formation. A, Representative macroscopic images of colony formation assay in shNSC, shBRG1#1 and shBRG1#4 infected NB cells, and **B** average quantification of three independent experiments ±s.e.m. (n=3/condition). *P<0.05, **P<0.01, ***P<0.001. Abbreviation: shRNA, short hairpin RNA. *P<0.05, **P<0.01, ***P<0.001

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4.3.2. Loss of BRG1 expression induced apoptotic cell death

Cell proliferation experiments revealed not only a reduction in cell number but also an abrupt change in the slope of the growth curve. Therefore, we proceeded to analyze whether the effects of BRG1 knockdown on cell proliferation were due to cell cycle arrest or to loss of viability. Chromatin staining of shBRG1-infected cells showed an apoptosis-consistent pattern of condensed and/or fragmented chromatin (Figure 25B and 24C, white arrowheads) at 72 and 96 h post-infection, while non-silencing control (NSC)-infected cells displayed uniform chromatin staining (Figure 25C, left images). The activity of executor caspases 3/7 was analyzed to further confirm the apoptotic process. BRG1 knockdown induced up to a 4 fold increase in the cleavage of the DEVD-AFC caspase-3/7 substrate (Figure 25D), thereby confirming apoptotic cell death.

A

B

C

D
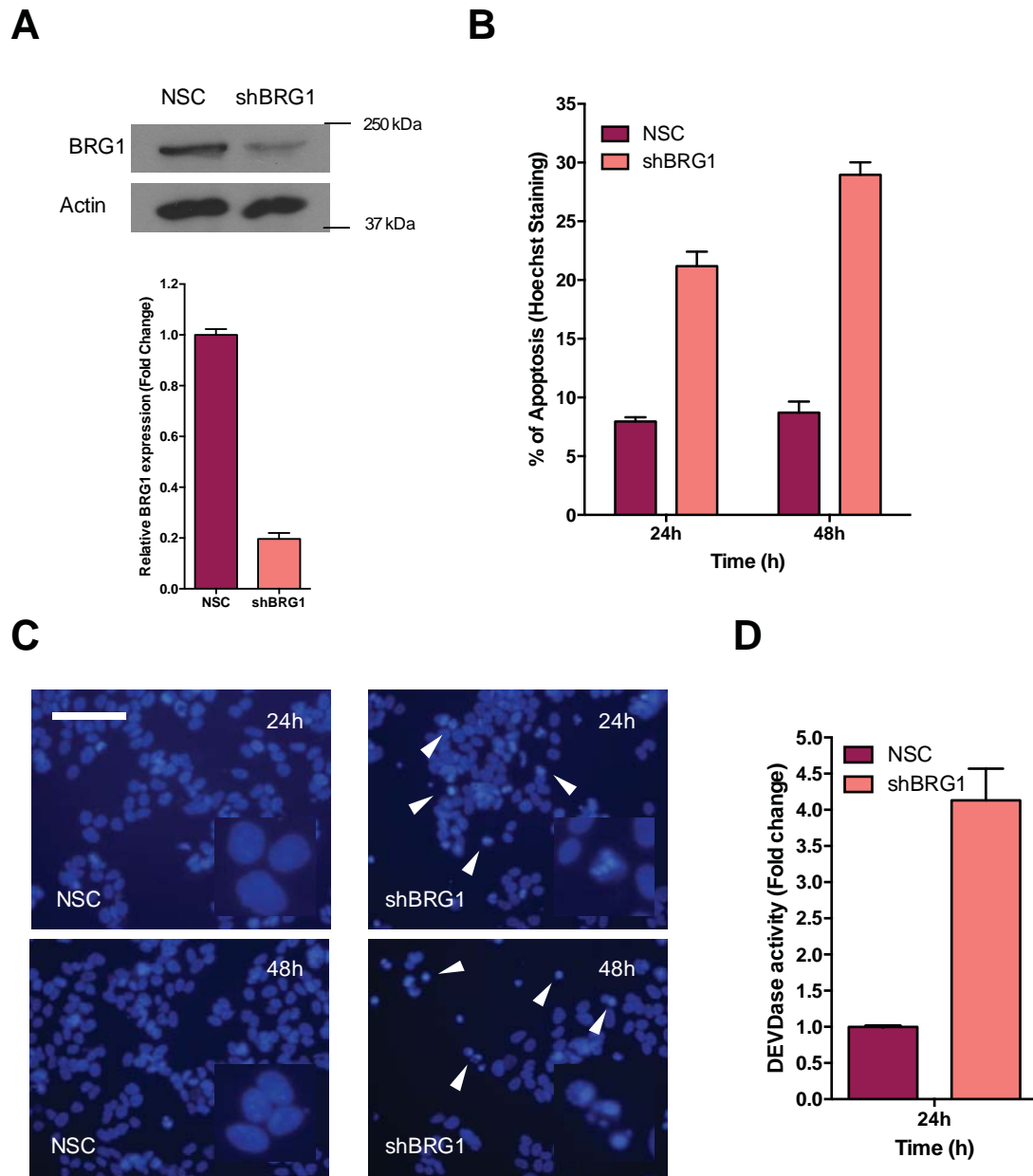
Figure 25: BRG1 induces caspase-dependent apoptosis. A, Representative immunoblot (upper panel) or quantitative real-time PCR (lower panel) of BRG1 levels after BRG1 shRNA-mediated knockdown. **B**, Cell death assay in SK-N-BE(2)C cells transduced with shNSC or shBRG1 quantified by analysis of fragmented/condensed chromatin at the indicated times post-seeding (n=3/condition). Data are mean of three independent experiments±s.e.m. **P<0.01, ***P<0.001. **C**, Hoechst staining images of shNSC or shBRG1-infected cells. Arrowheads point at nuclei with condensed or fragmented chromatin. Bar represents 100 μm. **D**, Caspase-3/7 activity assay in NB cells infected with shNSC or shBRG1 at 24 h post-seeding (n = 4/condition). Graph represents an average of three independent experiments ± s.e.m. ***P<0.001.

4.3.3. BRG1 inhibition impairs Neuroblastoma tumor growth *in vivo*

In an attempt to validate the *in vitro* results, we proceeded to analyze the effects of silencing BRG1 in a NB xenograft model. The aggressive NB cell line SK-N-BE(2)C was infected with shNSC and shBRG1 lentiviral particles for 48h and $5x10^6$ of viable cells were then injected into the flank of 8 week-old female NMRI-nude mice (n=10/group). A portion of infected cells was used to monitor BRG1 expression by Western blot (Figure 26A). Two weeks post-injection, all mice bearing shNSC-infected cells developed tumors whereas only 20% of mice bearing shBRG1-infected cells did (Figure 25B, p< 0.001). Moreover, the tumors detected in the shBRG1 group did not progress or did so at a very low pace (Figure 26C). All mice were euthanized at day 18 post-injection. The excised tumors of the shNSC group were larger in size and heavier compared to shBRG1 tumors (Figure 26D-E). Histologic analysis confirmed the presence of tumor cells in the extracted tissue fragments of both groups (Figure 26F).
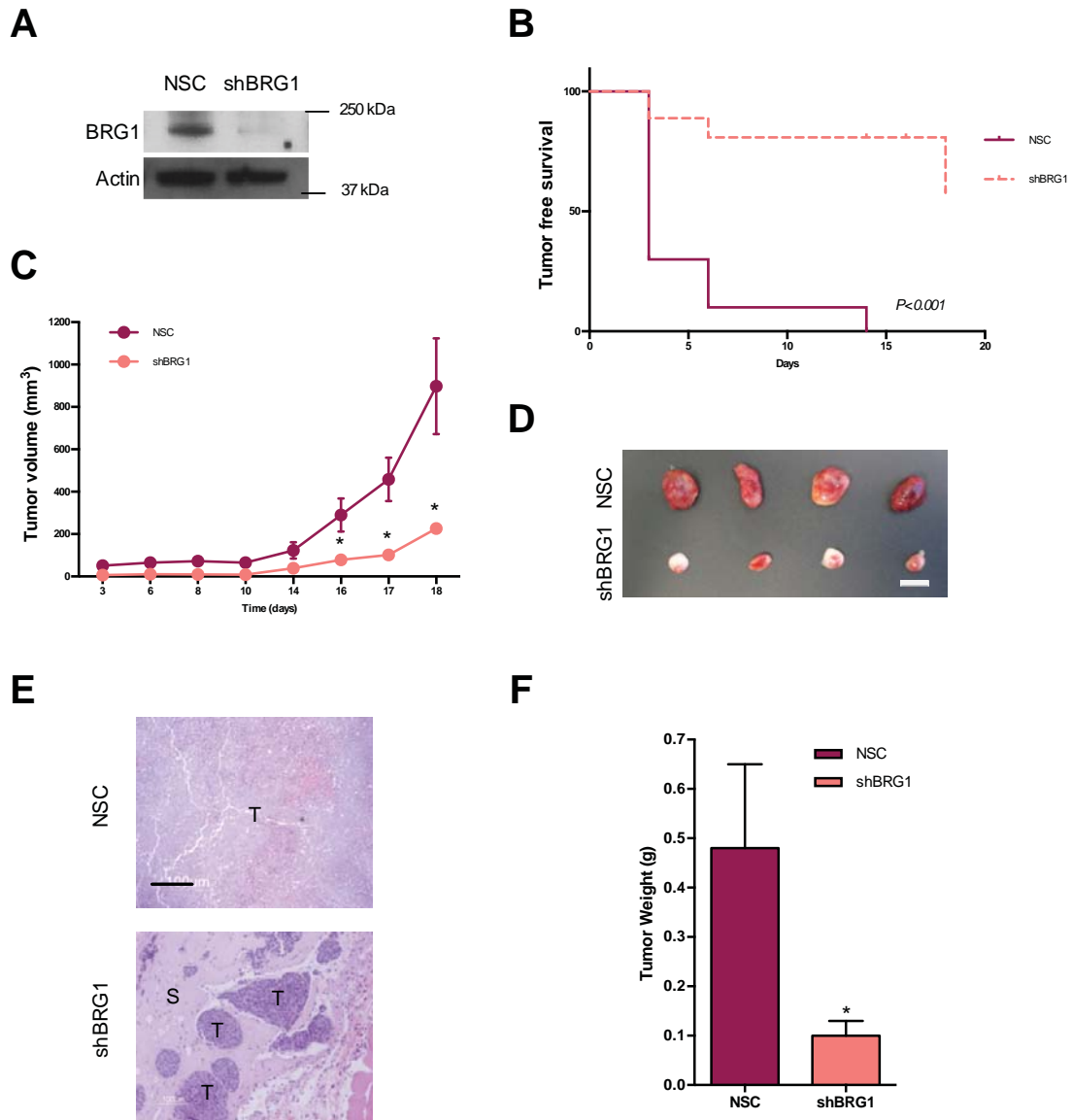
Figure 26: BRG1 is necessary for growth of NB cells *in vivo*. **A**, Western blot showing BRG1 downregulation after infection with shNSC or shBRG1 lentiviruses. **B**, Tumor-free survival curve of mice bearing NB xenografts transduced with shNSC (continuous line) or shBRG1 (dotted line). **C**, Tumor volume of mice injected with either NSC- or shBRG1-transduced SK-N-BE(2)C cells (n = 10 group), measured for 18 days. **D**, Macroscopic image of resected tumors at the conclusion of the experiment. Bar, 1 cm. **E**, Representative microscopic hematoxylin and eosin-stained images of NB xenografts. Bar, 100 µm. **F**, Average weight of resected tumors. *P<0.05.

4.4.   BRG1 controls de expression of multiple cancer-related genes

Induction of c-MYC oncogene or the repression of PTEN tumor suppressor has been observed in experimental paradigms where BRG1 behaves as an oncogene [18, 23]. However, we observed no changes in c-MYC or PTEN levels in NB cells at different time points when *BRG1* was silenced (Figure 27), thereby indicating that BRG1 targets may differ among different cell types or lineages.
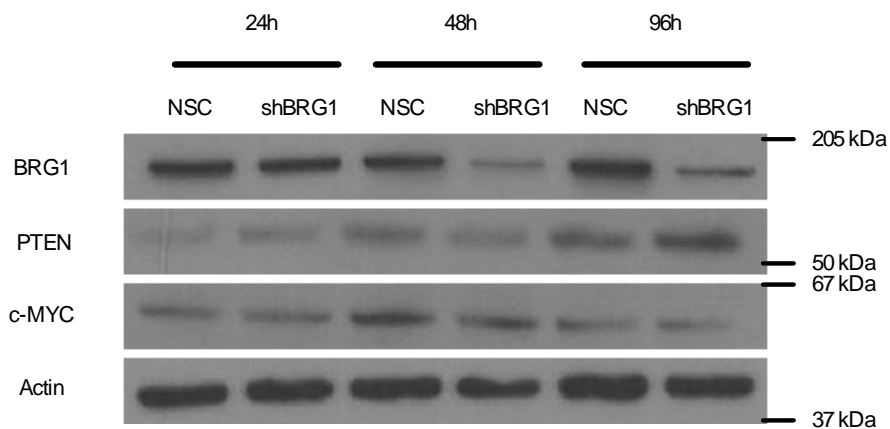
Figure 27: BRG1 knockdown does not modulate PTEN or c-MYC protein levels. SK-N-BE(2) cells were transduced with shNSC or shBRG1#4. At the indicated times, cell lysates were prepared and BRG1, PTEN and c-MYC protein expression was analyzed by Western blot. Actin was used as loading control.

Therefore, to identify potential BRG1 target genes in NB, a whole genome expression analysis was performed. Principal component analysis segregated samples on the basis of treatment (control versus shBRG1), indicating a consistent transcriptional impact of BRG1 targeting (Figure 28A). After BRG1 knockdown, 128 genes were found to be downregulated whereas 54 genes were upregulated (Table Annex2, Fold Change >2, p-value <0.001).

According to QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City,www.qiagen.com/ingenuity), a significant number of genes were associated with cancer, cell growth and proliferation, or cell death and survival, among others (Figure 28B, with a minimum of 10 genes, p-value <0.05). The expression of several upregulated and downregulated genes analyzed by qPCR validated the array results (Figure 28C). Interestingly, KEGG pathway analysis revealed that BRG1 knockdown affected the expression of several key genes,

such as *PI3KCA*, *CTNNB1* and *BCL2*, related to cell death and survival, and cell growth and proliferation (Figure 28D and Table 17). Furthermore, gene sets composed of genes involved in apoptosis, proliferation and cell-cycle progression were found to be negatively enriched in shBRG1 cells, indicating impairment in these processes upon BRG1 knockdown at the transcriptional level (Figure 28E).

