Chapter

The Scaffold Protein p140Cap as a Molecular Hub for Limiting Cancer Progression: A New Paradigm in Neuroblastoma

Giorgia Centonze, Jennifer Chapelle, Costanza Angelini, Dora Natalini, Davide Cangelosi, Vincenzo Salemme, Alessandro Morellato, Emilia Turco and Paola Defilippi

Abstract

Neuroblastoma, the most common extra-cranial pediatric solid tumor, is responsible for 9–15% of all pediatric cancer deaths. Its intrinsic heterogeneity makes it difficult to successfully treat, resulting in overall survival of 50% for half of the patients. Here we analyze the role in neuroblastoma of the adaptor protein p140Cap, encoded by the *SRCIN1* gene. RNA-Seq profiles of a large cohort of neuroblastoma patients show that *SRCIN1* mRNA levels are an independent risk factor inversely correlated to disease aggressiveness. In high-risk patients, *SRCIN1* was frequently altered by hemizygous deletion, copy-neutral loss of heterozygosity, or disruption. Functional assays demonstrated that p140Cap is causal in dampening both Src and Jak2 kinase activation and STAT3 phosphorylation. Moreover, p140Cap expression decreases *in vitro* migration and anchorage-independent cell growth, and impairs *in vivo* tumor progression, in terms of tumor volume and number of spontaneous lung metastasis. p140Cap also contributes to an increased sensitivity of neuroblastoma cells to chemotherapy drugs and to the combined usage of doxorubicin and etoposide with Src inhibitors. Overall, we provide the first evidence that *SRCIN1*/p140Cap is a new independent prognostic marker for patient outcome and treatment, with a causal role in curbing the aggressiveness of neuroblastoma. We highlight the potential clinical impact of *SRCIN1*/p140Cap expression in neuroblastoma tumors, in terms of reducing cytotoxic effects of chemotherapy, one of the main issues for pediatric tumor treatment.

Keywords: p140Cap, *SRCIN1* gene, Src kinase, Signal transducer and activator of transcription 3, chemotherapy, neuroblastoma, Src inhibitors

1. Introduction

Neuroblastoma (NB) is the most frequent embryonic malignancy among children particularly before 5 years of age [1]. It originates from primitive sympathetic neural precursor cells of the peripheral nervous system [2]. The majority of these tumors develop in the adrenal medulla; however, NB can arise anywhere along the

sympathetic nervous system (neck, chest, abdomen or pelvis). Primary tumors in the neck or upper chest can cause Horner's syndrome (ptosis, miosis, and anhidrosis). Tumors arising along the spinal column can expand through the intraforaminal spaces and cause cord compression, with resulting paralysis [3].

NB is a complex disease with different outcomes, going from metastasis to one or more distant sites [4] to spontaneous regression or differentiation, even in the absence of any specific treatment [5]. Given the high heterogeneous features of NB, the International Neuroblastoma Staging System (INSS), considers a plethora of criteria to rank patients. Namely, the degree of surgical excision of primary tumor, lymph node involvement, dissemination to distant organs, degree of bone marrow involvement and the age of infant [6]. Accordingly, stages 1, 2A and 2B include patients with localized tumor, without propagation to lymph nodes. Stage 3 and stage 4 comprehends patients with metastatic disease. Stage 4S specifies a metastatic disease in children under the age of one year, which may undergo spontaneous regression, usually associated with 90% survival rate at 5 years [7].

The genetic etiology of NB includes some established markers such as the presence of segmental chromosome abnormalities (chromosomes 1p, 3p, 4p, 11q loss and of 1q, 2p, 17q gains) [8] and DNA ploidy [9]. At the molecular level, the Anaplastic Lymphoma Kinase (*ALK*) oncogene is the most frequently mutated gene in hereditary familial NB, where it is amplified or constitutively activated in its tyrosine kinase domain [10, 11]. Amplification of the *N-MYC* oncogene (*MYCN*) occurs in 20% of NB, representing a poor prognostic factor for this embryonic malignancy [12, 13]. Tropomyosin receptor kinase B (*TrkB)* and Brain-Derived Neurotrophic Factor (*BDNF)* are both expressed in aggressive NB with *MYCN* amplification [14]. In addition, driver mutations in the lin-28 homolog B (*LIN28B*) [15] and in the Paired-like Homeobox 2b (*PHOX2B*) [16] genes have been reported.

NB therapeutic standard of care worldwide is based on multi-modality therapy including chemotherapy, surgery, radiation therapy, myeloablative therapy with stem cell transplant, immunotherapy and differentiation therapy [17–19]. However, a more accurate stratification of patients based on newly identified prognostic markers would allow the development of additional therapeutic strategies with increased effectiveness and reduced toxicity.

p140Cap (Cas-associated protein), also known as SNIP (Snap25-interacting protein) [20], is a scaffold protein codified by the gene *SRCIN1*. It is highly expressed in the brain, testis and epithelial rich tissue [21]. In human cancer patients, p140Cap/*SRCIN1* is a new favorable prognostic marker in HER2-related breast cancer, where p140Cap expression is associated with good prognosis [22]. At the molecular level, p140Cap impairs breast cancer growth and metastatic progression, interfering with both Src kinase [23] and Rac1 GTPases [22] activation.

More recently we have investigated p140Cap/*SRCIN1* relevance in NB. This chapter aims to present data supporting p140Cap/*SRCIN1* as a key biological determinant of NB outcome, representing a new independent prognostic marker for patient outcome and treatment. We highlight the potential clinical impact of *SRCIN1*/p140Cap expression in NB tumors in terms of reducing cytotoxic effects of chemotherapy, one of the main issues for pediatric tumor treatment.

2. The p140Cap adaptor protein

The human *SRCIN1* gene, located on chromosome 17q12, includes 27 exons, and it is highly conserved in vertebrates and mammals [24]. The genomic region immediately bordering *SRCIN1* contains several genes involved in breast cancer onset and progression such as *ERBB2* (17q12), *BRCA1* (17q21), retinoic acid receptor-α

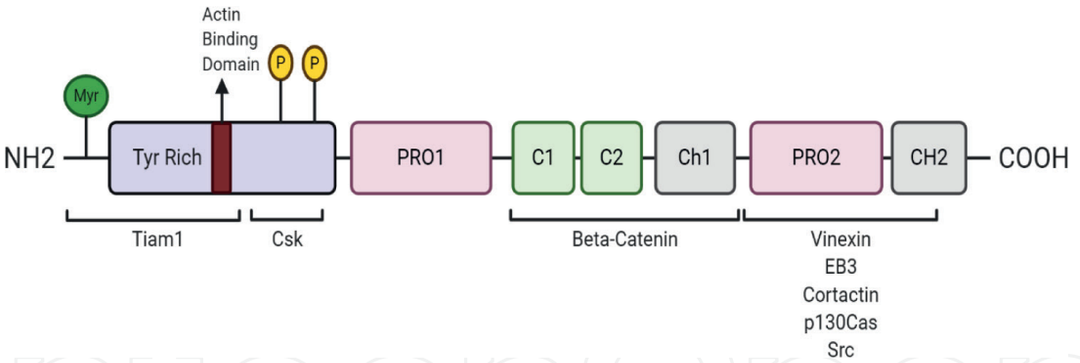


Figure 1.
The structure of the adaptor protein p140Cap. p140Cap protein analysis reveals the presence of a putative N-terminal myristoylation site, a tyrosine-rich region (Tyr-rich), an actin-binding domain (ABD), a proline rich domain (Pro1), a coil-coiled region (C1-C2), two domains rich in charged amino acids (CH1, CH2) and a C-terminal proline-rich domain (Pro2). Tyrosine phosphorylation (PY) EPLYA and EGLYA are shown. The interactors Tiam1, Csk, β-catenin, Vinexin, EB3, Cortactin, p130Cas and Src are associated with specific domains.

(*RARA*; 17q21) and signal transducer and activator of transcription 3 (*STAT3*; 17q21). These genes often undergo a gain of function role in human tumors [25]. Moreover, the 17q gain occurs in 50–70% of all high stage NB and is associated with poor prognosis as an independent marker of adverse outcome [26–29].