Table 17: Altered Genes in PI3K and WNT pathways

Pathway	Gene Symbol	Fold Change	p-value
PI3K/AKT pathway	PIK3CA	-1.095	$1.37 \cdot 10^{-4}$
	CTNNB1	-1.158	$1.09 \cdot 10^{-4}$
	BCL2	-1.667	$1.02 \cdot 10^{-4}$
	CXCR4	-1.141	$2.77 \cdot 10^{-3}$
Wnt/β-Catenin pathway	TLE3	-1.2	$1.07 \cdot 10^{-4}$
	FZD6	-1.413	$3.73 \cdot 10^{-4}$
	BCL9	1.053	$4.95 \cdot 10^{-4}$
	DKK1	1.032	$4.09 \cdot 10^{-4}$
	CTNNB1	-1.158	$1.09 \cdot 10^{-4}$
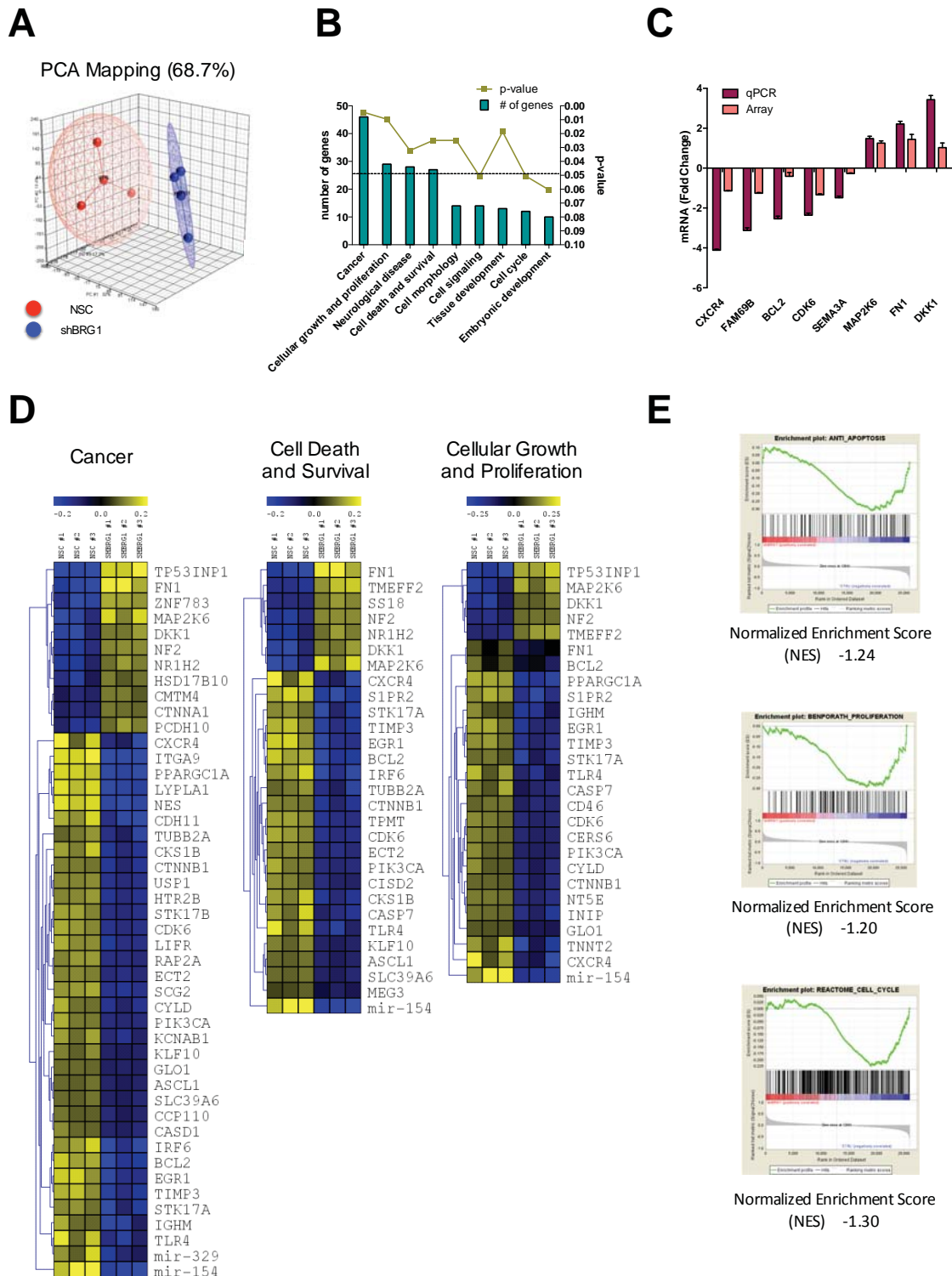
Figure 28: BRG1 downregulation alter several genes involved in cell death, proliferation and survival pathways. A, Principal component analysis shows segregation of distinct expression profiles for the shBRG1 and control groups. **B**, Ingenuity disease function enrichment analysis of differentially expressed genes between shNSC and shBRG1-transduced SK-N-BE(2)C cells (n = 3/group). **C**, Array validation. Quantitative real-time PCR using primers for five downregulated genes (CXCR4, FAM69B, BCL2, CDK6 and SEMA3A) and three upregulated genes (MAP2K6, FN1 and DKK1). Values are represented as fold change versus shNSC and are the mean ± s.e.m. of three independent experiments. **D**, Heatmap shows selected differentially expressed genes grouped in categories of functionally relevant pathways. **E**, Gene set enrichment analysis performed on genes differentially expressed between shNSC and shBRG1 SK-N-BE(2)C cells.

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To confirm the involvement of these pathways in our system, the activity of the PI3K/AKT and WNT pathways was analyzed by phosphorylation of AKT_{Ser473} and β-catenin levels respectively. BRG1 silencing resulted in reduced levels of p110α, Phospho-AKT_{Ser473}, Phospho-S6 and β-catenin total levels while total AKT and Actin remained unaltered. The role of apoptotic pathway was determined by a rapid increase in the levels of the cell-cycle inhibitor p27, a reduction of the antiapoptotic BCL2 protein, and an increase in hallmarks of apoptosis such as the presence of cleaved caspase-3 active fragments and the processing of the caspase-3 substrate PARP (Figure 29).
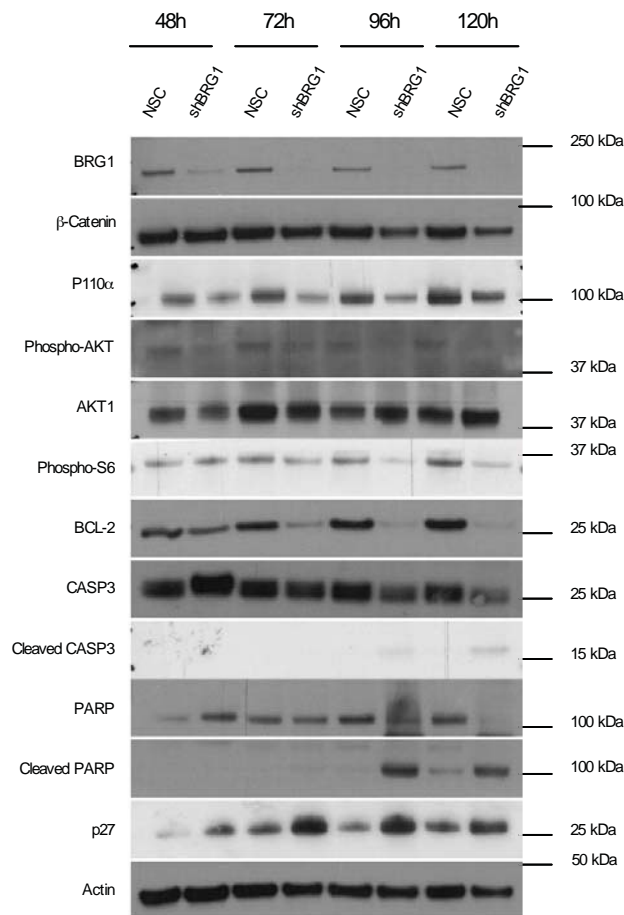
Figure 29: BRG1 inhibition alteres AKT/WNT/Apoptotic pathways at the protein level. SK-N-BE(2) cells were infected with NSC and shBRG1 virus and cells where collected at the indicated time points. Western blot of the indicated proteins to verify the alteration in PI3K signaling, cell cycle and apoptosis signaling. Actin was used as a loading control.

4.5. PI3K and WNT inhibitors show additive effects on NB cell growth

Since different elements of the PI3K/AKT and WNT pathways were found modulated by BRG1 silencing, we sought to ascertain whether the inhibition of these pathways alone or in combination could result in beneficial therapeutic effects in NB. To that end, we used the PI-103 (PI3K inhibitor) and the iCRT14 (WNT inhibitor)[368] molecules alone or in combination and evaluated their effects on the proliferation of NB cells. As expected, inhibition of the PI3K pathway resulted in loss of proliferation even at low doses (0.25µM). By contrast, a significant reduction in cell proliferation was only observed with the WNT inhibitor at higher concentrations (Figure 30). Interestingly, the combination of both drugs resulted in a greater impact on cell proliferation at all concentrations tested (Figure 31A,B). The Combination Index (CI) calculated by the Chou-Talay method showed a very strong synergism between the two drugs (Figure 31C). These data suggest that the combined inhibition of the PI3K/AKT and WNT pathways could be a suitable therapy for high-risk NB.
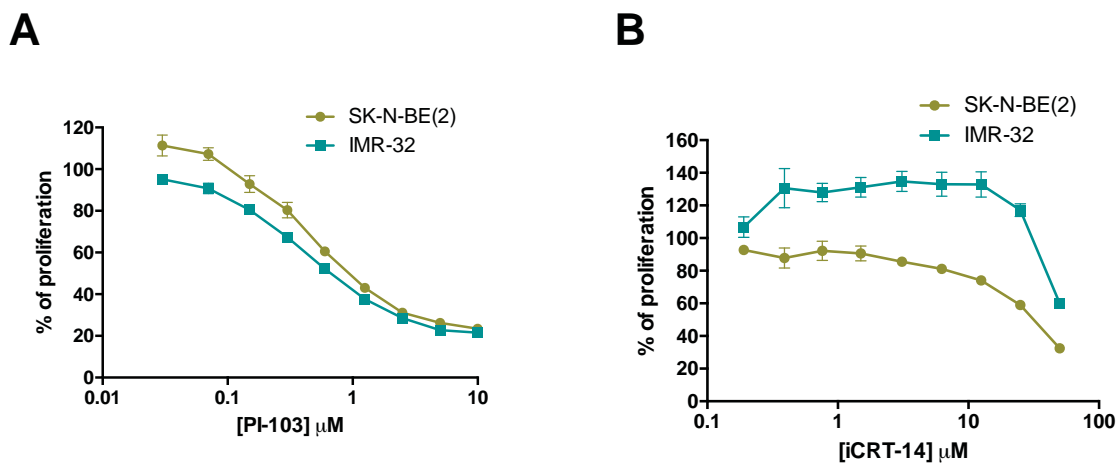
Figure 30: PI3-K and WNT inhibitors reduce the proliferation of NB cells. Normalized proliferation curves of the indicated NB cell lines treated with the PI3-K inhibitor PI-103 **A**, or the WNT inhibitor iCRT-14 **B**. Data represent an average quantification of three independent experiments ± SEM (n=6/condition).

A
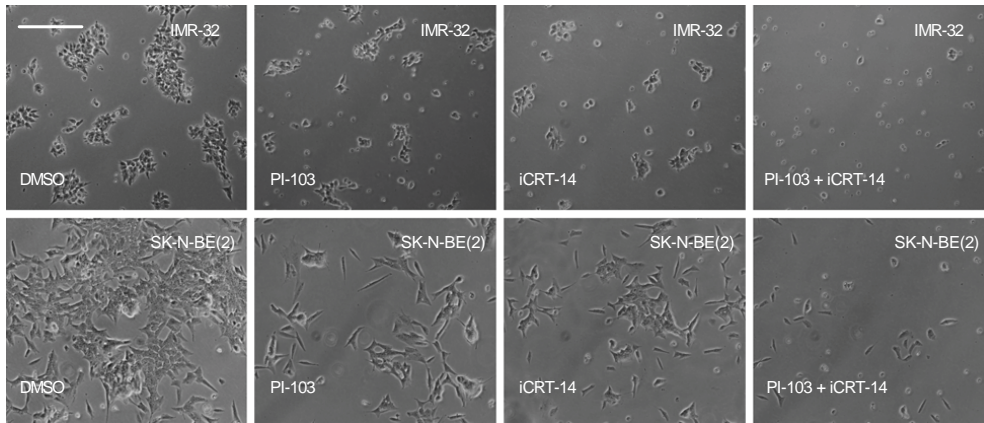
B
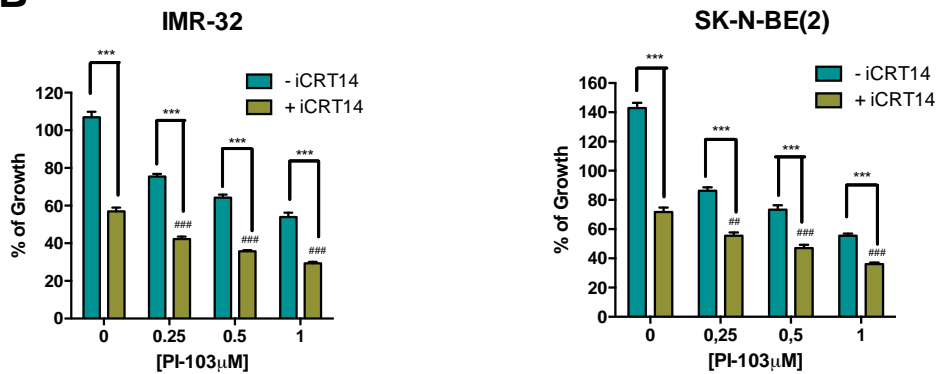
C
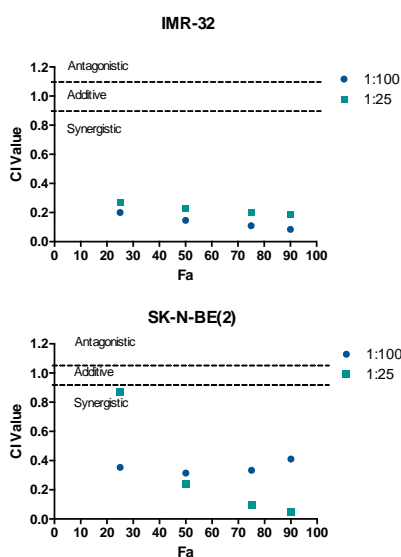
D
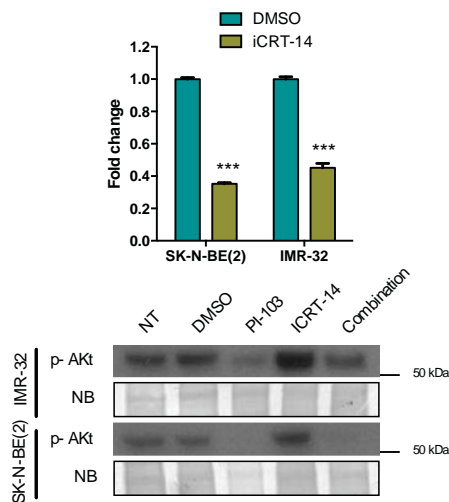
Figure 31: PI3K and WNT inhibitors cooperate to impair NB growth. A, Representative microscopic images of the indicated NB cell lines treated with PI-103 (1μM), iCRT14 (25μM) or the combination of both. Bar, 100μm **B**, Normalized proliferation curves of the indicated NB cell lines treated with the PI3-K inhibitor PI-103 ± the WNT inhibitor iCRT14 (25μM) for four days, measured by crystal violet staining (n=6/condition). Graphs are the average of 3 independent experiments ± SEM. * compares ± iCRT-14 treatment and # compares all conditions versus vehicle (DMSO)-treated cells. **, P<0.01, *** and ###, P<0.001. **C**, Combination index (CI) of PI-103 and iCRT-14, at two different constant ratios. CI was calculated by the Chou-Talay method. Data plotted are CI values at 25%, 50% and 75% of fraction affected. **D**, Controls for the activity of the drugs over WNT and AKT pathways. Up Luciferase Reporter Assay for TCF/LEF binding sites. Down, western blot of phospo-AKT (ser-473).