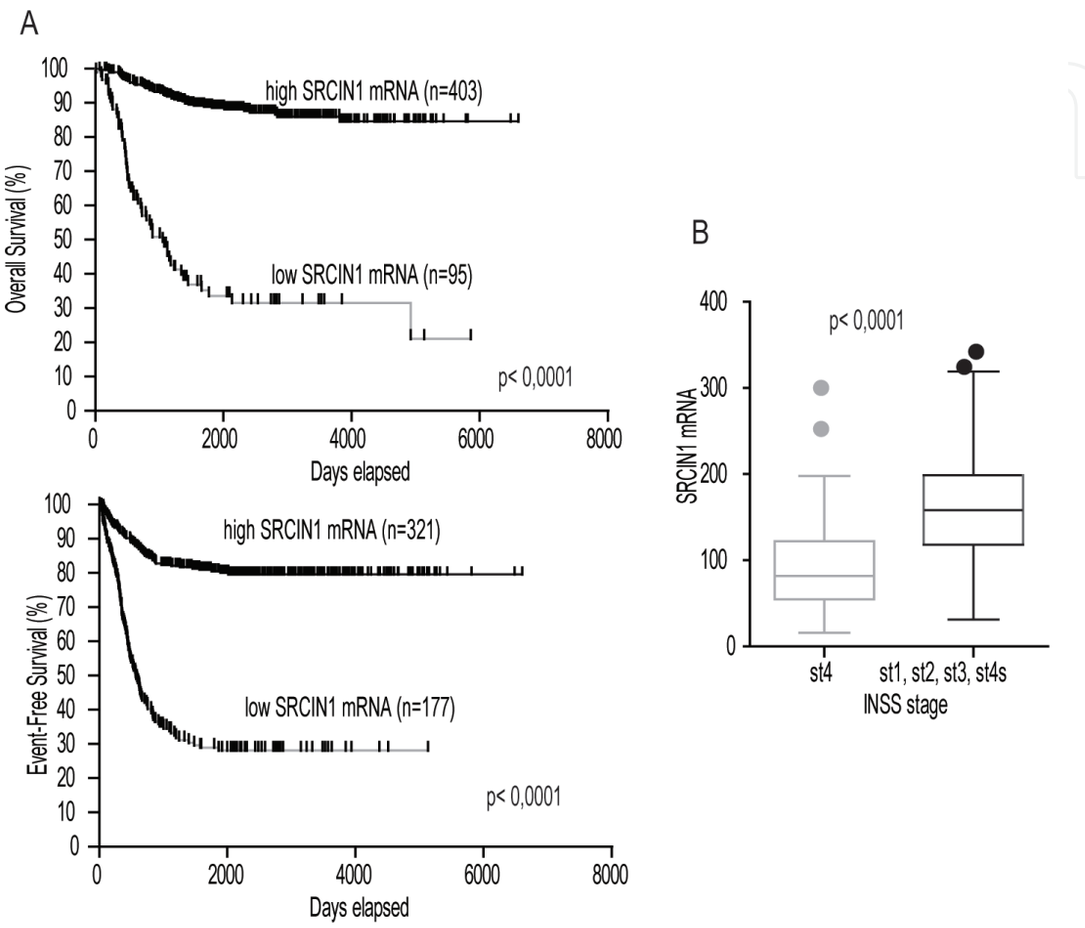
p140Cap shares different Intrinsically Disordered Regions (IDRs) that classify p140Cap as "Intrinsic Disorder Protein" (IDP) [30, 31]. The IDP features of p140Cap could allow the interaction with several partners and promote protein–protein interactions that are the elected functions for a scaffold protein. The p140Cap protein can interact with multiple partners [30] (**Figure 1**). In particular, p140Cap associates with the tyrosine kinases Csk and Src. This macromolecular complex triggers Csk activity to phosphorylate Src on its inhibitory tyrosine, resulting in Src inactivation and in the suppression of downstream pathways regulating motility and invasion of cancer cells [23]. Indeed, at the structural level, p140Cap contains a tyrosine rich domain, important for the interaction with Csk [32], two coiled coil regions, that can mediate the binding with beta-catenin [10] and two different proline rich domains responsible for the association with the microtubule associated protein EB3 [33], Cortactin [34] and Vimentin [35].

The physiologic role of p140Cap has been mainly investigated in the brain [35], where it is expressed in neurons both in the presynapse [10, 36] and in the postsynapse [10, 37–41]. In differentiated neurons, it controls synaptic plasticity [33, 40], and regulates GABAergic synaptogenesis and development of hippocampal inhibitory circuits [36]. In particular, p140Cap enters and accumulates in the dendritic spine (DS) through EB3 binding [33]. In this compartment p140Cap acts as hub interacting with Cortactin, a protein that regulates actin branching and new filament polymerization [42] and with Citron-N [40] resulting in mature DS stabilization. In both the pre- and post-synaptic regions, p140Cap is involved in a network of protein–protein interactions as confirmed by its interactome in synaptosomes. p140Cap interactors converge on key synaptic processes, including transmission across chemical synapses, actin cytoskeleton remodeling and cell–cell junction organization [41].

3. *SRCIN1* mRNA expression is an independent prognostic marker for NB

To address the involvement of p140Cap in NB patients, we first investigated the relationship between *SRCIN1* mRNA levels and patient outcomes, by using the R2 genomics analysis and visualization platform (R2: Genomics analysis and

Visualization Platform (http://r2.amc.nl)). The bioinformatics analysis performed on a dataset containing clinical and gene expression data of 498 NB patients revealed that *SRCIN1* positively impacts on patients' outcome. In fact, the Kaplan–Meier analysis showed that high expression of *SRCIN1* is associated with good prognosis in 403 patients, whereas low expression is observed in 95 poor prognosis patients (**Figure 2A**). Furthermore, high *SRCIN1* expression was significantly



C

Covariate	Multivariate cox analysis (OS)				Multivariate cox analysis (EFS)			
	coefficient	HR	95% CI	P-value	coefficient	HR	95% CI	P-value
SRCIN1 (Low vs High)	-1.07	0.34	(0.2,0.5)	1.20E-05	-1.3	0.3	(0.1,0.3)	2.30E-11
Age group (<=12 months vs > 12 months	0.9	2.5	(1.3,4.7)	4.00E-03	-	-	-	
INSS stage (1, 2, 3, 4s vs 4	1.3	3.7	(1.5,5.5)	3.10E-07	-	-	-	
MYC status (normal vs amplified	0.7	2.2	(1.3,3.5)	1.00E-03	0.1	1.1	(0.8,1.6)	1.00E-04

Figure 2.
Stratification by SRCIN1 mRNA expression in primary NB patients and SRCIN1 gene status. A) Kaplan–Meier curves for overall (upper panel) and event-free (bottom panel) survival stratified by SRCIN1 expression in a cohort of 498 NB patients. Cut off for high or low SRCIN1 expression was chosen by Kaplan–Meier scan method. Survival curves were compared by log-rank test. P-values were corrected for multiple hypotheses testing by Bonferroni method. Each plot reports the corrected P-value (P). Corrected P-values lower than 0.05 were considered statistically significant. The number of patients with high or low expression of SRCIN1 mRNA is reported in every curve. B) Box and whisker plot for the expression of SRCIN1 mRNA in the two risk groups defined by INSS stages (st1, st2, st3, st4s vs. st4). The significance of the mean expression was measured by unpaired student t-test. A P-value lower than 0.05 was considered significant. C) Multivariate cox regression analysis for overall survival (OS) and event-free survival (EFS). The prognostic value of SRCIN1 mRNA expression (high and low) was tested in the context of known risk factors: Age at diagnosis (>12 months vs. <12 months) MYCN amplification (normal vs. amplified) INSS stages (st1, st2, st3, st4s vs. st4). Cut off for high or low SRCIN1 expression was chosen by Kaplan–Meier scan method. Cox regression coefficient (coefficient), hazard ratio (HR), 95% of confidence interval (95% CI) and P-value are shown for each variable in the OS and EFS panel. Significant P-values are lower than 0.05 (OS: HR 0.34 95% CI 0.2–0.5 P < 0.0001; EFS: HR 0.27 95% CI 0.1–0.4, P < 0.0001).

NB patients	***SRCIN1* gene status**	**CHROMOSOMAL COORDINATES**
Case 1	disrupted in the breakpoint	Chr17: 36696338–81029941 Cytoband: 17q12-q25.3 Size: 44.33 Mb
Case 2	disrupted in the breakpoint	Chr17: 36694901–80943345 Cytoband: 17q12-q25.3 Size: 44.24 Mb
Case 3	loss	Chr17: 25311574–36777884 Cytoband: 17q11.1-q12 Size: 11.46 Mb
Case 4	disrupted in the breakpoint	Chr17: 36696338–80969424 Cytoband: 17q12-q25.3 Size: 44.27 Mb
Case 5	disrupted in the breakpoint	Chr17: 36696279–81029941 Cytoband: 17q12-q25.3 Size: 44.33 Mb
Case 6	copy neutral LOH	Chr17: 25569094–42949451 Cytoband: 17q11.1-q21.31 Size: 17.38 Mb
Case 7	copy neutral LOH	Chr17: 29149425–45297941 Cytoband: 17q11.1-q21.31 Size: 16.14 Mb
Case 8	copy neutral LOH	Chr17: 31571877–40588363 Cytoband: 17q11.2-q21.2 Size: 9.01 Mb
Case 9	loss	Chr17: 25278114–37876263 Cytoband: 17q11.1-q12 Size: 12.59 Mb
Case 10	loss	Chr17: 25278114–68301170 Cytoband: 17q11.1-q24.3 Size: 43.02 Mb
Case 11	disrupted in the breakpoint	Chr17: 36696279–81029941 Cytoband: 17q12-q25.3 Size: 44.33 Mb
Case 12	disrupted in the breakpoint	Chr17: 36696338–81029941 Cytoband: 17q12-q25.3 Size: 44.33 Mb
Case 13	disrupted in the breakpoint	Chr17: 36740844–80943189 Cytoband: 17q12-q25.3 Size: 44.20 Mb
Case 14	disrupted in the breakpoint	Chr17: 36740903–80993001 Cytoband: 17q12-q25.3 Size: 44.25 Mb
Case 15	loss	Chr17: 25278114–81029941 Cytoband: 17q11.1-q25.3 Size: 55.75 Mb
Case 16	disrupted in the breakpoint	Chr17: 36672992–77470237 Cytoband: 17q12-q25.3 Size: 40.79 Mb
Case 17	disrupted in the breakpoint	Chr17: 36694044–81099040 Cytoband: 17q12-q25.3 Size: 44.40 Mb

Table 1.
SRCIN1 loss/cn-LOH or disruption in the breakpoint on 17 NB patients.

associated with event-free survival (EFS) (321 patients) whereas a low expression was significantly associated with reduced metastatic recurrence (177 patients) (**Figure 2B**). *SRCIN1* mRNA expression was a favorable prognostic factor, both in terms of overall survival (OS) and EFS, regardless of the other known risk factors, including *MYCN* amplification, INSS stage, and age at diagnosis (**Figure 2C**).

To date, p140Cap expression by immunohistochemistry (IHC) on NB samples has not been studied owing to the lack of available cancer tissues, but *SRCIN1* mRNA expression correlates with a good outcome and is an independent prognostic marker for NB.

The *SRCIN1* gene is located on chromosome 17q12, a genomic region frequently involved in genetic abnormalities in NB. Therefore, a large cohort of 225 NB patients of all stages with 17q gain with poor prognosis, was analyzed by high-resolution oligonucleotide array-Comparative Genomic Hybridization (a-CGH) and Single Nucleotide Polymorphism - array (SNP-array). *SRCIN1* was hemizygously deleted in four NB tumors and it was subjected to copy-neutral Loss Of Heterozigosity (cn-LOH) in three specimens. Moreover, ten tumors displayed *SRCIN1* loss due to a breakpoint involved in the generation of 17q gain [43] (**Table 1**). However, because of the limited number of analyzed cases, survival differences between patients harboring these alterations did not reach statistical significance. Similar results come out in NB cell lines, as shown in SK-N-SH cells, where cn-LOH (8.72 Mb) that included *SRCIN1* gene, correlates with weak protein expression, suggesting an effective partial knockout of gene expression, originally proving that in NB patients the *SRCIN1* gene status may affect p140Cap expression, affecting prognosis.

4. p140Cap negatively affects tumorigenic features

The data obtained in NB patients support the hypothesis that p140Cap may curb the intrinsic biological aggressiveness of NB tumors. NB originates from the developing sympathetic nervous system, with a preferential localization in sympathetic ganglia and adrenal glands. Interestingly, we found that p140Cap is expressed in the main site of origin of NB tumors, in the medulla of normal human neonatal adrenal glands (**Figure 3A**). p140Cap is also expressed in a board panel of human NB cell lines which represent valid surrogate models for NB research [44]. Among these cell lines, p140Cap level was highly detected in HTLA-230, IMR-5, IMR-32, LAN-1 and SH-SY-5Y cell lines, weakly in SK-N-SH cells and undetectable in ACN cell line, a neuroblast-like cell line derived from bone marrow metastasis [45] (**Figure 3B**). According to the protein level analysis, the genomic profiling revealed a wide spectrum of *SRCIN1* gene abnormalities. Indeed, the *SRCIN1* gene was lost in ACN, while a single copy was found in SH-SY-5Y, IMR-32, and HTLA-230 cell lines. Moreover, a genomic gain was observed in the LAN-1 cell line, whereas SK-N-SH cells displayed a cn-LOH.

The absence of p140Cap protein renders the ACN cell line a suitable tool for the generation of a p140Cap-expressing NB cell line via retroviral infection that might be leveraged for further functional investigations (**Figure 3C**). It is well established that p140Cap inhibits breast cancer cell features such as migration and proliferation [22]. Consistently, p140Cap-overexpressing ACN (p140Cap-ACN) cells exhibited decreased migration properties in a Wound Healing assay, and impaired anchorage-independent growth of NB cells, one of the main hallmarks of cancer. Cancer cells are known to avoid apoptosis by increasing or decreasing the expression of apoptotic and anti-apoptotic genes, respectively [46]. A specific type of apoptotic process, called anoikis, occurs in cells in response to loss of adhesion to the extracellular matrix.

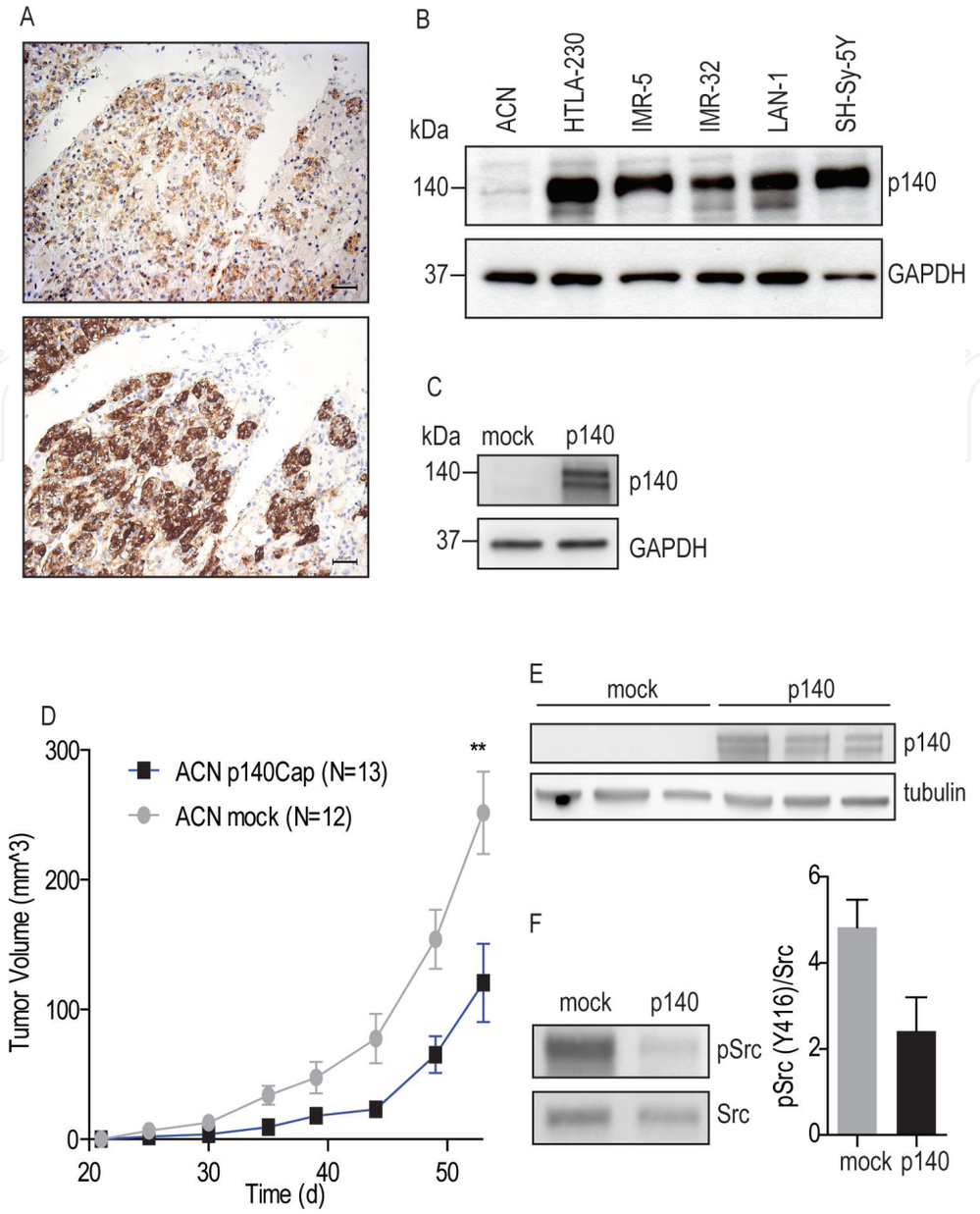


Figure 3.
*In vitro expression and in vivo role of p140Cap. (A) p140Cap staining is visible in the chromaffin cells of the adrenal medulla (upper panel), as confirmed by the chromogranin a staining (lower panel). Scale bar: 50 μM; (B) p140Cap expression in NB cell lines, by western blot of equal amounts of proteins from the indicated cell lines; (C) p140Cap expression in a pool of clones of ACN cells upon viral infection; (D) p140Cap limits in vivo tumor growth. Mock and p140 cells (2 × 10^5 cells in 0.2 ml of PBS) were subcutaneously injected into the dorsal region of male NSG mice. Average tumor volume. The size of the tumors was evaluated twice a week using digital calipers in blind experiments and significance was quantified by unpaired t-test (**P < 0.01); (E) WB analysis of p140Cap expression on explanted tumors by SDS-PAGE. Antibodies to p140Cap and tubulin (as loading control) were used; (F) Src kinase activation in tumor extracts. Tyr 416 phosphorylation (Y416) and Src protein level is shown. Quantification on the right is the ratio between phosphorylated Src and total Src protein in 5 tumors per group, as mean ± SEM (right) (unpaired t-test **P < 0.01).*