4.5.1.   WNT pathway is not constitutively active in Neuroblastoma

There are multiple available pharmacological WNT inhibitors, which target the pathway at different levels. For example ICRT-14 disrupts β-catenin-DNA interaction, and XAV939 a tankryase inhibitor that stimulates β-catenin degradation by stabilizing axin, the concentration-limiting component of the destruction complex[369] (Figure 32).
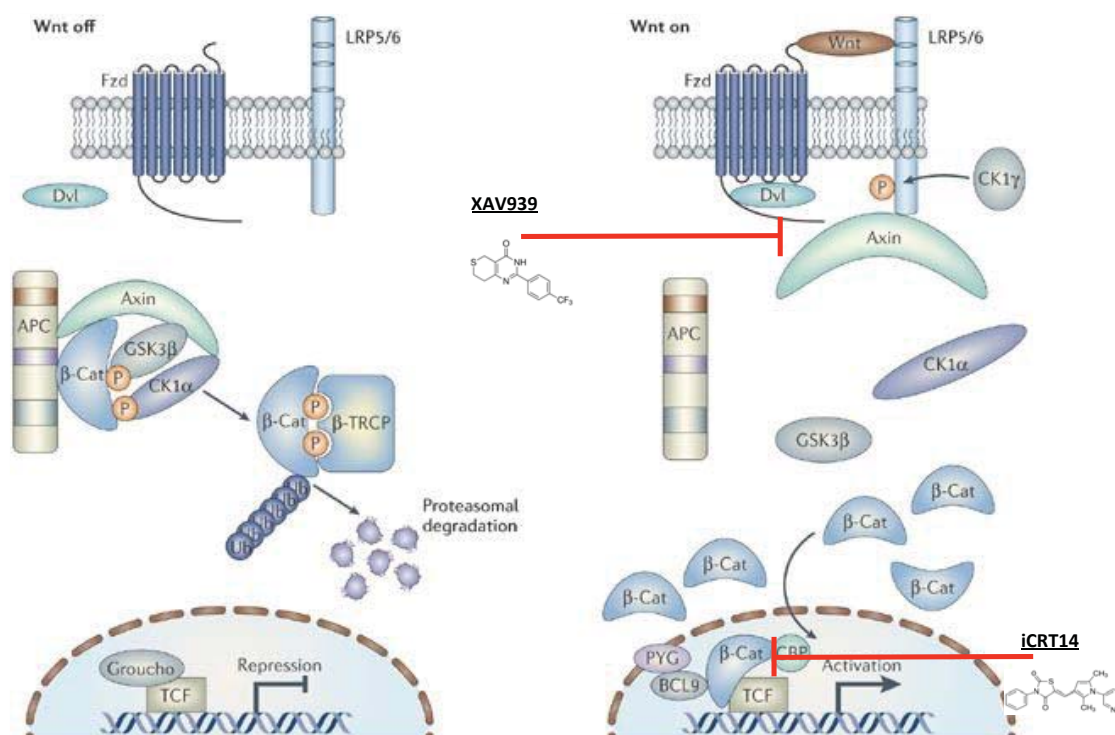
Figure 32: WNT pathway. In the absence of a Wnt signal, β-catenin is captured by APC and axin within the destruction complex, facilitating its phosphorylation and the binding of the β-TRCP, which subsequently mediates the ubiquitinylation and efficient proteasomal degradation of β-catenin. Without β-catenin, the presence of the nuclear DNA-binding proteins of the Tcf/Lef transcription factor family (TCF1, TCF3, TCF4 and LEF1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their promoters and/or enhancers. **Interaction of a Wnt ligand** with its specific receptor complex containing a Frizzled family member and LRP5 or LRP6 triggers the formation of Dvl–Fzd complexes and the phosphorylation of LRP, facilitating relocation of axin to the membrane and inactivation of the destruction box. This allows β-catenin to accumulate and enter into the nucleus where interacts with members of the Tcf/Lef family. In the nucleus, β-catenin converts the Tcf proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting an array of coactivator proteins. This ensures efficient activation of Tcf target genes such as *c-MYC*, which instruct the cell to actively proliferate and remain in an undifferentiated state. **The WNTpathway** can be inhibited through different molecules. In this study we used the iCRT14, an inhibitor of β-catenin and DNA binding and XAV939, a tankyrase inhibitor. (Adapted from N.Barker et al[370])

Therefore, in order to select the best WNT inhibitor for *in vivo* experiments we proceeded to analyze whether the WNT pathway was constitutively active or needed to be activated in a ligand-dependent manner in

NB. We used a cell-based assay that consists of transducing a lentiviral reporter construct, which contained 7 putative TCF4/LEF binding sites for β-catenin upstream of the GFP gene and *mcherry* under an independent promoter to monitor infection efficiency in our NB cells (Figure 33A). This tool provides the possibility of detect active β-catenin in individual alive cells.

Surprisingly, β-catenin activity, i.e. GFP-positive cells, was absent in the great majority of cells and only detected when the cells were incubated with the WNT3A ligand. This fact indicates that the pathway is generally inactive in our culture system and becomes activated only in the presence of exogenous ligands; therefore, our functional effects observed with the iCRT-14 inhibitor in the absence of ligand must be off-target (Figure 33B).

Nevertheless, we analyzed whether BRG1 knockdown may affect the ligand dependent-WNT pathway activation. We observed that the loss of BRG1 did not block the activation of the pathway triggered by WNT3A (Figure 33C). Since β-catenin may also have WNT-independent functions, we proceeded to overexpress constitutively-active β-catenin in BRG1-depleted cells. Again, the overexpression of active β-catenin did not prevent the reduction in proliferation/viability caused by loss of BRG1 (Figure 33D). Moreover, another proliferation assay with a different WNT inhibitor (i.e. XAV939) showed similar effects (Figure 33E).

Overall, we can conclude that, despite the clear effects of BRG1 knockdown on β-catenin levels and some other components of the WNT pathway, its inhibition is not necessary for the functional consequences of BRG1 inhibition.
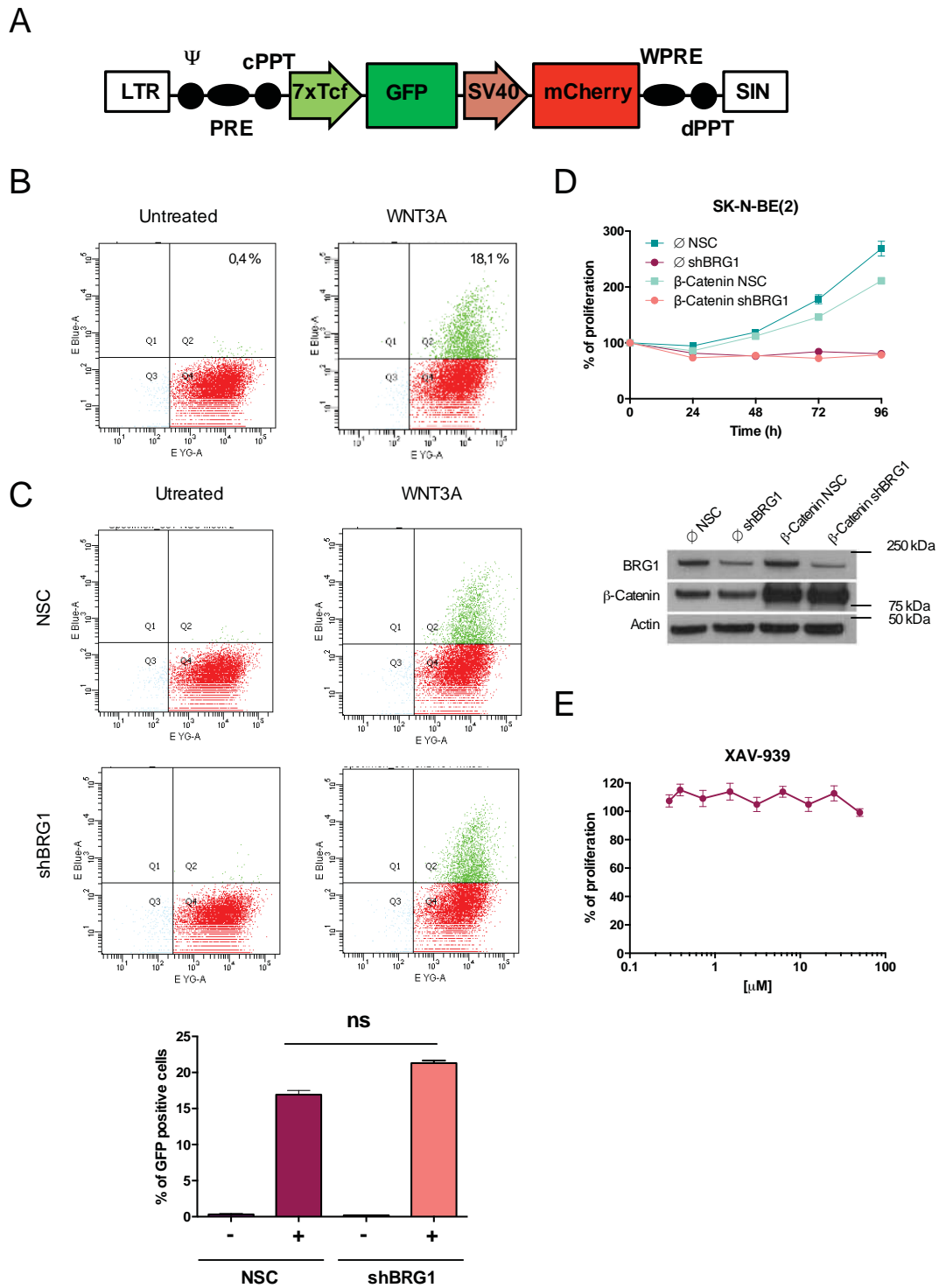
Figure 33: BRG1 does not alter WNT signaling in NB. A, Scheme representation of β-catening activity reporter construct. When β-catening binds to TCF sequences drives the expression of GFP, while m*cherry* expression to monitor transduction efficiency is driven by an independent promoter. **B**, FACS analysis in SK-N-BE(2) cells transduced with the β-catenin reporter vector in basal conditions (untreated) or stimulated the WNT pathway with the WNT3A ligand. **C**, β-catening activity assay in SK-N-BE(2) cells transduced with Non Silencing Control (NSC) or shBRG1. Graph (on the right) represents the average of three independent experiments ± SEM. ns = non significant. **D**, Growth curve in SK-N-BE(2) cells transduced with β-catening and shBRG1 constructs. Western-blot confirms the efficiency of BRG1 knockdown and β-catenin overexpression. **E**, Cell viability assay in SK-N-BE(2) cells treated with the indicated concentrations of the WNT inhibitor XAV939.

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4.6. PI3K and BCL2 inhibitors exert synergistic effects on NB cell growth

Among the apoptosis-associated genes, the well-known antiapoptotic gene *BCL2* was one of the most downregulated genes upon BRG1 silencing. BCL2 expression has been shown to be elevated in high-risk neuroblastoma and its pharmacological inhibition reduces tumor growth *in vitro* and *in vivo*[371].

Since BCL2 is a master regulator of apoptosis and also regulated by the PI3K pathway, we proceeded to analyze whether the overexpression of BCL2 could revert the phenotypic effects caused by BRG1 knockdown. Interestingly, the overexpression of BCL2 only transiently prevented BRG1 silencing-induced cell death (Figure 34A), suggesting that BRG1 inhibition leads to non-redundant, simultaneous regulation of multiple cell cycle/cell death effectors, thereby resulting in attenuated proliferation and increased cell death. This indicates that inhibiting different BRG1 targets, such as apoptotic or PI3K pathway components, would be necessary to obtain the same results as with BRG1 inhibition.
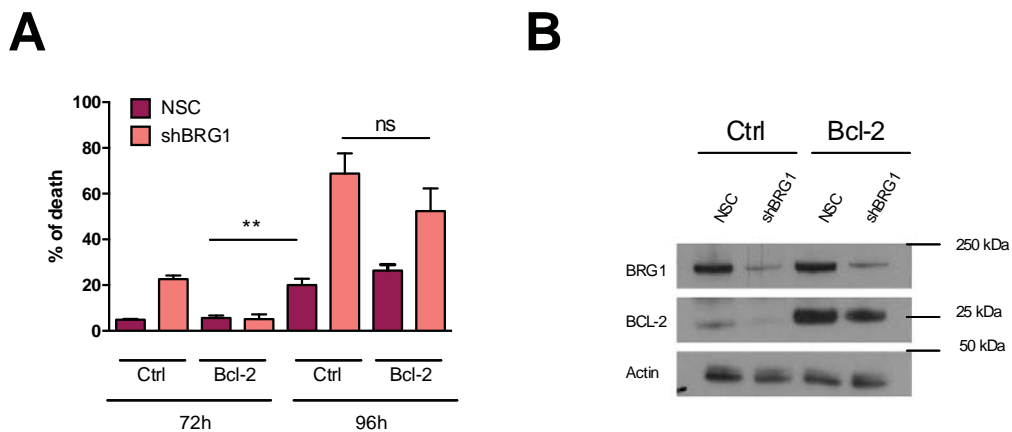
Figure 34: Bcl-2 transiently rescues BRG1-induced cell death. A, Stably BCL2-transfected SK-N-BE(2) cells were transduced with control or shBRG1 and viability was measured 48 and 72 h later with trypan blue. **B**, Western blot confirmed BRG1 silencing and BCL2 overexpression. Actin was used as a loading control.