Upon anoikis, p140Cap-ACN cells showed both a lower upregulation of the anti-apoptotic protein Bcl-2 compared to mock cells, and a significantly higher percentage of apoptotic cells detected by annexin V labeling. Overall, p140Cap can limit anchorage-independent growth, migration and apoptosis of NB cells, suggesting a causal involvement of this protein in curbing NB cancer cell properties.

To date, the *in vivo* models commonly used for NB research and drug efficacy studies ranges from genetically engineered mouse models to xenograft murine systems [47] and from syngeneic mice to zebrafish and chick embryo chorioallantoic membrane [48, 49]. Each animal model has its own strengths and limitations, and

provides insights to specific biological questions. Immunodeficient mouse models such as the NOD Scid Gamma (NSG) mice represent a valuable tool for the study of engrafted human cell lines and PDX tumors [50]. In particular, NSG mice exhibit a complete deficiency in the adaptive immunity and a severe deficiency in the innate immunity as a consequence of mutations in the IL2-receptor common gamma chain, the *Prkdc* gene, which determines the so-called "scid" mutation, and the Rag1 or Rag2 null mutation [50]. Therefore, the NSG preclinical model was a suitable candidate to investigate the *in vivo* tumor-suppressing role of p140Cap. Upon subcutaneous injection into the dorsal region of the NSG mice, p140Cap cells gave origin to smaller tumors, compared to mock cells. p140Cap tumors were also extensively poorly proliferative, in terms of proliferation marker KI67 (**Figure 3D, E**).

In NB, angiogenesis has a prominent role in determining tumor phenotype. A study published by Meitar D *et al.* demonstrated that higher vascularity in NB correlates with metastasis, unfavorable histology, and poor outcome [51]. p140Cap tumors showed a slight but significant lower number of vessels positive for CD31 and CD105 endothelial cell markers compared to control. Histological sections were also stained for AML and NG2 markers of mature or young pericytes, respectively, in order to evaluate the pericyte coverage of vessels. In line with the idea that p140Cap limits the angiogenic activity of cancer cells leading to the formation of larger and more stable vessels, p140Cap tumors exhibited higher pericyte coverage of the endothelium compared to control.

As already mentioned, p140Cap has been widely demonstrated to limit breast cancer cells growth and metastasis formation [22, 23]. The ability of p140Cap to inhibit cancer cell adhesion, migration and proliferation may contribute to the overall reduced occurrence of metastatic events. p140Cap tumors gave rise to a significantly reduced number of lung metastases compared to control. Overall, p140Cap impairs NB tumor growth and spontaneous metastasis *in vivo*, with a significant decrease in proliferation markers and an increase in tumor vessel pericyte coverage. These results are in line with those obtained in HER2 positive breast cancer patients and preclinical models [22], where p140Cap dampens the aggressiveness of these highly aggressive tumors.

Further evidence supporting the biological relevance of p140Cap in curbing NB aggressiveness was provided by the recent work of Yuan XL *et al*. [52]. Yuan XL *et al*. demonstrated that *SRCIN1* is a direct target of the microRNA-373 (miR-373) and that their expression has a negative correlation in both NB human samples and cell lines. miR-373 functions as an oncomiRNA promoting proliferation, migration and invasion of NB cells. Inhibition of miR-373 by using a specific anti-miRNA in SK-N-BE(2) cells led to a significant decrease of tumor growth in a mouse xenograft model that was paralleled with increased p140Cap mRNA and protein levels in the resected tumors. Silencing of *SRCIN1* partially abrogated the inhibitory effect of antimiR-373 in NB cell proliferation, migration and invasion.

The molecular mechanisms underpinning the tumor-suppressive properties of p140Cap in NB may rely on the modulation of specific intracellular signaling pathways that will be dissected in the next sections.

5. Molecular mechanisms and therapeutic targets in neuroblastoma

Over the last years, genomic analysis, exome and whole-genome sequencing, genome-wide association studies, transcriptomics and drug screenings have shed light on NB biology [53]. The ongoing phase relies on translating NB biology and genetics into improved prognostic stratification and precision medicine. New druggable targets could come out from the identification of predictors for response and

outcome as well as from the discovery of molecular aberrations in the tumors (for a recent review see [53]). Of the genetic aberrations described in NB only *MYCN* overexpression and activating mutations of the tyrosine kinase receptor (RTK) *ALK* have been proven to be de novo oncogenic drivers as mutation or overexpression of these molecules give rise to NB in genetically engineered mouse models [54, 55]. The oncogenic transcription factor *MYCN* is a hallmark of poor prognosis in NB patients [56]. However, the compounds that can interfere with *MYCN* interaction with its partner MAX as a way to block its transcriptional action, were not efficient *in vivo* [57], dampening their development in clinical testing. Strategies to exploit *MYCN* as a tumor-associated antigen for immunotherapy deserve further functional validation [58]. The *ALK* gene is altered by gain-of-function point mutations in around 14% of high-risk NB and represents an ideal therapeutic target given its low or absent expression in healthy tissue postnatally [59]. ALK signaling can be blocked in ALK-mutant NB cell lines and mouse models by different means, including RNA interference and small-molecule inhibitors. Moreover, the STAT3, PI3K/AKT and Ras/MAPK are the main pathways involved in full-length ALK signaling [60]. In particular, Mass Spectrometry-based phosphotyrosine profiling of signaling events associated with the full-length ALK receptor, showed robust activation of STAT3 on Tyr705 in a number of independent NB cell lines. STAT3 silencing reduces *MYCN* protein levels downstream of ALK signaling, together with inhibition of NB cell growth in the presence of STAT3 inhibitors. Overall these data suggest that activation of STAT3 is important for ALK signaling activity in NB [61]. On the other hand, ERK5 can mediate ALK-induced transcription of *MYCN* and proliferation of NB, suggesting that targeting both ERK5 and ALK may be beneficial in NB patients [62].

In addition to ALK, signaling through the EGFR and ERBB2 RTK, both found to be non-mutational activated in subsets of NB, converge at MAPK, with increased MAPK signaling. Further, MAPK/ERK kinase (MEK) inhibitors have been shown to inhibit the growth of NB cells *in vitro* [63] and *in vivo*, alone or in synergy with the CDK4/6 inhibitor, ribociclib to suppress tumor growth in a panel of murine xenograft models of NB [64]. However, a recent preclinical study advises against trametinib as monotherapy in ALK-addicted NB due to increased feedback activation of other signaling pathways including PI3K/AKT in both cell lines and mice xenografts [65].

Interestingly, high-risk NBs without *MYCN* amplification may deregulate *MYC* and other oncogenic genes via altered beta-catenin signaling providing a potential candidate pathway for therapeutic inhibition [66]. XAV939, a tankyrase 1 inhibitor, promotes cell apoptosis in NB cell lines by inhibiting Wnt/beta-catenin signaling pathway, by reducing the expression of anti-apoptotic markers and decreasing colony formation *in vitro* [67]. O6-methylguanine-DNA methyltransferase (MGMT) is commonly overexpressed in cancers and is implicated in the development of chemoresistance. A significant correlation between Wnt signaling and MGMT expression was found in several cancers, including NB. Further, immunofluorescence analysis on human tumor tissues showed co-localization of nuclear beta-catenin and MGMT in subtypes of NB. Pharmacological or genetic inhibition of Wnt activity downregulates MGMT expression and restores chemosensitivity of DNA-alkylating drugs [68].

6. p140Cap impairs the Src/p130Cas and the STAT3/Jak2 signaling pathways

Focal adhesion kinase (FAK) and Src are two non-receptor intracellular kinases highly expressed in a number of human tumors including NB, and together regulate both cellular adhesion and survival. Both FAK and Src play a role in protecting NB

cells from apoptosis, and dual inhibition of these kinases may be important when designing therapeutic interventions for this tumor [69]. Immunohistochemical staining showed FAK to be present in 73% of human NB specimens examined. In addition, p125FAK staining was significantly increased in stage IV tumors with amplification of the *MYCN*. Src expression in NB patients has been associated with poor outcomes [70, 71]. Indeed, Src family kinases promote cell survival/proliferation and reduce cell aggregation of NBs. Conversely, its inhibition results in decreased proliferation and enhanced apoptosis in NB cells [72, 73], suggesting that Src family kinase inhibitors may be good candidates for molecular targeted therapy [74]. Of note, dasatinib, a well-known Src kinase inhibitor, is a potent inhibitor of NB cell viability with an IC(50) in the submicromolar range. As a consequence, dasatinib decreased anchorage-independent growth, affecting senescence and apoptosis. Interestingly, in the HTLA-230 NB model, dasatinib decreased c-Kit and Src activation together with a strong MAPK and Akt impairment. Dasatinib was also tested *in vivo* in a murine orthotopic model, where NB cells were injected directly in the adrenal gland in a microenvironment that closely mimics the human tumors conditions. HTLA-230 tumors were reduced in size and cellularity, with proliferation disease. Drug treatment in the orthotopic model utilizing HTLA-230 cells produced a significant reduction of tumor burden. Nevertheless, dasatinib activity *in vivo* was also significantly inhibited, but complete tumor eradication was not achieved [75]. Recently, the scaffold protein PAG1 was involved in the regulation of Src family kinase (SFK) signaling in NB. The NB cell line expressing PAG1™⁻ lacks the membrane-spanning domain of PAG1 and is located in the cytoplasm. PAG1™⁻ cells exhibited higher amounts of active SFKs and increased growth rate. Under differentiation conditions, PAG1™⁻ cells continued to proliferate and did not undergo differentiation. Activated FYN was sequestered in PAG1™⁻ cells, suggesting that disruption of FYN localization led to the observed defects in differentiation. Overall, PAG1 is an additional example of how a scaffold protein may control SFK intracellular localization, impacting on their activity and signaling that induces differentiation events, that may be crucial in the control of NB aggressiveness [76].

IL-6-dependent activation of STAT3 [77] has already been reported in NB, where STAT3 is critical in mediating increased survival and drug resistance [78–80]. Interestingly, very recently, the antiapoptotic and prometastatic JAK-STAT3 pathway was activated in chemoresistant tumors, generated in the Th-*MYCN* ^{CPM32} model. This model derives from a multicycle treatment with cyclophosphamide of the Th-*MYCN* genetically engineered mice which develop rapidly progressive chemosensitive NB, but lack clinically relevant metastases. Copy number aberrations in these tumors reflect the genomic alterations typical of human *MYCN*-amplified NB, e.g. copy number gains at mouse chromosome 11, syntenic with gains on human chromosome 17q. The Th-*MYCN* ^{CPM32} model is characterized by chemoresistance and progression in spontaneous bone marrow metastatic events. NB tumors show a huge remodeling of the immune microenvironment, with augmented tumor-associated fibroblasts and stroma. Treatment with the JAK1/JAK2 inhibitor CYT387 reduced progression of chemoresistant tumors and increased survival, highlighting that under treatment conditions that mimic chemotherapy in human patients, Th-*MYCN* ^{CPM32} mice develop genomic, microenvironmental, and clinical features reminiscent of human chemorefractory disease, with dysregulation of signaling pathways such as JAK-STAT3 that could be targeted to improve treatment of aggressive disease [81].

Our recent data show that p140Cap expression in NB cells is sufficient to down-modulate the tyrosine phosphorylation of Src Tyr 416 (p-Src), a marker of active Src, as well as of p130Cas, a well-known Src substrate [82] (**Figure 4A, B**).

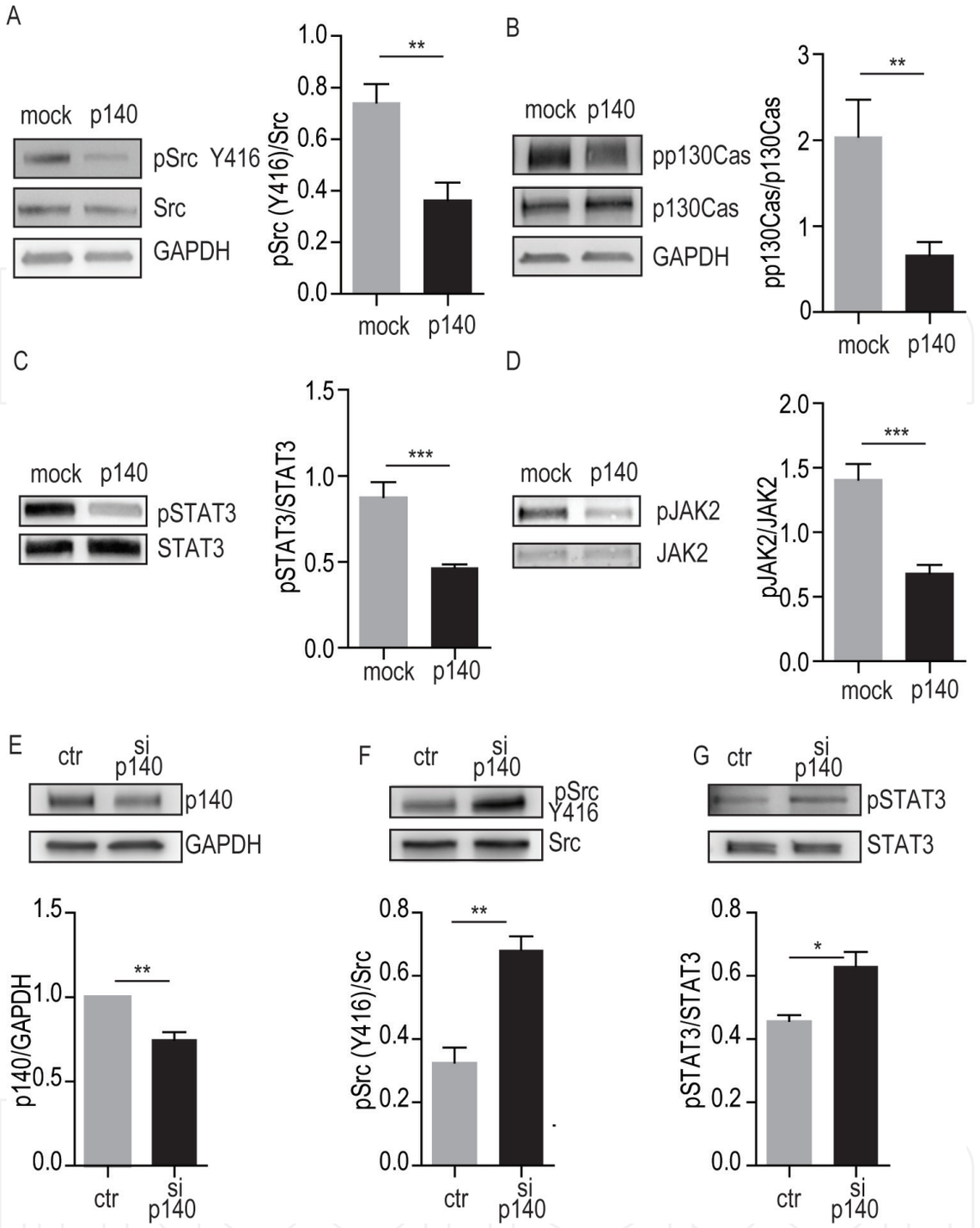


Figure 4.
p140Cap affects signaling pathways in NB cells. (A) Src activation was evaluated on mock and p140 ACN cells by WB analysis of Tyr 416 phosphorylation (Y416) and Src protein level as loading control. Antibodies to GAPDH (as loading control) were used. Quantification on the right is the ratio between phosphorylated Src and total Src protein; (B) p130Cas phosphorylation was evaluated with antibodies to phosphorylated p130Cas at Tyr 410, p130Cas and GAPDH antibodies for loading control. Quantification on the right is the ratio between phosphorylated p130Cas and total p130Cas; (C) STAT3 phosphorylation was evaluated with antibodies to phosphorylated STAT3 at Tyr705 and STAT3 antibodies for loading control. Quantification on the right is the ratio between phosphorylated STAT3 and total STAT3 protein from three independent experiment; (D) Jak2 activation was evaluated with antibodies to phosphorylated Jak2 at Tyr1007/1008 and Jak2 antibodies for loading control. Quantification on the right is the ratio between phosphorylated Jak2 and total Jak2 protein; in E-G p140Cap silenced cells were tested for p140Cap WB, and GAPDH as loading control (E), for Src activation at Tyr 416 phosphorylation (Y416) and Src protein level as loading control (F), and for phosphorylated STAT3 at Tyr 705 and STAT3 protein level as loading control (G). Quantification is shown on the left as the ratio between phosphorylated Src/STAT3 and total Src/STAT3 protein.