Therefore, we followed the same rationale as with PI3K and WNT pathways. We used the PI-103[372] (PI3K inhibitor) and the ABT-199[373] or ABT-263[374] (BCL2 inhibitors) molecules alone or in combination and evaluated their effects on the proliferation of NB cells. Inhibition of the PI3K and BCL2 pathways resulted in loss of proliferation (Figure 35). Between the two tested

BCL2 inhibitors, we selected ABT-263, because it was more effective on inhibiting cell proliferation.
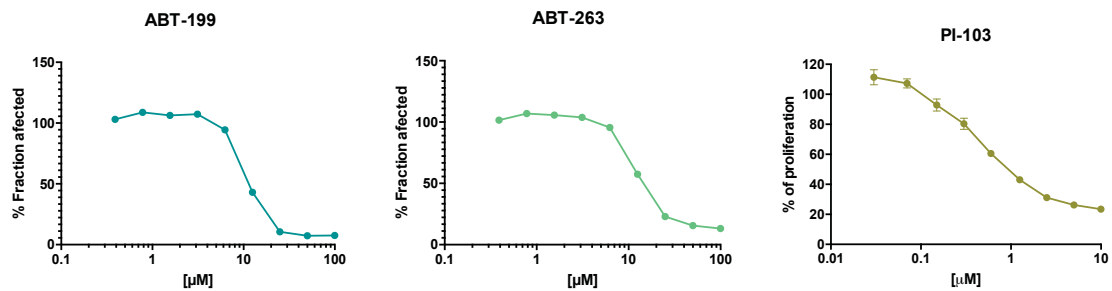
Figure 35: PI3-K and BCL-2 inhibitors reduce the proliferation of NB cells. Normalized proliferation curves of SK-N-BE(2) NB cell line treated with two different BCL-2 inhibitors, ABT-199 and ABT-263 and the PI3-K inhibitor PI-103.

Interestingly, the combination of PI-103 and ABT-263 drugs resulted in a greater impact on cell proliferation (Figures 36A and 36B). The combination index (CI) calculated by the Chou–Talalay method showed very strong synergism between the two drugs (Figure 36C). Western blot analysis confirmed an increased amount of active cleaved caspase-3 forms and processing of PARP when both drugs were combined (Figure 36D).

A
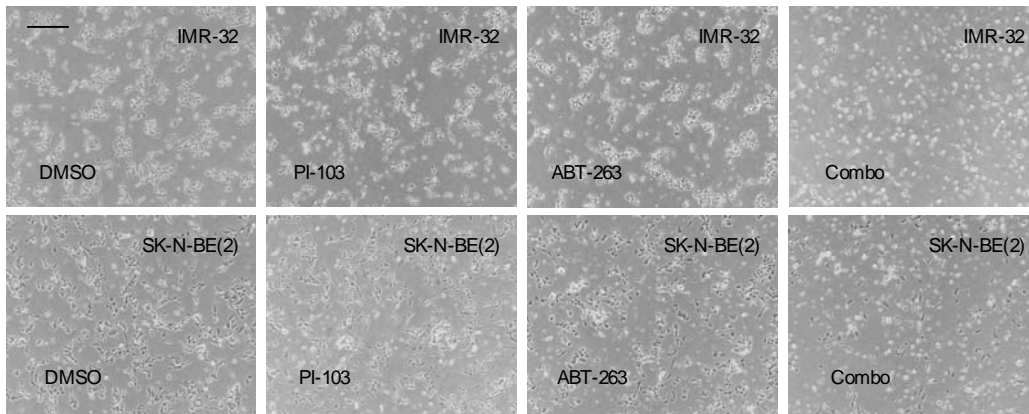
B
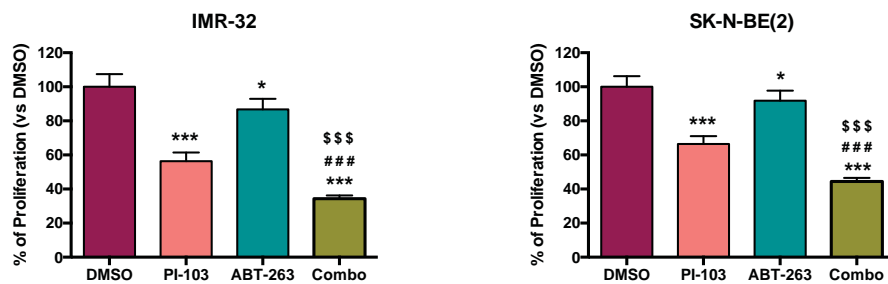
C
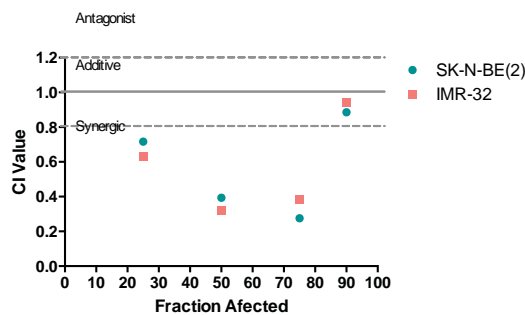
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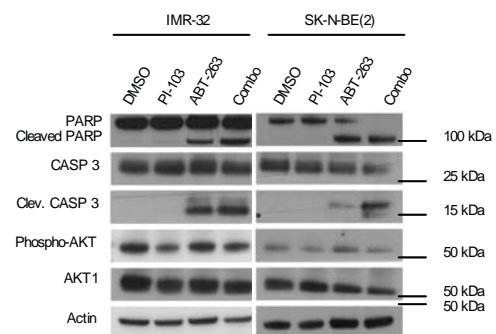
Figure 36: PI3K and BCL2 inhibitors cooperate to impair NB growth. A, Representative microscopic images of the indicated NB cell lines treated with PI-103 (1 µM), ABT-263 (3 µM for IMR-32 and 6 µM for SK-N-BE(2)) or the combination (Combo). Bar, 100 µm. **B**, Proliferation/viability assay of the indicated NB cell lines treated with the PI3K inhibitor PI-103 (1 µM) and the BCL2 inhibitor ABT-263 (12 µM) and the combination of both (Combo) for 24 h, measured by crystal violet staining (n = 6/condition). Graphs represent one of three independent experiments. *compares Combo treatment versus vehicle (DMSO); #Combo versus PI-103; $Combo versus ABT-263. *P<0.05; ***P<0.001, *** and ###P<0.001. **C**, Combination index (CI) of PI-103 and ABT-263 at 1:12 constant ratio. CI was calculated by the Chou–Talalay method. Data plotted are CI values at 25, 50, 75 and 90% of fraction affected. **D**, Western blot of the indicated proteins to confirm the efficacy of the inhibitors.

In order to verify whether this approach may be effective *in vivo*, we repeated the analysis of the pharmacologic compounds using multicellular tumor spheroid cultures, which have proved to better mimic the characteristics of *in vivo* growth of solid tumors compared with 2D cultures[375]. Treatment with the PI-103 and ABT-263 inhibitors individually impaired tumor spheroid growth and exerted a more dramatic effect when combined (Figures 37A and 37B). These data suggest that the combined inhibition of PI3K/AKT and BCL2 may be a suitable therapy for high-risk NB.

A
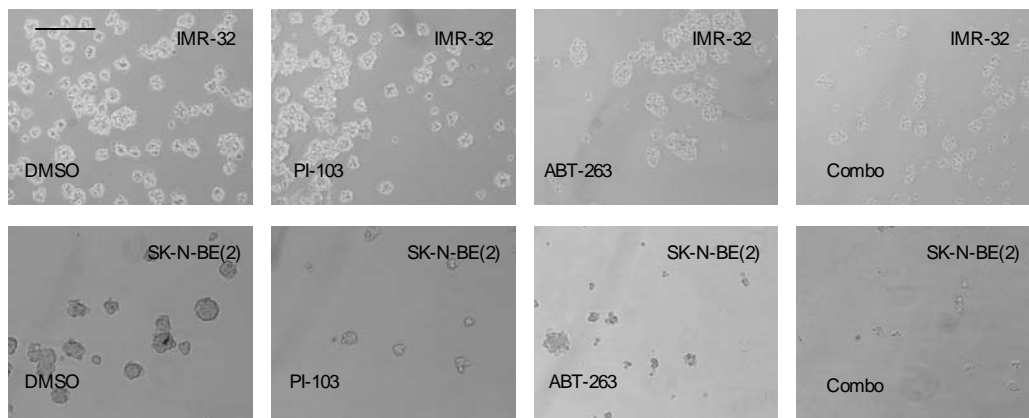
B
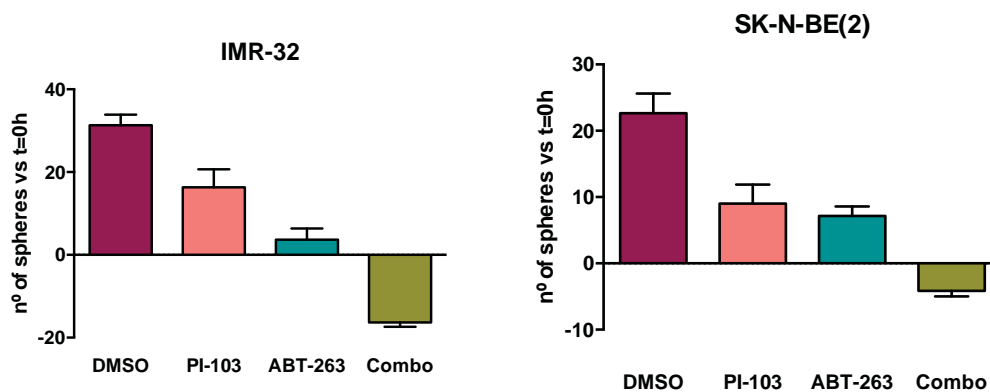
Figure 37: PI3K and BCL2 inhibitors reduce the viability of NB tumor spheroids. A, Representative microscopic images of tumor spheroids treated with PI-103 (1 μM), ABT-263 (3 μM for IMR-32 and 6 μM for SK-N-BE(2)) or the combination of both (Combo). Bar, 100 μm. **B**, Viability assay of the indicated NB cell lines treated with the PI3K inhibitor PI-103 (1 μM) and the BCL2 inhibitor ABT-263 (3 and 6 μM for IMR-32 and SK-N-BE(2), respectively) and the combination of both (Combo) measured by counting of spheroids $\geqslant$ 40 μm in diameter (n = 6/condition). Graphs are the average of three independent experiments ± s.e.m.

DISCUSSION

5. DISCUSSION

5.1. Epigenetic regulators as new therapeutic targets in Neuroblastoma

Neuroblastoma displays abundant alterations in the epigenetic landscape of the genome such as aberrant methylation patterns of specific genes (e.g. CASP8)[143] or in large genomic areas[376], and anomalous post-translational modification of histones.

Alterations in many epigenetic regulators have been observed in NB (See section 1.2). Proteins from DNMT, HMT, HDM, HAT, HDAC and BRD families have been shown to play important roles in this disease, such as patient outcome[302,311], cell differenciation[303,377], proliferation[335,340], invasion[317,322], metastasis[378] and resistance to therapy[343]. In an attempt to restore the normal conformation of the epigenome, inhibitors for these proteins have been developed.

DNMT INHIBITORS

General increased genomic methylation is associated with poor outcome in NB[379]. Therefore, the use of DNMT inhibitors (DNMTi) may offer new alternatives for patients who do not respond to current therapies. One of the first DNMTi tested in NB cells was 5-Aza-deoxycytidine (5-Aza or decitabine), a chemical analog of the nucleoside cytidine. Treatment of NB cells with 5-aza showed induced cell differentiation[380], reduced proliferation and colony formation [380,381]. Further studies demonstrated that 5-Aza can potentiate the cytotoxic effects of current chemotherapies such as of doxorubicin, cisplatin and etoposide[382], thereby suggesting that a combination of 5-Aza with standard therapies can lead to more effective and safer treatments. However, a phase I clinical study of decitabine with doxorubicin showed that only low-doses of decitabine with this combination were tolerable and those capable of producing clinically significant biologic effects were not well tolerated[383] .

These results suggest that more specific DNMT-inhibitors may offer better safety profiles. Recently, two new DNMT inhibitors, SGI-1027 (selective

for DNMT1, DNMT3A/B) and Nanaomycin A (DNMT3B specific), displayed higher cytotoxic effects alone or in combination with doxorubicin but without altering general genome methylation[384]. The use of these new inhibitors will presumably result in less side effects.

HMT INHIBITORS

On of the most studied HMT is EZH2, a PKMT with elevated expression levels in aggressive NB. EZH2 is the key enzymatic component of the Polycomb Repressive Complex 2 (PRC2), an important player in regulating gene expression during development and tissue differentiation[377]. Several clinical trials with EZH2 inhibitors (e.g. tazemetostat, CPI-1205) are currently recruiting patients for adult malignancies but are lacking for pediatric tumors.
Other PKMT inhibitors are showing therapeutic potential in NB. This is the case of BIX-01294, a specific inhibitor of EHMT2, a protein frequently overexpressed in several tumor types. Treatment of NB cells with BIX-01294 showed decreased cell proliferation, inhibition of cell mobility and invasion[385], induction of apoptosis *in vitro* and reduced tumor growth in preclinical mouse models [386].

HDM INHIBITORS

Regarding to NB, only KDM1A (an HDM that is classified inside of the KDM1 family) inhibitors have been designed; however, just trans-2-phenylcyclopropylamine (TCP) derivatives have been advanced into early phase clinical trials. NB preclinical experiments showed that TCP alone was able to reduce NB cell proliferation and induce the tumor-suppressor gene p21. Interestingly, the combination of TCP with a c-myc/NMYC inhibitor (i.e. 10058-F4) showed synergistic effects and induction of apoptosis[387]. However, the therapeutic potential of these compounds in animal models or in clinical trials remains to be addressed.

HAT INHIBITORS

Three HAT inhibitors have been tested in NB models: PU139 (a HAT pan-inhibitor[388]), PU141 (a CBP and p300 selective inhibitor[388]), and BF1 (an H3 -acetylation protein inhibitor[389]). All reduced NB cell growth *in vitro*[388,389], but only PU139 and PU140 were demonstrated to reduce tumor growth *in vivo*[388]. Furthermore, PU139 treatment had synergistic effects with doxorubicin *in vivo* and blocked tumor growth[388].

HDAC INHIBITORS

Owing to the relevance of HDAC proteins in cancer, many inhibitors have been developed in recent decades. These inhibitors are classified depending on the targeted HDAC class. The first developed HDAC inhibitors (HDACi) were those targeting Classes I, II and IV. They can be classified into six basic types depending on the structure of the inhibitor (reviewed in[390]). On the other hand, Class III HDAC are inhibited with derivatives of NAD[391].

Multiple studies showed the therapeutic potential of HDACi in NB in preclinical studies (Table ##), but few of them reached clinical trials (Table 18).

Table 18: HDAC inhibitors studied in NB

Name	Alias	Effective *in vivo*	Reference
m-Carboxycinnamic Acid bis-Hydroxamide	CBHA	+	392–394
suberoyl-3-aminopyridineamide hydroxamic acid	Pyroxamine	n.d.	395
MS-275	Entinostat	+	396–399
Sodium Butyrate	NaB	n.d.	400–405
BL1521	BL1521	n.d.	406–408
Trichostatine A	TSA	+	409–415
Glycerin Tributyrate	Tributyrin	n.d.	416
M344	M344	n.d.	397
HKI 46F08	HKI 46F08	n.d.	417
Helminthosporium carbonum-toxin	HC-toxin	n.d.	418
Romidepsin	Istodax	+	419
C149	C149	n.d.	420
LBH-589	Panobinostat	+	421,422
PCI-24781	Abexinostat	+	423,424
BRD8430	BRD8430	n.d.	425
CAS 14513-15-6	Cambinol	+	30
Salermide	Salermide	n.d.	31
PCI-35051	PCI-35051	+	335,426
Tubacin	Tubacin	n.d.	342,343
1-naphthohydroxamic acid	Cpd2	+	335

n.d.: not determined

One of the most studied HDACi in NB is valproic acid (VAP), discovered by B.S. Burton in 1882[427]. This inhibitor has higher, but not exclusive, selectivity to Class I HDAC. Initially, this compound was used to treat seizures, bipolar disorders or migraines. Different studies later showed that VAP inhibited HDAC proteins (reviewed in[428]), thereby opening a door to cancer treatment.

When NB cells are treated with VAP, a strong inhibition of cell proliferation and induction of differentiation and apoptosis is observed[416,429]. Other studies showed the therapeutic potential of VAP in combination with current therapies such as ABT-510 (an angiogenic inhibitor)[430] or with OGX-011[431] (inhibitor of clusterin), resulting in tumor growth impairment. However, in some cases, VAP combination effects are subject to administration order. When VAP is combined with conventional chemotherapeutic agents such as etoposide or cisplatin, these drugs must be administered in first place[432,433].

Another well-studied HDACi in NB is vorinostat (also called SAHA). Vorinostat is a selective class I and II HDACi and is being used in multiple clinical trials in NB. Treatment of NB cells with vorinostat resulted in cell cycle arrest in G2/M phase followed by the activation of the intrinsic apoptotic pathway[434]. Vorinostat was also shown to impair VEGF secretion by NB cells, thereby suggesting a potential antiangiogenic effect[435].

Vorinostat has also been shown to potentiate the antitumor activity of different drugs such as flavopiridol (a pan-Cdk inhibitor) [436], fenretinide (a synthetic retinoid) [437] and radiotherapy[438,439].

The latest HDACi to reach clinical trials was 4PB (4-phenylbutyrate), also selective for HDAC class I and II. In 1998, M.A. Pelidis *et al.* described for the first time the effectiveness of 4PB in NB. These authors demonstrated that 4PB reduced the proliferation and induced differentiation of NB cell lines. Moreover, 4PB demonstrated additive cytotoxic effects when administered with the chemotherapeutic drug vincristine[440]. Concurring with these results, Tang *et al* showed that 4PB induced the expression of several genes associated with favorable outcome (i.e. EPHB6, EFNB2, EFNB3, NTRK1 and CD44) and impaired tumor growth and metastasis *in vitro* and *in vivo*[441].

BROMODOMAIN INHIBITORS

The crystallization of the BRD structure and the feasibility of designing small molecules to target this domain placed the BRD inhibitors in the spotlight as new therapeutic targets for cancer. In 2010, two small-molecule inhibitors against BET-bromodomains (a family of BRD called Bromodomain and extra-terminal domain, which consists of four different proteins) were described by two independent groups (JQ1 and I-BET[442,443]) with high affinity for BRD2, BRD3 and BRD4. Both compounds showed that BRD inhibition resulted in antitumor effects in mixed lineage leukemia[444], multiple myeloma[445] or lung adenocarcinomas[446].

The therapeutic potential of BRD inhibition in NB was first analyzed by Puissant *et al.* The treatment of NB cells with the BET inhibitor (iBET) JQ1 resulted in a reduction in MYCN levels, reduced cell growth and induction of apoptosis *in vitro* and *in vivo*[447,448]. The JQ1 inhibitor also showed synergistic

effects when combined with the HDACi panobinostat. This drug combination showed reduced MYCN protein expression and impaired tumor growth *in vivo*[449].

Recently, another BRD inhibitor (i.e. OTX015), was tested in NB. The administration of OTX015 showed reduction in MYCN expression and loss of interaction of MYCN with the promoter of their target genes. Furthermore, BRD4 was also shown to be preferentially displaced from DNA super-enhancers regulated by MYCN[450].

Despite the vast literature in epigenetic regulators inhibitors, only 6 of them reached clinical trials in NB (Table 19). Most of these new drugs are not specific and may therefore result in modest effects, which, at least, could be improved if the main contributors to the oncogenic phenotype are identified.

Table 19: Current epigenetic drugs clinical trials in NB

Name of the drug	Type of drug	Phase	Estate	Number
Decitabine	DNMT pan-inhibitor	Phase I	complete	NCT01241162
		Phase I	complete	NCT00075634
Genistein	DNMT pan-inhibitor	Phase II	recruiting	NCT02624388
Vorinostat	HDACi class I and II inhibitor	Phase I	complete	NCT00217412
		Phase I	complete	NCT01132911
		Phase II	ongoing but not recruiting	NCT02035137
		Phase I	recruiting	NCT02559778
		Phase I	complete	NCT01019850
		Phase I	complete	NCT01208454
VAP	HDACi class I and II inhibitor	Phase I	complete	NCT01204450
4-PB	HDACi pan-inhibitor	Phase I	complete	NCT00001565
GSK525762	iBET	Phase I	recruiting	NCT01587703

To discover the main epigenetic regulator contributors in NB we built a list with the more relevant epigenetic factors and performed an *in silico* analysis of mRNA expression public available databases. We selected 3 NB tumor databases with stage and MYCN amplification information, and the highest

number of samples at that moment. One of them, GSE16476, also contained survival information for each sample. Taken altogether this information, we chose the most significantly deregulated genes in the NB tumor database that studied the highest number of genes and then we compared with the information of the rest of datasets. We built a list with the ten most deregulated genes, among which we chose BRG1 as the most consistently deregulated one in all 3 datasets, thereby indicating that BRG1 plays an important role in the disease and is a good candidate for developing a NB targeted therapy. The other genes on the list have been also described to be altered in other tumors or in NB. For example, PRMT5 and EZH1 are altered in Leukemias[451,452], MLL3 is altered in gastric cancers[453], or WHSC1 and PRDM2 which are altered in NB[454,455].

5.2.   Why BRG1 is overexpressed in Neuroblastoma?

We demonstrated that BRG1 was widely expressed in NB and higher levels of the protein correlates with advanced stages of the disease. Furthermore, patients with higher BRG1 levels showed shorter event-free survival and worse overall survival, thereby suggesting a role as a potential prognostic marker. Still, a question remains to be determined: why BRG1 is overexpressed in NB?

BRG1 is located on the 19p13.2 genomic locus. In NB, chromosome 19 deletions have been described, although none of them are in 19p13.2[456–459]. No amplifications of chr19 have been found in NB; nevertheless, 19p13 gains have been described in ovarian cancer, lymphomas, ependymoma and head and neck squamous cell carcinomas[460–463]. Furthermore, in some cases such as ovarian cancer, BRG1 expression correlates with worse patient outcome. Thus, further research must be done to determine whether 19p13.2 amplifications can explain the overexpression of BRG1 in some cases of NB.

Gene promoter hypo- or hypermethylation are common events in cancer that help to initiate or promote the disease through upregulation or downregulation of protein expression. Hypermethylation of CASP-8 promoter in NB[143] is an example of the importance of this type of regulation. Thus, it is not unreasonable to think that BRG1 expression could be regulated through this

type of mechanisms. Unfortunately, no studies have determined that a hypomethylation can rule the expression of BRG1, and one study determined that hypermethylation of BRG1 promoter in lung cancer was not the mechanisms of which BRG1 is donwregulated in these type of cancers[464], thereby indicating that BRG1 may have other mechanisms of regulation. This question could be addressed by the study of the methylation status of BRG1 in patient samples from different types of cancers.

One of the main gene regulation mechanisms in the cell is through transcription factor (TF) regulation. Unfortunately no studies have determined the regulation of BRG1 by TF; therefore, we performed an *in silico* analysis in three different databases: GeneHancer, a database of regulatory elements of genes, which have predicted TF binding sites[465]; R2 visualization platform, using GSE45547 samples we correlated SMARCA4 against all TF[466] and GTRD, a data base containing ChIP-seq of several TF[467]. Table 19 shows potential BRG1 regulatory elements. Some of them have already established functions in NB, such as MYCN which is one of the most well known oncogenes in NB. Other TF are related to the retinoic acid (RA) pathway and RA-induced differentiation.

Table 20: Possible BRG1 regulating TF

Gene symbol	NB relation	References
NMYC	Amplification correlates with poor NB prognosis	28,32,33
NFYC	N/A	
MITF	N/A	
JUND	N/A	
ATF4	Coordinates glutamine metabolism in NMYC amplified NB	468
RARA	Involved in differentiation and chemoresistance	469–471
GATAD2B	N/A	
GATAD2A	Part of the NuRD chromatin remodeling complex in NB along with CHD5	472
CEBPG	Related to RA induced NB differentiation	473
FOXA2	Related to RA induced NB differentiation	474

N/A: not determined

One of the most important post-transcriptional gene regulators are microRNAs (miRNA). DICER and DROSHA, the proteins in charge of post-

transcriptional maturation of miRNA, are lost or mutated in advanced NB, thereby causing a global reduction of miRNA. Thus, upregulation of BRG1 in advanced NB may be due to a reduction of the miRNAs that are targeting 3'UTR of the gene. Cuadros, M. *et al.* demonstrated that miR-155 regulate BRG1 in leukemia cells[475]. Since no more studies of BRG1 miRNA regulation have been carried out in NB, we performed an analysis of BRG1 3'UTR with five different miRNA binding sites prediction algorithms: TargetScan[476], miRWalk[477], miRANDA[478], miRDB[479,480] and miRMap[481] (Figure 38A). We found that miR-155was predicted in all three algorithms. Additionally, other miRNA that acts as tumor suppressor in NB, such as let-7 family miRNAs[482], have been predicted as possible regulators of BRG1 (Figure 38B).

miRNA	ID	Family	Sequence	Locus
miR_5695	MIMAT0022488	miR_5695	ACUCCAAGAAGAAUCUAGACAG	19p13.13
miR_582_5p	MIMAT0003247	miR_582	UUACAGUUGUUCAACCAGUUACU	5q12.1
miR_1185_1_3p	MIMAT0022838	let_7	AUAUACAGGGGGAGACUCUUAU	14q32.31
miR_5190	MIMAT0021121	miR_5190	CCAGUGACUGAGCUGGAGCCA	18p11.21
miR_4768_5p	MIMAT0019920	miR_4768_5p/6833_3p	AUUCUCUCUGGAUCCCAUGGAU	Xp22.13
miR_1185_2_3p	MIMAT0022713	let_7	AUAUACAGGGGGAGACUCUCAU	14q32.31
let_7f_2_3p	MIMAT0004487	let_7	CUAUACAGUCUACUGUCUUUCC	Xp11.22
miR_4699_5p	MIMAT0019794	miR_4699_5p	AGAAGAUUGCAGAGUAAGUUCC	12q21.31
miR_155_5p	MIMAT0000646	miR_155	UUAAUGCUAAUCGUGAUAGGGGU	21q21.3

Figure 37: Predicted miRNA to target BRG1. A, Venn diagram representing the overlap of predicted target genes between five miRNA-target prediction algorithms (miRWalk.2.0, miRanda.2010, miRDB.4.0, miRMap and TargetScan.6.2). **B**, Table containing the miRNA that are predicted to target BRG1 in the five algorithms.

5.3. BRG1: a two-faced protein

5.3.1. BRG1 as a tumor supressor

BRG1 is one of the core proteins of the SWI/SNF complex. It is found mutated in 3% across all cancers, rendering BRG1 as the most mutated core protein of the complex[367]. Additionally, knockout mouse studies showed that a null mouse was embryonic lethal, whereas heterozygotes males and female presented a mammary tumor incidence of 9% (Reviewed in[483]). Together these results suggest a tumor suppressive role of BRG1. Furthermore, this protein has been postulated as a tumor suppressor in many cancers such as lung cancer, medulloblastoma or rhabdoid tumors among others (Table 21).