Moreover, we showed that STAT3 Tyr 705 (pSTAT3) is less phosphorylated, and JAK2 kinase is less active in p140Cap cells (**Figure 4C, D**). Consistent with these data, silencing of the endogenous p140Cap in SH-SY-5Y cells RNA [22] caused increased Src activation of STAT3 phosphorylation, confirming that p140Cap can regulate these two signaling pathways (**Figure 4E-G**). Overall, p140Cap ability to influence the Src/p130Cas and the JAK2/STAT3 pathways could be causal for the impairment of NB progression observed in patients [70, 72, 78–80, 83]. p140Cap also impairs Src kinase activity in breast cancer cells upon integrin-mediated adhesion or growth factor treatment stimulation [23, 84]. Overall, p140Cap may negatively regulate Src activity at least two tumor types, as a key event in dampening their migratory and invasive phenotype.

Based on the pro-survival role of STAT3 in NB, we also performed anoikis assays, showing that p140Cap-expressing cells were characterized by a significant decrease in the level of pSTAT3. Only the forced expression of the constitutive active STAT3C mutant is able to decrease p140Cap sensitivity to anoikis-dependent death. Overall, our data indicate that in NB cells, p140Cap expression may affect cell death, by impairing the pro apoptotic signaling sustained by the JAK2/STAT3-Bcl2 survival pathway.

7. p140Cap increases NB cell sensitivity to chemotherapeutic treatment

Despite advances in the molecular exploration of pediatric cancers, approximately 50% of children with high-risk NB lack effective treatment [85]. NB treatments are designed on the basis of a risk classification, which takes into account a subset of prognostic factors associated with a patient's outcome. Clinical features (for instance, the tumor stage or patient's age at diagnosis) and biological tumor properties (such as histology, genetic alteration and molecular markers) can be used as prognostic factors [9, 19] to classify NB patients in low risk, intermediate risk (IR) or high risk (HR) groups [19].

Non-high-risk represent slightly more than half of newly diagnosed patients. Outcomes are generally excellent for these children, with variable treatment strategies including observation alone, surgical resection, or moderate doses of chemotherapy [86, 87]. On the other hand, high-risk NB are very difficult to treat and require multi-modal therapy. Intensification of therapy has vastly improved survival rates, and research is focused on novel treatments to further improve survival rates [88].

Children with an intermediate or high risk often receive chemotherapy, namely carboplatin, cyclophosphamide, doxorubicin, etoposide, busulfan, ifosfamide or vincristine [9]. However, the side effects of chemotherapy and the outcome depend on the individual and the dose used. In this context, we demonstrated that p140Cap correlates with an increased sensitivity to chemotherapy. Namely, we tested five chemotherapeutic drugs commonly used in NB patients (ciclophosphamide, carboplatin, doxorubicin, etoposide, and vincristine) in dose viability assays. NB cell lines overexpressing p140Cap showed significantly increased sensitivity to low doses (10 nM, 100 nM) of cyclophosphamide, vincristine, doxorubicin and etoposide (**Figure 5A-C**). Consistently, in SH-SY-5Y cells, p140Cap silencing resulted in increased viability to both doxorubicin and etoposide [43] (**Figure 5D**).

Both etoposide and doxorubicin prevent ligation of the DNA strands, stopping the process of replication. The number of foci/cells of phosphorylated histone H2AX (gamma H2AX), an established marker of DNA damage [89], was counted

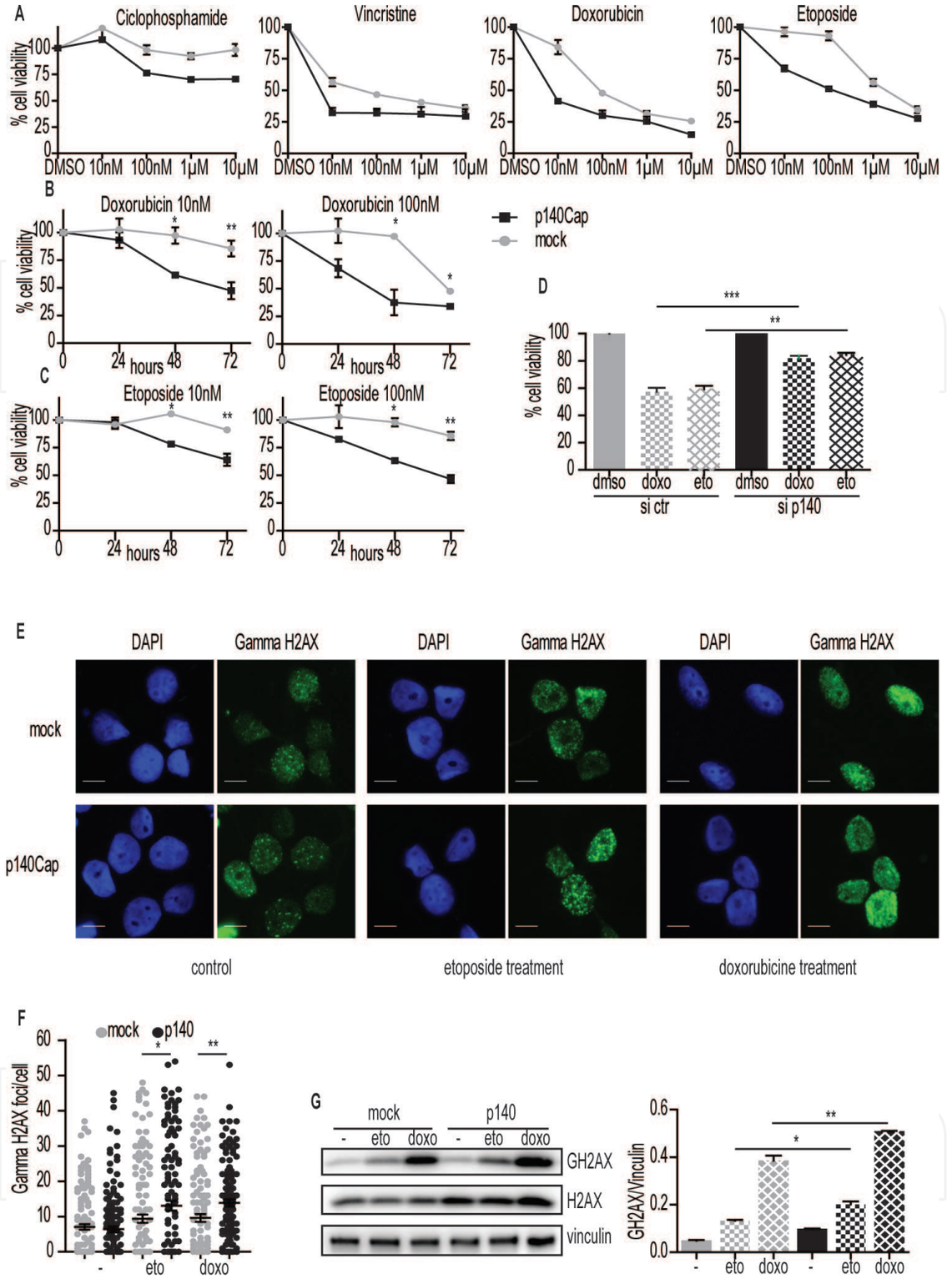


Figure 5.
p140Cap regulates cell viability to chemotherapeutic drugs. (A) Dose dependence viability to chemotherapy drugs. Mock and p140 cells were treated with the four indicated doses and cell viability was quantified at 72 h of treatment; (B-C) time dependent viability. Mock and p140 cells were processed as in (a); (D) cell viability in SH-5YSY cells silenced for p140Cap. Cells were transfected with appropriate siRNA and after 24 h treated with 1 μM etoposide or doxorubicin. Cell viability was quantified at 48 h; (E) visualization of nuclear foci for gamma H2AX histone as a marker of DNA damage. Mock and p140 cells on glass slides were treated for 6 h with 1 μM etoposide and doxorubicin. Green: Gamma H2AX foci; blue: DAPI for nuclear staining. Scale bar: 10 μm; (F) quantification of gamma H2AX foci/cell. Mock: Red; p140: Green. 50 nuclei were evaluated for each experiment; (G) gamma H2AX levels upon chemotherapy treatment. Mock and p140 cells were acutely treated with 1 μM etoposide or doxorubicin for 6 h. extracts were analyzed by western blot with antibodies to gamma-H2AX, H2AX and vinculin for loading controls. Quantification on the right is the ratio between gamma-H2AX and vinculin.

after an acute 6 h treatment with 1 μM etoposide and doxorubicin. p140Cap cells showed a significant increase in this marker over mock cells, indicating that the increased sensitivity of p140Cap cells to these drugs was associated with increased DNA lesions (**Figure 5E-G**). Overall, our study indicates that p140Cap NB cells display a significant decrease in cell viability upon drug treatment, with an increased sensitivity to drug-dependent DNA damage [43].