Table 21: BRG1 roles in cancer

Acts as	Cancer type	Evidence	References
Oncogene	Small Cell Lung Cancer	Functional evidence	484
	Acute myeloid leukemia	BRG1 overexpressed; functional evidence	485,486
	Melamona	BRG1 overexpressed; functional evidence	487–489
	Medulloblastoma	Functional evidence	490
	Colorectal cancer	BRG1 overexpressed; functional evidence	491
	Gastric cancer	BRG1 overexpressed; association with prognosis and drug resistance	492,493
	Prostate cancer	BRG1 overexpressed; association with prognosis; functional evidence	494,495
	Glioma	BRG1 overexpressed; functional evidence; susceptibility SNPs	496,497
	Pacreatic cancer	BRG1 overexpressed; association with stage and drug resistance; functional evidence	498–500
	Breast cancer	BRG1 overexpressed; association with survival; functional evidence	501–503
Tumor Suppressor	Small cell carcinoma of the ovary, hypercalcemic type	BRG1 mutated; loss of expression; functional evidence	504,505
	Non-Small Cell Lung Cancer	BRG1 mutated; loss of expression; association with prognosis; functional evidence	506–509
	Rhabdoid tumors	BRG1 mutated; loss of expression; predisposition mutations	510,511
	Medulloblastoma	BRG1 mutated	512
	Burkitt Lymphomas	BRG1 mutated	513
	Clear cell renal cell carcinoma	BRG1 mutated	513
	Hepatocellular carcinoma	BRG1 mutated	514,515
	Endometrioid tumors	BRG1 loss of expression	516,517
	Pancreatic cancer	BRG1 mutated; loss of expression; functional evidence	500,518,519
	Acute lymphoblastic leukemia	BRG1 loss of expression; functional evidence	520

5.3.2. BRG1 as an oncogene

BRG1 was first postulated as a tumor suppressor, but Watanabe et al observed that BRG1 was overexpressed in colorectal cancer patients and the supression of BRG1 impaired cellular growth[491]. Despite that BRG1 heterozygous mice develop mammary tumors, later studies unveiled an oncogenic role of BRG1 in breast cancer. In fact, knock-down of the protein resulted in reduced *in vivo* and *in vitro* tumor growth[521]. Together with breast

cancer, other cancers such as colon cancer, glioma, prostate cancer, melanoma, etc. show an oncogenic like role of BRG1 (Table 21).

It seems that BRG1 role varies from tumor suppressor to oncogenic depending on the type of cancer. Furthermore, even in the same tumor type BRG1 can act promoting or repressing tumoral activity, depending on the tumoral stage.

In small cell lung cancers that contain mutations in MAX, a c-MYC associated factor, BRG1 knockdown caused a reduction of cell growth (in contrast to what happens in non-mutated MAX small cell lung cancer in which BRG1 acts as a tumor suppressor), thereby indicating a synthetic lethality between the two proteins.

Sonic-hedgehog type medulloblastoma showed an attenuated tumor formation and progression upon BRG1 depletion[490]. Nevertheless, other types of medulloblastoma have been associated with heterozygous missense or in-frame In/Del mutations in BRG1[490,522].

In pancreatic cancer, numerous reports have indicated a tumor suppressive role of BRG1; lower expression was found in precancerous lesions and pancreatic cancer itself (reviewed in[483]), and loss of BRG1 in inhibited KRAS-driven formation of precancerous pancreatic cancer lesions[519]. However, other studies correlated the higher expression of BRG1 and BRM (the mutually exclusive core subunit of the SWI/SNF complex) with advanced states of the disease and poor survival[498] and functional studies showed that knockdown of BRG1 in pancreatic cancer cell lines reduced tumor formation *in vivo* and *in vitro*[523]. Further studies unveiled the distinct roles of BRG1 in pancreatic cancer, as BRG1 prevents and promotes this cancer in a stage-specific manner; BRG1 functions as a tumor suppressor at precancerous lesions preventing dedifferentiation and tumor initiation, but when pancreatic cancer is formed, BRG1 promotes its progression[500].

In our study, we found that BRG1 was expressed in NB and higher levels correlated with shorter event-free survival and worse overall survival, independently of MYCN amplification, rendering it as a good candidate for disease progression marker, though further studies must be carried to characterize this function. By contrast, BRM protein levels were barely

detectable in NB cell lines and, at the mRNA level, no association was found between BRM expression and patient outcome. BRM has been reported to be absent in neural precursor cells and increase during neuronal differentiation[524]. Thus, not surprisingly, the low levels of BRM observed in our panel of cell lines may result from the undifferentiated status of NB cells. These results appear to indicate that BRG1 is the main core component of the SWI/SNF complex in NB.

Our data also indicate that the high expression of BRG1 plays an essential role in maintaining the proliferation and viability of NB cells. Contrary to what occurs with other embryonic cancer cell lines, BRG1 silencing impairs cell growth and triggers caspase-dependent apoptosis in multiple NB cell lines, regardless of their MYCN status, p53 functionality or ALK mutations. This evidence points to BRG1 as a new therapeutic target for NB. Our transcriptomic analysis after BRG1 depletion in NB cells using different platforms (i.e. Ingenuity Pathway Analysis, KEGG, Gene Set Enrichment Analyses) defined BRG1 as a master regulator of cell proliferation and viability in NB cells.

Therefore, and also supported by the fact that BRG1 depletion induces cell death even in NB cells considered to be chemoresistant[525], targeting BRG1 may overcome resistance to current treatments. Moreover, independence of MYCN, p53 or ALK status suggests BRG1 inhibition as a possible new line of treatment also for patients for whom no targeted therapy currently exists.

Notwithstanding, we found one cell line that was not affected upon BRG1 depletion, the SK-N-AS cell line. Depletion of the protein through shBRG1 did not reduced or increased proliferation or colony formation. Additionally, targets that were regulated in BRG1-dependent cell lines showed no change in SK-N-AS BRG1 knockdown cells (Figure 39). Thus, these results render SK-N-AS cell line as a perfect model to analyze BRG1 and SWI/SNF complex in BRG1 dependent or independent context, in order to shed light on how the same protein can act as an oncogene or a tumor suppressor depending on the tumoral context.

A
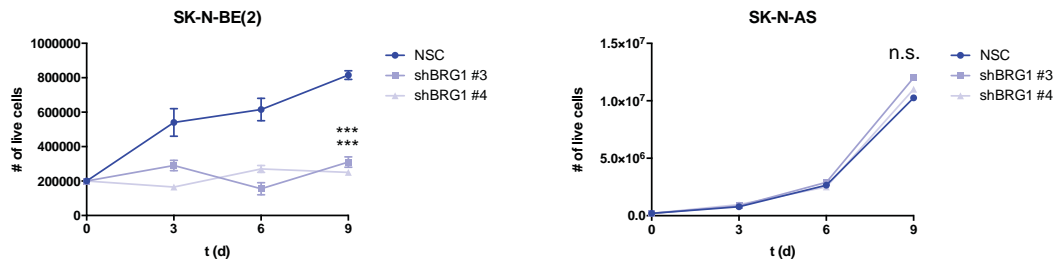
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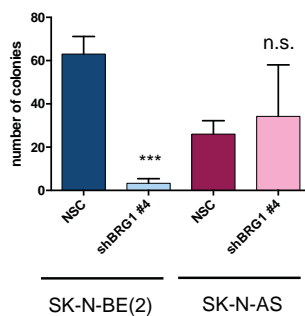
C
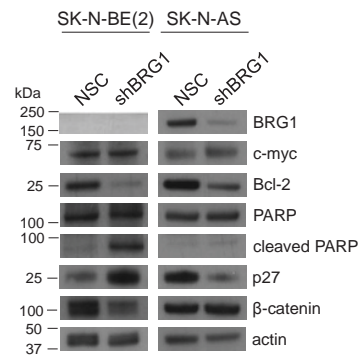
Figure 39: SK-N-AS BRG1 downregulation do not affect cell growth. A, Growth curve of SK-N-BE(2) and SK-N-AS stably transduced with NSC or shBRG1, as BRG1-dependent and –independent NB cell lines respectively. **B**, Colony formation assay comparing in the indicated NB cells transduced with NSC or shBRG1 lentiviral particles for 10 days. n.s.: not significant; *** p<0.001. **C**, Western-blot analysis of the indicated proteins after infection of NB cells with NSC or shBRG1 lentivirus in the two indicated cell lines

5.4.   How to target BRG1: Different strategies for one purpose

The elevated expression of BRG1 in Neuroblastoma renders it as a potential target for therapeutic purposes. Thereby, different strategies can be carried out to achieve this goal. I will discuss them in the following sections.

5.4.1.   BRG1 structure

BRG1 share a 75% of amino acid sequence identity with BRM, the other core subunit of the SWI/SNF complex[526]. They share the conserved ATPase domain, which pertains to the SNF2 ATPase family and is in charge of translating the energy from ATP in to mechanical motion of DNA templates[527]. Despite their similarity, these proteins exert different roles in the cell and their

functional specificity has been linked to sequence variations near the N-terminal region, which have the interaction specificities for transcription factors[528].

BRG1 has different domains which include:

- QLQ domain: the function of this domain is still unknown but it has been postulated that mediates protein-protein interactions[529,530].

- HSA domain: this domain is in charge of the protein-protein interactions among BAF250a and ARID1A SWI/SNF complex subunits and is required by BRG1-dependent transcriptional activation by nuclear hormone receptors[531,532].

- Snf2 ATP coupling (SnAC) domain: this domain is conserved through Snf2 ATPases and binds directly to the histone proteins when SWI/SNF are bound to the nucleosomes, though this has only described in yeast[533,534].

- AT hook: this region binds to AT-rich DNA sequences in a non-sequence specific manner. In BRG1 it has been shown that AT-hook region binds to linear DNA by unwinding it[535].

- Bromodomain: it consists of 110 amino acids and can interact specifically with acetylated lysines on H3 and H4 tails[536,537].

5.4.2.   BRG1 inhibitors

The use of small inhibitors is widely used in the clinics because of the production costs and the fact that they can accept multiple modifications to be given in different administration routes and/or pass different biological barriers such as the blood-brain barrier or nuclear membrane. Below are described some of these molecules.

BRD INHIBITORS

In order to develop an specific inhibitor is usually needed to have the crystallographyc structure of the protein or domain. After BRG1 bromodomain structure was resolved[538] Structural genomic consortium/Pfizer developed a BRD inhibitor for BRG1, the PFI-3[539]. However, when we treated NB cell lines

with this compound, we did not observed changes in cell proliferation (Figure 40).
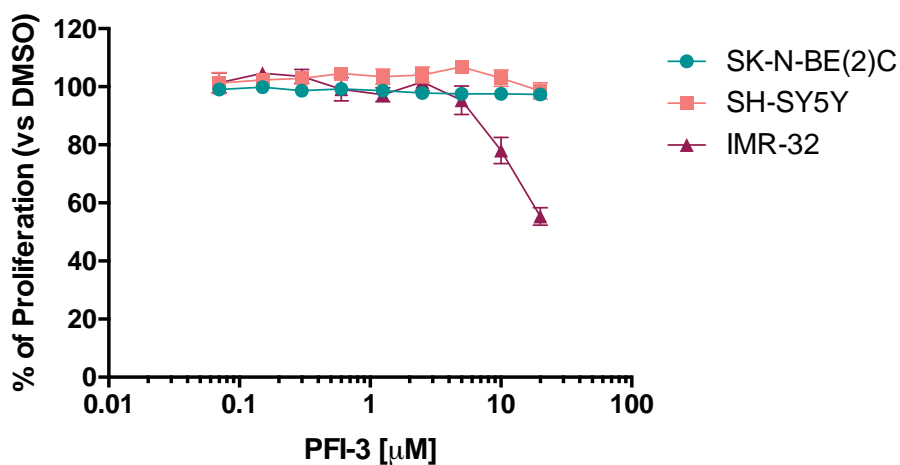
Figure 40: PFI-3 inhibitor do not affect NB proliferation. Three different NB cell lines were treated during 48h with PFI-3 BRG1 inhibitor. Data was normalized against DMSO control.

Concurring with our results, a report in breast cancer showed no effects of the compound in cell proliferation; however, PFI-3 could recapitulate the effects of BRG1 knockdown sensitization to chemotherapy through ABC transporters regulation[502]. Additionally, it was shown that BRG1 BRD domain association to nucleosomes plays a regulatory role rather than a targeting role in SWI/SNF complex in cancer[540] and the catalytic activity of the ATPase domain is the one required to define the transcriptional programs[541].

ATPASE DOMAIN INHIBITORS

Recent findings point towards a crucial relevance of the BRG1/BRM ATPase domain in the SWI/SNF function[541]. Thereby, the use of specific inhibitors against this domain could be effective on the treatment of neuroblastoma. ADAADi (Active DNA-dependent ATPase A Domain inhibitor) is a minor byproduct of a reaction catalyzed by bacterial aminoglycoside-3'-phosphotransferase APH (3')-III enzyme that can be separated by chromatographic steps[542,543]. It is an inhibitor of the ATPase activity of the SNF2 family of ATPases that has an increased affinity for BRG1[542]. It was shown that ADAADi breast cancer treated cells reduced proliferation, due to a reduction of the BRG1 activity[502]. Therefore, this provides the first proof of principle that

BRG1 inhibitors can directly inhibit cancer proliferation. However, targeting an ATPase domain may be perilous due to the extent localization and the importance of this type of domains. Thus, and despite that ADAADi may have a preference for BRG1, the inhibitor can also affect other ATPase domains and lead to severe secondary effects *in vivo*. Thereby, testing this inhibitors and/or seeking for alternative methods to inhibit BRG1 is a need.

5.4.3. Mimicking BRG1 inhibition through its downstream effectors

While new compounds are being developed to inhibit BRG1 function, an alternative is to seek for currently 'druggable' genes or pathways among its immediate downstream effectors. Our transcriptome analysis after BRG1 silencing showed a significant modulation of genes involved in cell viability and proliferation, among which central players of the PI3K/AKT and WNT pathways such as p110α, BCL2, and β-catenin genes were found to be downregulated after BRG1 knockdown.

Concurring with our results, BRG1 silencing has proved to inhibit the phosphorylation of AKT and inhibit pancreatic cancer cell growth *in vitro* and *in vivo*[523]. Furthermore, BRG1 knockdown has also been reported to inhibit the PI3K/AKT pathway in colorectal cancer, though in this case is through PTEN[491]. In NB, BRG1 regulates the PI3K catalytic subunit, p110α, and other components of the pathway. AKT activation correlates with aggressive NB variables such as MYCN amplification, 1p36 loss and shorter event-free and overall survival[544]. Moreover, pharmacologic inhibition of PI3K has already been shown to reduce NB progression *in vitro* and *in vivo* NB-mouse models[545]. Other reports showed the efficacy of PI3K inhibitors as single agents to be mild; however, they did increase the sensitivity to other treatments such as chemotherapy[546] or TRAIL[547].