8. p140Cap increases NB cell sensitivity to Src kinase inhibitors

As already said above, Src family kinases are proto-oncogene tyrosine-protein kinases which are involved in tumor progression in several cancer types and are considered as a target for a low toxic anti-tumor treatment. High Src levels are generally associated with a poor prognosis and play an important role in the differentiation, cell-adhesion and survival of NB cells. Indeed, the inhibition of such kinase is an effective approach for NB treatment and several Src- inhibitors have been developed, holding a promising antiproliferative effect, cell cycle arrest, apoptosis induction and decreased adhesion/invasiveness [69, 72, 73].

Since active Src was significantly down-regulated in p140Cap tumors over mock tumors and p140Cap overexpressing cells showed lower levels of active Src (**Figure 3F** and **4A**), we hypothesized that Src activity may be involved in NB cell viability [89]. In mock cells, Src activity was highly sensitive to two well-known Src inhibitors, saracatinib (which also inhibits the Abl kinase [90] at 100 nM), and sugen (used in preclinical NB models [91] at 1 μM). At 72 h, in mock cells both inhibitors decreased cell viability of 20–25%. Interestingly, the same treatment in p140Cap cells leads to a reduction in viability of nearly 40%. Moreover, viability to Src inhibitors was increased in cells silenced for p140Cap compared to p140Cap overexpressing cells. However, the partially silenced cells were still more sensitive than mock cells, indicating that there is a direct correlation between p140Cap expression and the augmented sensitivity to Src inhibitors.

We observed a decreased viability in mock cells upon treating them with Src inhibitors coupled with drugs that induce a DNA damage (in particular, doxorubicin or etoposide have been used at a concentration of 10 nM and 100 nM in association with saracatinib and sugen).

The decreased viability of mock cells (approximately at 50%) in these conditions indicates that may Src inhibitors concur in increasing chemotherapy cytotoxic effect in those cells which do not express p140Cap.

In addition, the use of both genotoxic drugs and Src inhibitors in the same treatment confers to p140Cap overexpressing cells a lower viability, in particular in cells treated with doxorubicin. Taken together, our data suggest that a combined treatment with Src inhibitors could increase NB cells sensitivity to etoposide and doxorubicin.

Upon a treatment with augmented doses of etoposide and doxorubicin (in a range of 1 nM-1 mM) used alone or in association with the same concentrations of Src inhibitors, we observed that the combined experimental setting was synergistic in both the cell lines (mock and p140Cap overexpressing cells).

Indeed, the Combination Index (CI) values computed for the different combinations of drugs were < 1 in all the experimental settings [92]. The p140Cap overexpressing cells still showed an increased sensitivity to the Src inhibitors in the combined treatment, with a shift of the sensitivity to lower doses (**Figure 6**). Therefore, our data show that chemotherapy and Src inhibitors combination synergistically decreases NB cell viability and this effect can be further increased by p140Cap expression [43].

treatment	Cell line	CI	DRI50	r	Cell line	CI	DRI50	r
Doxo, Sug	mock	0.08838	Doxo -12.39; Sug -130.442	0.97494	p140	0.17652	Doxo -5.814; Sug -221.354	0.93442
Doxo, Sara	mock	0.1775	Doxo -9.021; Sara -14.904	0.98575	p140	0.3368	Doxo -3.219; Sara -38.115	0.95530
Eto, Sug	mock	0.277	Eto -5.606; Sug -10.041	0.99194	p140	0.07017	Eto -18.881; Sug -58.115	0.96845
Eto, Sara	mock	0.08899	Eto -28.921; Sara -18.377	0.97889	p140	0.07185	Eto -26.208; Sara -29.676	0.92729

Figure 6.
Synergistic analysis of combined treatments with chemotherapy drugs and Src inhibitors. The CI (combination index to reduce viable cells to 50%) and the DRI50 (the dose-reduction necessary to decrease viable cells to 50%, were calculated using the CalcuSyn software (www.biosoft.com/w/calcusyn.htm); r: Linear regression coefficient.

9. Conclusions

This chapter highlights the original involvement of *SRCIN1*/p140Cap in NB, providing evidence that *SRCIN1* gene expression may be exploited as a marker of good outcomes in NB (**Figure 7**). *SRCIN1* mRNA levels are clinically relevant in NB

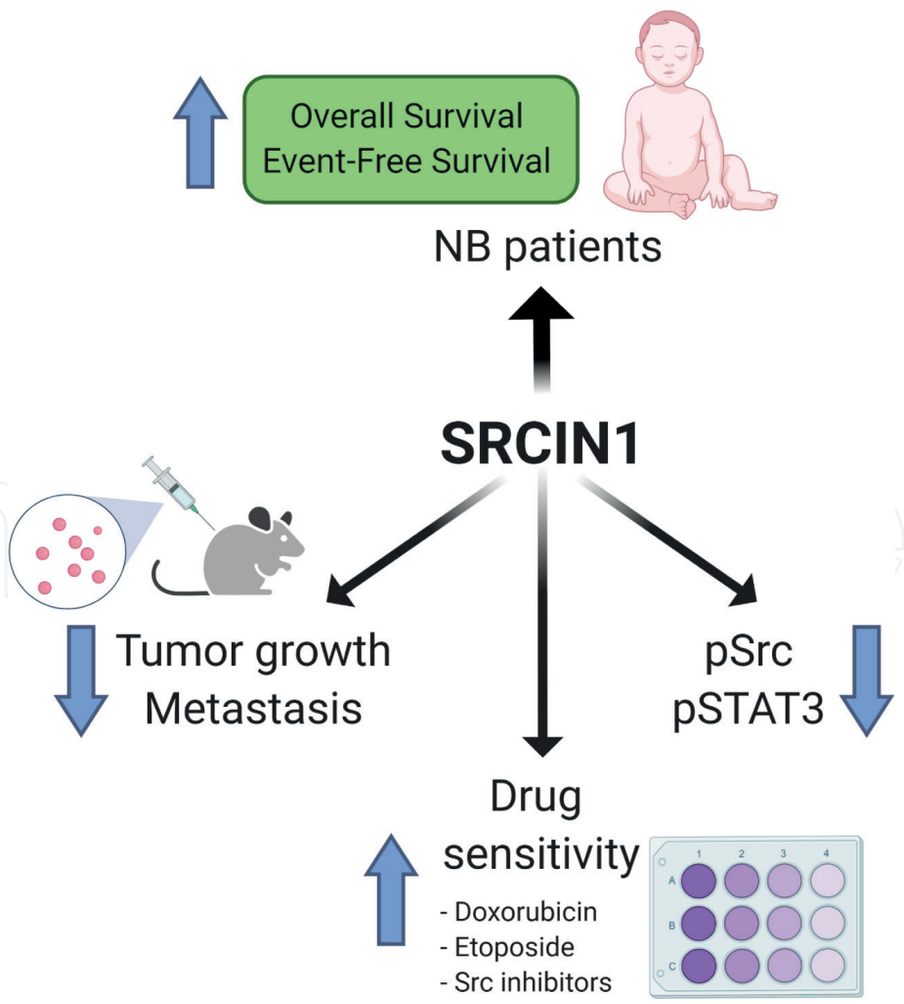


Figure 7.
Overview of SRCIN1 *involvement in NB. The data reported here indicate a key causal role of* SRCIN1/*p140Cap in dampening cell signaling, tumor growth, metastasis and drug sensitivity in NB cells, leading to a good outcome in NB patients.*

patients, with high levels of expression positively correlating with good prognosis and high survival rate. Of note, *SRCIN1* mRNA behaves as an independent risk factor, thus providing evidence that *SRCIN1* is a useful, additional marker for better stratifying NB patient cohorts.

Overall, the protein p140Cap acts as a tumor suppressor gene in NB tumors, dampening tumor volume and decreasing progression towards distant metastasis. This might occur because of an increased ability to undergo apoptosis and a decreased capability of p140Cap NB cells to proliferate *in vivo*. Tumor microenvironment could also play a role, as shown by decreased permeability of p140Cap tumor vessels, likely due to the increased presence of pericytes, as shown by their specific marker NG2 [93].