These results indicate that targeting the PI3K/AKT pathway as a single or combined treatment will be beneficial for NB patients. Therefore, to find the best combination possible we examined which other genes or pathways could be contributing to the oncogenic activities of BRG1. One of the first pathways that

called our attention was the WNT pathway, since the levels of β-catenin were shown to be reduced upon BRG1 silencing.

WNT3A, an activator ligand of the WNT pathway, has been correlated with BRG1 in colon cancer cells; in fact, BRG1 drives the expression of WNT3A levels to promote cancer cell progression[548] and BRG1 knockout suppressed WNT-driven tumor initiation in mice[549]. Furthermore, BRG1 knockdown promoted metastasis in colon cancer through regulation of WNT/β-catenin signaling pathway[550,551]. These results show an important role of BRG1 in WNT-dependent tumors such colorectal cancer. Nevertheless, many WNT pathway components, such as DKK1 or β-catenin, have shown to be regulated by BRG1 in NB. Despite our efforts to validate the role of WNT pathway in NB, we found that WNT inhibitors do not affect NB proliferation at concentrations that are specific to its targets. Also, WNT-pathway is not constitutively active in NB cell lines *in vitro* but can be activated with WNT3A ligand. Despite this activation, when BRG1 was inhibited did not affect the activity of β-catenin. Moreover, overexpression of a constitutively active form of β-catenin did not rescue the phenotypic effects of BRG1 inhibition, not even partially or temporally.. In addition, analysis of mRNA datasets showed that β-catenin and WNT3A expression do not correlate with event-free survival (Figure 41A).

Altogether this data indicated that BRG1-WNT pathway regulation may be significant in cancers where the WNT pathway play a main role whereas, in WNT-independent cancers, such as NB, BRG1 regulation of this pathway is not the responsible of causing the main effects. Nevertheless, we found that DKK1 and FZD6 (an inhibitor and a receptor of WNT pathway respectively) are regulated in NB by BRG1 and they show a significant correlation with event-free survival in NB tumor samples (GSE45547) (Figure 41B). Interestingly, FZD6 has been shown to be a marker of aggressive neuroblastoma cells with stem cell-like features[552].

FZD6 has an important role in the non-canonical WNT pathway; The activation of this receptor in kidney epithelial cells did not induce the activation of TCF/LEF reporters[553] and in HelaS3 downregulation of FZD6 did not resulted in β-catenin nuclear localization changes[554]. Additionally, FZD6-overexpressing NB cells have higher levels of JNK phosphorylation (a downstream effector of the non-canonical pathway) than NB cells with FZD6 low expression[552]. These

facts suggest that the non-canonical WNT pathway may have a role in NB in a β-catenin independent manner. This would explain why iCRT14 and XAV939 had no effects on NB cell proliferation, since both of them are affecting putative targets of WNT canonical pathway.

Whether BRG1 is altering the non-canonical WNT pathway in NB or not must be further determined through the study of downstream effectors of this pathway upon BRG1 depletion. If the alteration of non-canonical WNT pathway by BRG1 is confirmed, a combination of PI3K inhibitors and non-canonical WNT pathway inhibitors would may result in an increase inhibition of NB proliferation. Until now, only have been developed inhibitors against downstream effectors of the pathway such as JNK inhibitors. The development of DKK1 mimics or FDZ6 inhibitors could shed light in the future of NB treatment.

A
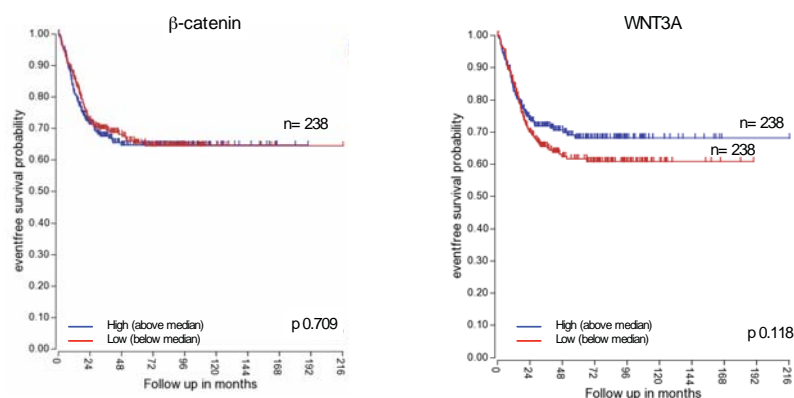
B
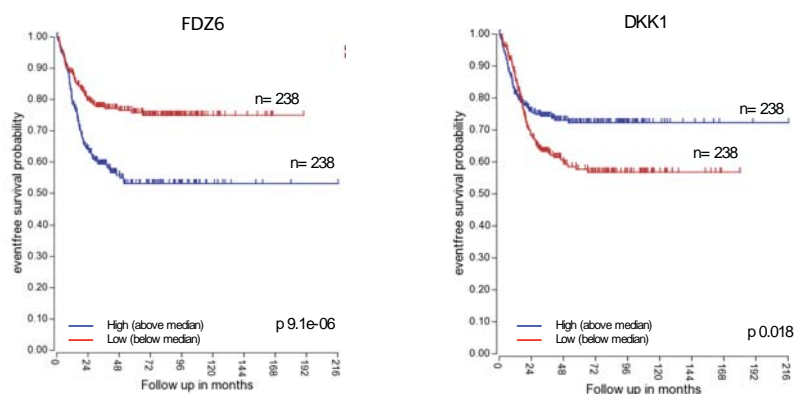
Figure 41: WNT pathway components and their correlation with NB event-free survival. A, β-catenin and WNT3A are two mediators of the canonical WNT pathway. Non-of them show significant correlation with NB event-free survival. **B**, FDZ6 and DKK1 mediate the activation of non-canonical WNT pathway. Both of them show significant correlation with NB event-free survival. GSE45547 data base was used to generate the Kaplan-Meier curves.

DISCUSSION

Another gene of pathway that could be directly related to the oncogenic functions of BRG1 is the regulation of BCL2, a well-known antiapoptotic protein. BCL2 has been shown to be highly expressed in NB tumors although a clear correlation with clinical variables has not been established. Lamers *et al.* reported that NB cells are sensitive to BCL2 inhibitors *in vitro* and *in vivo*[371], thereby establishing BCL2 as a promising drug target in neuroblastoma. Furthermore, a reduction in BCL2 levels was proven to be an important contributor to the therapeutic effects of another epigenetic modulator, the I-BET726 bromodomain inhibitor, in NB[555]. Currently there are several BCL-2 inhibitors, such as ABT-199 and ABT-263. ABT-199 is more BCL-2 specific, whereas ABT-263 may also target other members of the family such as BCL-X. In NB, both inhibitors reduced cellular proliferation, but ABT-263 was more efficient than ABT-199; thereby we decided to use ABT-263 in combination with PI-103 to recapitulate BRG1 inhibition in NB models. ABT-263 and PI-103 combination resulted in a strong synergism, not only recapitulating BRG1 inhibition in 2D cellular growth, but also in a tumor neurosphere NB model. This makes a proof of principle of the use of these inhibitors in NB patients. This drugs are currently on clinical trials in adults. ABT-263 or Navitoclax has four clinical trials recruiting patients for the phase I/II (NCT02143401; NCT02520778; NCT02079740; NCT01989585) and PI-103 has no clinical trial on course, but currently are 45 clinical trials of other PI3K inhibitors such as BKM120, Copanlisib, LY3023414 or Gedatolisib among others, that could be used in combination with Navitoclax. Therefore, approval of the usage of this inhibitor combination in NB patients will be faster than developing a BRG1 inhibitor and patients could benefit of these discoveries before an specific drug against BRG1 Is commercialized.

BRG1 non-dependent NB cell line discovery will help to new inhibitors or combinations development. Determining BRG1 genome occupation through ChIP-seq technique together with an RNA-seq analysis in BRG1 dependent and independent models will help to discriminate the BRG1 direct targets that specifically contribute to the oncogenic function of BRG1.

5.4.4. Blocking interactions with other SWI/SNF complex components

BRG1 is one of the core elements of SWI/SNF complex, a chromatin-remodeling complex that has been described as a key element in cancer[356,357] and neurodevelopmental disorders[556]. It is a large multiproteic complex of at least 10 subunits, which can adopt several compositions depending on the cellular context. The SWI/SNF complexes (also known as BAF complexes in mammals) are powered by two mutually exclusive ATPase subunits: SMARCA 4 (BRG1) and SMARCA2 (BRM). Despite, that they are mutually exclusive, both subunits coexist in most cell types, except in embryonic stem cells in which BRG1 is the only ATPase expressed[557].

Genes encoding SWI/SNF complexes were first identified in yeast[558,559]. Later, it was shown its ability of breaking DNA-histone interactions, in order to slide the histone octamers or eject them from the DNA[560–563]. It has been postulated that ATPase subunit of SWI/SNF binds to a specific site on the nucleosome and uses 3' translocase activity to move DNA in a directional wave[564].

Functional experiments defined its role in controlling cell fate, lineage specification and cell proliferation *in vivo*, likely through its role as context-scpecific master transcriptional regulator (reviewed in[353]).

Diversity of mammalian SWI/SNF complex subunit compositions is essential for determining cell fate, and while ATPase subunit perform the catalytic activities, the associated subunits exerts the role in modulating the catalytic activity. Thus, SWI/SNF or BAF complexes can be classified depending on their composition[565] and each type of complex is able to perform different activities.

Among SWI/SNF complexes conformation, BAF and PBAF (also called SWI/SNF A and SWI/SNF B respectively) complexes are two of the best defined (Figure 42). In vivo complexes may contain more than 15 subunits, although the chromatin remodeling activity can be reconstituted *in vitro* with a set of four core subunits: BRG1, SNF5, BAF155 and BAF170[566]. Interestingly, despite being a core subunit, SNF5 is not required for the complex integrity, but when is lost, several oncogenic signaling pathways are found altered[567] .

A   B

BAF (SWI/SNF-A)   PBAF (SWI/SNF-B)
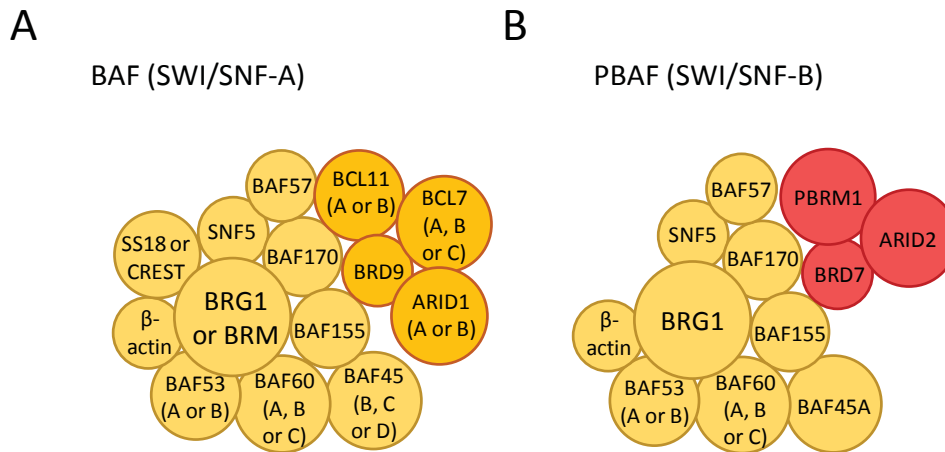
Figure 10: BAF and pBAF complex overview. A-B, Examples of known SWI/SNF configurations, both types can exist in the same cell type. Adapted from Hohmann et al[567]

PROTEIN-PROTEIN INTERACTION DISRUPTION

A feasible alternative of blocking BRG1 activity is the disruption of the protein-protein interactions of intra-complex proteins. This strategy is being developed for c-MYC, an undruggable protein by small molecules. MAX dimerizes with c-MYC to bind its target regions; thereby, disrupting this interaction can lead to c-MYC dysfunction. Soucek L. et al described a peptide, the Omomyc, which can interact with c-MYC and prevent its dimerization with MAX. That resulted in impaired growth and formation of MYC dependent tumors[568–571].

The experiments to find the BRG1 interactors that functionally contribute to its oncogenic activity in NB are ongoing.

5.4.5. The use of ncRNA as a therapeutical approach to target BRG1

A lot of efforts are being invested in the development of nanoparticles that can be conjugated with therapeutic agents and molecules that direct these compounds to the tumors. NcRNAs can be conjugated to nanoparticles and be directed right to the tumors. Therefore, delving on the regulation of BRG1 through ncRNA can lead to the discovery of new therapeutic agents that will be

able reach the clinics in the following years. Among the ncRNA that can be used as therapeutic agents, miRNA and lncRNA are emerging as promising candidates.

A study on leukemia showed that miR-155 regulates BRG1[475]. This study together with the *in silico* analysis pave the way of future experiments to validate and determine which miRNA are regulating BRG1 in NB. Thereby, finding and describing these miRNA can lead to the development of new therapeutic agents for NB.

In the case of lncRNA, UCA1 (urothelial carcinoma associated 1) was shown to interact with BRG1 in bladder cancer and regulate p21[572]. Another lncRNA, Mhrtwas shown to regulate BRG1 interaction with myosins during heart stress[573]. These two lncRNA set a precedent for investigating their use as therapeutic agents in NB.

In summary, our findings unveil BRG1 as a pro-oncogenic factor in neuroblastoma which regulates multiple key processes such as cell cycle, survival and proliferation. Our experimental results offer the possibility of inhibiting the expression of BRG1 or a combination of its immediate downstream effectors to halt the proliferative capacity of NB cells and therefore improve the outcome of high-risk NB patients. Nevertheless further investigation must be carried out to develop a selective inhibitor for BRG1.

CONCLUSIONS

6. CONCLUSIONS

First: Multiple epigenetic genes are differentially expressed in advanced stages of neuroblastoma.

Second: BRG1 is overexpressed in advanced stages of Neuroblastoma and high levels correlate with worse event-free and overall survival. This correlation is independent of other NB prognostic factors such as MYCN amplification, thereby suggesting that BRG1 could be an independent NB biomarker.

Third: BRG1 is indispensable for the growth and viability for most neuroblastoma cell lines *in vitro* and *in vivo*.

Fourth: BRG1 regulates essential cell survival signaling pathways in NB such as AKT, WNT and apoptotic pathways.

Fifth: The combination of the PI3K inhibitor PI-103 and the BCL2 inhibitor ABT-263, mimics BRG1 knockdown on cell lines and tumor spheres; thereby, rendering this combination therapy as a potential new therapy against high-risk neuroblastoma.

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7. BIBLIOGRAPHY

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ANNEX

8. ANNEX

8.1.   ANNEX 1: Tables

Annex Table 1: Epigenetic Genes studied in NB

HMT	Bromo Domains	HAT	HDAC	HDM	DNMT	Others	Function description
ASH1L	ASH1L	ATF2	HDAC1	KDM1A (AOF2)	DNMT1	MBD1	
CARM1	ATAD2A	CDY1	HDAC10	KDM2A (FBXL11)	DNMT3A	MBD2	
EHMT1	ATAD2B	CDYL	HDAC11	KDM2B (FBXL10)	DNMTRB	MBD3	DNA demethylases
EHMT2	BAZ1A	EP300	HDAC2	KDM3A (JMJD1A)		MBD4	
EZH1	BAZ1B	HAT1	HDAC3	KDM4A (JMJD2A)		MECP2	
EZH2	BAZ2A	CREBBP	HDAC4	KDM4C (JMJD2C)		MECOM (PRDM3)	TF
MLL	BAZ2B	GTF3C4	HDAC5	KDM5B (JARID1B)		PADI4	histone arginine residues to citrulline residues
MLL2	BRD1	KAT2A (GCN5L2)	HDAC6			SRCAP	core catalytic of SRCAP complex
MLL3	BRD2	KAT2B (PCAF)	HDAC7			TAF1	TATA binding protein
MLL4	BRD3	KAT5 (HTATIP)	HDAC8			TAF1L	
NSD1	BRD4	KAT6A (MYST3)	HDAC9			TRDMT1	RNA methyltransferase
SETD7	BRDT	KAT6B (MYST4)	SIRT1				
SETD8	BRD8	KAT7 (MYST2)	SIRT2				
SETDB1	BRD9	KAT8 (MYST1)					
SETDB2	BRPF1	NCOA1					
PRDM1	BRPF3	NCOA3					
PRDM10	BRWD3						
PRDM11	CECR2						
PRDM12	CREBBP						
PRDM13	EP300						
PRDM14	BPTF (FALZ)						
PRDM15	KAT2A (GCN5L2)						
PRDM16	MLL						
PRDM2	PBRM1						
PRDM4	KAT2B (PCAF)						
PRDM5	PHIP						
PRDM6	ZMYND8 (PRKCBP1)						
PRDM7	SMARCA2						
PRDM8	SMARCA4						
PRDM9	SP100						
PRMT1	SP110						
PRMT5	SP140						
PRMT7	TAF1						
PRMT8	TRIM24						
SMYD3	TRIM33						
SUV39H1	BRWD1 (WDR9)						
SUV39H2	ZMYND11						
WHSC1							
WHSCIL1							

TF: transcription factor

Annex Table 2: Significantly deregulated genes after BRG1 knockdown

Gene Symbol	Log Fold Change	Adjusted P Value
B4GALT6	-2.62	3.50E-05
GCH1	-2.21	2.91E-05
SMARCA4	-2.10	2.91E-05
ITGA9	-1.87	4.03E-05
MBNL3	-1.80	6.29E-05
FAM69A	-1.79	1.39E-04
TWSG1	-1.78	3.31E-04
VAMP3	-1.77	6.10E-05
RIMS3	-1.71	6.69E-05
PM20D2	-1.68	6.29E-05
LYPLA1	-1.67	4.69E-05

BCL2	-1.67	1.02E-04
HABP4	-1.66	1.20E-04
RN5S295	-1.65	1.94E-03
NES	-1.65	4.03E-05
SCG2	-1.65	3.76E-05
RPN2//EEF1A2	-1.64	3.50E-05
SNORD114-10	-1.62	1.36E-03
RN5S221	-1.62	3.89E-04
SNORD114-24	-1.58	1.00E-03
EGR1	-1.57	1.13E-04
CENPN	-1.54	8.67E-05
SNORD114-19	-1.49	1.20E-04
S1PR2	-1.49	1.03E-04
MEG3	-1.48	8.28E-05
SCML1	-1.48	7.32E-05
PPARGC1A	-1.45	6.10E-05
C18orf54	-1.43	1.03E-04
BEGAIN	-1.42	7.58E-04
RHPN2	-1.42	8.42E-05
FZD6	-1.41	3.73E-04
FYTTD1	-1.41	6.10E-05
LIFR	-1.40	6.74E-05
CERS6	-1.40	6.29E-05
INIP	-1.40	1.15E-04
NT5E	-1.40	6.29E-05
CD46	-1.38	6.29E-05
PLEKHA6	-1.36	8.67E-05
OGFRL1	-1.36	1.85E-04
SNORD114-31	-1.35	6.58E-04
USP1	-1.35	6.10E-05
CDH11	-1.35	1.44E-04
CDK6	-1.34	6.30E-05
E2F7	-1.32	6.85E-05
SNORD113-1	-1.32	7.92E-04
SNORD114-27	-1.31	2.63E-04
MIR376B	-1.31	2.26E-03
TIMP3	-1.31	9.86E-05
LIMS3//LIMS3L//LIMS3-LOC440895//LIMS1	-1.30	6.85E-05
ECT2	-1.29	6.10E-05
SNORD114-30	-1.29	2.58E-04
SNORD114-2	-1.28	5.88E-04
PANX1	-1.28	6.35E-05
FAM69B	-1.26	6.30E-05
GALNT1	-1.25	7.32E-05
IGSF3	-1.25	1.17E-04
TPMT	-1.25	6.30E-05

PDLIM3	-1.25	4.67E-04
CKS1B	-1.24	1.40E-04
MIR154	-1.24	5.60E-04
PNPLA8	-1.23	2.56E-04
ASCL1	-1.23	6.69E-05
GNG5P2	-1.23	9.84E-05
GPR64	-1.22	6.69E-05
SNORD113-3	-1.22	1.51E-04
MORC4	-1.21	8.67E-05
PCGF5	-1.21	1.41E-04
GABRQ	-1.21	1.27E-04
CACNG4	-1.20	1.45E-04
TLE3	-1.20	1.07E-04
INO80C	-1.20	8.42E-05
SNORD114-16	-1.18	1.46E-03
STK17B	-1.18	7.45E-05
SLC39A6	-1.18	8.42E-05
GPR22	-1.18	1.60E-04
SLC25A39	-1.18	9.84E-05
MIR770//MEG3	-1.18	2.12E-04
HIGD1C	-1.17	1.62E-03
NETO2	-1.17	1.33E-04
GPR155	-1.17	1.73E-04
CISD2	-1.17	1.03E-04
SNORD114-17	-1.17	1.45E-04
ESF1	-1.17	1.60E-04
MIR323B	-1.16	5.90E-04
STAM	-1.16	1.06E-04
VAPA	-1.16	8.28E-05
CTNNB1	-1.16	1.09E-04
GLO1	-1.16	6.30E-05
ERLIN1	-1.15	1.42E-04
HTR2B	-1.15	8.19E-05
MAN1A2	-1.15	8.28E-05
RAP2A	-1.14	7.32E-05
CXCR4	-1.14	2.77E-03
CLOCK	-1.13	8.42E-05
STAC	-1.13	1.45E-04
SLC7A14	-1.12	2.21E-04
KIAA0125	-1.11	1.06E-04
DGKK	-1.11	1.37E-04
STK17A	-1.11	4.91E-04
PIK3CA	-1.10	1.37E-04
SNORD114-13	-1.09	2.59E-04
ZCRB1	-1.09	8.67E-05
CASD1	-1.09	1.38E-04

WWP1	-1.09	9.69E-05
CCP110	-1.08	1.20E-04
SNORD114-25	-1.08	1.14E-02
H1F0	-1.07	1.02E-04
TUBB2A	-1.07	4.25E-04
TLR4	-1.06	6.41E-04
MIR1193	-1.06	1.20E-03
FRMD3	-1.05	1.20E-04
IRF6	-1.05	2.07E-04
MIR329-1	-1.05	9.24E-04
GJD2	-1.04	7.26E-04
KCNAB1	-1.04	1.98E-04
FAM64A	-1.04	2.34E-03
TNNT2	-1.04	1.32E-03
CCDC47	-1.04	8.42E-05
FAM20B	-1.04	6.95E-04
SNORD113-9	-1.02	3.97E-03
CASP7	-1.02	4.87E-04
TMEM51	-1.02	9.26E-04
IGHM	-1.02	7.02E-04
RNF152	-1.02	1.49E-03
GNG5	-1.01	3.30E-03
MEG8	-1.01	1.03E-04
CYLD	-1.00	3.15E-04
KLF10	-1.00	1.41E-04
MED24	1.00	3.15E-04
FAM227A	1.00	1.04E-03
PTGR2	1.01	1.51E-04
LOC90246	1.01	6.73E-04
TAS2R14	1.01	8.66E-04
C14orf39	1.01	9.26E-04
NR1H2	1.02	2.57E-04
LRRTM2//CTNNA1	1.02	1.09E-04
DKK1	1.03	4.09E-04
CMTM4	1.03	1.83E-04
LOC284023	1.04	1.37E-04
RPIA	1.04	2.00E-04
ZNF362	1.05	8.37E-04
BCL9	1.05	4.95E-04
FLJ14186	1.06	2.57E-04
USF2	1.06	6.31E-04
MIR1269B	1.06	1.37E-03
CBX6	1.06	4.14E-04
SLC36A1	1.06	6.23E-04
KDM6B	1.07	1.02E-04
HSD17B10	1.08	4.73E-04

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PCDHB6	1.10	1.98E-04
SFXN2	1.11	1.07E-04
AGBL5-AS1	1.12	2.26E-03
IRX6	1.14	5.15E-04
ZNF239	1.15	4.14E-04
MARVELD1	1.15	1.56E-04
MAPRE3	1.15	1.03E-04
SS18	1.16	7.45E-05
MANBA	1.16	1.47E-04
DDA1	1.18	9.86E-05
ANKRD52	1.20	6.30E-05
GABRB1	1.21	4.14E-04
PCDH10	1.21	1.63E-04
KLHDC3	1.22	1.20E-04
LINC00265	1.23	2.72E-04
TP53INP1	1.25	1.13E-04
MAP2K6	1.25	3.38E-04
LOC100507032	1.26	1.41E-04
TMEFF2	1.27	8.95E-05
SPC24	1.27	6.69E-05
RNY4P19	1.30	4.69E-04
NF2	1.31	7.12E-05
SEMA6D	1.33	1.41E-04
LPPR2	1.39	1.39E-04
ZNF783	1.39	1.29E-04
SLC16A6	1.39	6.30E-05
SLC16A14	1.41	1.37E-04
FN1	1.44	1.29E-04
SLC16A9	1.46	6.10E-05
LINC00473	1.46	9.03E-04
LINC00265	1.53	6.30E-05
SLITRK6	1.57	3.67E-04
SEMA3E	1.96	1.20E-04

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8.2.   ANNEX II: Publications

1. **Jubierre L,** Soriano A, Planells-Ferrer L, Paris-Coderch L, Tenbaum SP, Romero OA, Moubarak RS, Almazán-Moga A, Molist C, Roma J, Navarro S, Noguera R, Sanchez-Cespedes M, Comella JX, Palmer HG, Sánchez de Toledo J, Gallego S, Segura MF. BRG1/SMARCA4 is essential for neuroblastoma cell viability through modulation of survival pathways. **Oncogene. Mar 2016**. doi: 10.1038/onc.2016.50

2. Segura MF, **Jubierre L**, Li S, Soriano A, Koetz L, Gaziel-Sovran A, Masanas M, Kleffman K, Dankert JF, Walsh MJ, Hernando E. Krüppel-like factor 4 (KLF4) regulates the miR-183~96~182 cluster under physiologic and pathologic conditions. **Oncotarget.2017 Apr** 18;8(16):26298-26311. doi: 10.18632/oncotarget.15459

3. Soriano A, **Jubierre L**, Almazán-Moga A, Molist C, Roma J, de Toledo JS, Gallego S, Segura MF. microRNAs as pharmacological targets in cancer. **Pharmacol Res. 2013 Sep**;75:3-14. doi: 10.1016/j.phrs.2013.03.006. Epub 2013 Mar 25

4. Soriano A, París-Coderch L, **Jubierre L**, Martínez A, Zhou X, Piskareva O, Bray I, Vidal, I, Almazán-Moga A, Molist C, Roma J, Bayascas J. R, Casanovas O, Stallings R. L., Sánchez de Toledo J, Gallego S, Segura M. F. MicroRNA-497 impairs the growth of chemoresistant neuroblastoma cells by targeting cell cycle, survival and vascular permeability genes. **Oncotarget. Jan 2016**. DOI: 10.18632/oncotarget.7005

5. Segura MF, Fontanals-Cirera B, Gaziel-Sovran A, Guijarro MV, Hanniford D, Zhang G, González-Gomez P, Morante M, **Jubierre L**, Zhang W, Darvishian F,Ohlmeyer M, Osman I, Zhou MM, Hernando E. BRD4 Sustains Melanoma Proliferation and Represents a New Target for Epigenetic Therapy**. Cancer Res. 2013** Oct 15;73(20):6264-76. doi: 10.1158/0008-5472.CAN-13-0122-T. Epub 2013 Aug 15

6. Almazán-Moga A, Roma J, Molist C, Vidal I, **Jubierre L**, Soriano A, Segura MF, Llort A, Sánchez de Toledo J, Gallego S. Optimization of rhabdomyosarcoma disseminated disease assessment by flow cytometry. **Cytometry Part A. 2014** Aug 22. DOI:10.1002/cyto.a.22514

7. Qadeer ZA, Valle-Garcia D, Griffiths LM, **Jubierre L**, Anqi Ma, Soriano A, Sun Z, Filipescu D, Zeineldin M, Chowdhury A, Deevy O, Dekio F, Fowkes M, Maris JM, Cheung NKV, Jin J, Segura MF, Dyer MA, Bernstein E. ATRX mutant neuroblastoma is sensitive to EZH2 inhibition via modulation of neuronal differentiation. **Nature Medicine (Submitted)**

8. Feliciano A, Garcia-Mayea Y, **Jubierre L**, Mir C, Hummel M, Castellvi J, Hernández-Losa J, Paciucci R, Sansano I, Ramón y Cajal S, Kondoh H, Soriano A, Segura MF, Lyakhovich A, Lleonart M. miR-99a reveals two novel oncogenic proteins E2F2 and EMR2 and represses stemness in lung cancer. **Cell death and disease (Under second revision)**