An urgent need in NB is to increase the five-year OS rate of high-risk NB patients, which is still less than 40% [94]. Despite emerging new therapies, the impact of treatments is very heavy for affected children, which can have serious consequences for years to come [95]. The data showing that p140Cap expressing NB have significantly increased sensitivity to low doses (10 nM concentration) of doxorubicin and etoposide, two drugs used in first line NB treatment, open new perspectives. Further, the fact that a combo treatment with Src inhibitors and low doses doxorubicin or etoposide, sensitize mock cells, reducing cell viability to that of p140Cap cells treated with chemotherapy alone, is an encouraging result. Therefore, it would be interesting to set combinatorial approaches with low doses of both chemotherapy drugs and specific inhibitors, including also the Jak2 pathway, to quantify the additional/synergistic effects. Further, to increase the understanding of the mechanism of action of p140Cap on the sensitization to specific drugs, it would be very useful to identify the vital molecular signaling mechanisms involved. To achieve these results, both automated platforms for cell viability and genome-wide CRISPR-CAS9 technology are largely available. In conclusion, we believe that these data demonstrate the potential clinical impact of SRCIN1/p140Cap expression and of p140Cap-regulated pathways in NB tumors. These results pave the way to include *SRCIN1* mRNA in the NB patients' prognostic status, as a key marker for patient outcome.

Acknowledgements

The figures and Tables have been adapted from ref. [43] Grasso et al., 2020. We give here appropriate acknowledgment to the original authors of this publication (Silvia Grasso, Melissa Alzona, Alessia Lamolinara, Andrea Saglietto, Federico Tommaso Bianchi, Sara Cabodi, Iris Chiara Salaroglio, Federica Fusella, Marzia Ognibene, Manuela Iezzi, Annalisa Pezzolo, Valeria Poli, Ferdinando Di Cunto, Alessandra Eva, Chiara Riganti, Luigi Varesio). This work was supported by AIRC (Associazione Italiana Ricerca Cancro) to PD (IG- 20107), Compagnia San Paolo, Torino, Progetto DEFLECT to PD; Fondazione CRT 2020.1798 to PD.

Conflict of interest

There is no competing of interest to declare.

Rights and permissions

Abbreviations

Abl	Abelson Tyrosine-Protein Kinase
a-CGH	array - Comparative Genomic Hybridization
AKT	AKT serine/threonine kinase 1
ALK	Anaplastic Lymphoma Kinase
AML	Acute myeloid leukemia
Bcl-2	B-cell lymphoma 2
Bcl-2	B-cell lymphoma gene 2
BDNF	Brain-Derived Neurotrophic Factor
BRCA1	BRCA1 DNA repair associated
CAP	Cas-associated protein
CD105	endoglin
CD31	platelet and endothelial cell adhesion molecule 1
CDK4/6	cyclin D-cyclin-dependent kinase 4/6
CI	Combination Index
cn-LOH	copy-neutral Loss Of Heterozygosity
Csk	C-terminal Src kinase
DS	Dendritic spines
EB3	microtubule associated protein RP/EB family member 3
EFS	event free survival
ERBB2	erb-b2 receptor tyrosine kinase 2
ERK	extracellular signal-regulated kinase
ERK5	Extracellular signal-regulated kinase 5
FAK	focal adhesion kinase
FYN	FYN oncogene related to SRC, FGR, YES
H2AX	H2A histone family member X
IC50	the half maximal inhibitory concentration
IDP	Intrinsic Disorder Protein
IDR	Intrinsically Disordered Regions
IHC	Immunohistochemistry
IL-6	Interleukin 6
INSS	International Neuroblastoma Staging System
JAK	Janus kinase
Ki67	marker of proliferation Ki-67
LIN28B	lin-28 homolog B
MAPK	mitogen-activated protein kinase
MAX	myc-associated factor X
MGMT	O(6)-Methylguanine-DNA methyltransferase
MYCN	MYCN proto-oncogene
NB	Neuroblastoma

NG2	Neural/glial antigen 2
NSG	NOD Scid Gamma mice
OS	Overall survival
PAG1TM-	PAG1 fragment that lacks the membrane spanning domain
PDX	patient derived xenograft
PHOX2B	Paired-like Homeobox 2b
PI3K	Phosphoinositide 3-kinase
Prkdc	protein kinase, DNA-activated, catalytic subunit
Rag-1 and 2	Recombinant activating 1,2
RARA	retinoic acid receptor-α
Ras	Ras-related C3 botulinum toxin substrate
RTK	tyrosine kinase receptor
SFK	Src family kinase
SNIP	SNAP25 Interacting Protein
SNP-array	Single Nucleotide Polymorphism – array
Src	proto-oncogene tyrosine-protein kinase Src
SRCIN1	SRC kinase signaling inhibitor 1
STAT3	Signal transducer and activator of transcription 3
TrkB	Tropomyosin receptor kinase B
Wnt	Wingless-related integration site

Author details

Giorgia Centonze[1†], Jennifer Chapelle[1†], Costanza Angelini[1†], Dora Natalini[1†], Davide Cangelosi[2], Vincenzo Salemme[1], Alessandro Morellato[1], Emilia Turco[1] and Paola Defilippi[1*]

1 Department of Molecular Biotechnology and Health Science, University of Torino, Torino, Italy

2 Laboratory of Molecular Biology, Giannina Gaslini Institute, Genova, Italy

*Address all correspondence to: paola.defilippi@unito.it

† These authors equally contribute to this work.

References

[1] Gatta, G., et al., *Embryonal cancers in Europe.* Eur J Cancer, 2012. **48**(10): p. 1425-33.

[2] Maris, J.M., *Recent advances in neuroblastoma.* N Engl J Med, 2010. **362**(23): p. 2202-11.

[3] Farrell, P.A., A.L. Caston, and D. Rodd, *Changes in insulin response to glucose after exercise training in partially pancreatectomized rats.* J Appl Physiol (1985), 1991. **70**(4): p. 1563-8.

[4] DuBois, S.G., et al., *Metastatic sites in stage IV and IVS neuroblastoma correlate with age, tumor biology, and survival.* J Pediatr Hematol Oncol, 1999. **21**(3): p. 181-9.

[5] Brodeur, G.M. and R. Bagatell, *Mechanisms of neuroblastoma regression.* Nat Rev Clin Oncol, 2014. **11**(12): p. 704-13.

[6] Brodeur, G.M., et al., *Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment.* J Clin Oncol, 1993. **11**(8): p. 1466-77.

[7] Monclair, T., et al., *The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report.* J Clin Oncol, 2009. **27**(2): p. 298-303.

[8] Schleiermacher, G., et al., *Segmental chromosomal alterations have prognostic impact in neuroblastoma: a report from the INRG project.* Br J Cancer, 2012. **107**(8): p. 1418-22.

[9] Cohn, S.L., et al., *The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report.* J Clin Oncol, 2009. **27**(2): p. 289-97.

[10] Li, M.Y., et al., *A Critical Role of Presynaptic Cadherin/Catenin/p140Cap Complexes in Stabilizing Spines and Functional Synapses in the Neocortex.* Neuron, 2017. **94**(6): p. 1155-1172 e8.

[11] Ogawa, S., et al., *Oncogenic mutations of ALK in neuroblastoma.* Cancer Sci, 2011. **102**(2): p. 302-8.

[12] Zhu, S., et al., *Activated ALK collaborates with MYCN in neuroblastoma pathogenesis.* Cancer Cell, 2012. **21**(3): p. 362-73.

[13] Wang, L.L., et al., *Augmented expression of MYC and/or MYCN protein defines highly aggressive MYC-driven neuroblastoma: a Children's Oncology Group study.* Br J Cancer, 2015. **113**(1): p. 57-63.

[14] Nakagawara, A., et al., *Expression and function of TRK-B and BDNF in human neuroblastomas.* Mol Cell Biol, 1994. **14**(1): p. 759-67.

[15] Diskin, S.J., et al., *Common variation at 6q16 within HACE1 and LIN28B influences susceptibility to neuroblastoma.* Nat Genet, 2012. **44**(10): p. 1126-30.

[16] van Limpt, V., et al., *The Phox2B homeobox gene is mutated in sporadic neuroblastomas.* Oncogene, 2004. **23**(57): p. 9280-8.

[17] Owens, C. and M. Irwin, *Neuroblastoma: the impact of biology and cooperation leading to personalized treatments.* Crit Rev Clin Lab Sci, 2012. **49**(3): p. 85-115.

[18] Morgenstern, D.A., S. Baruchel, and M.S. Irwin, *Current and future strategies for relapsed neuroblastoma: challenges on the road to precision therapy.* J Pediatr Hematol Oncol, 2013. **35**(5): p. 337-47.

[19] Whittle, S.B., et al., *Overview and recent advances in the treatment of neuroblastoma.* Expert Rev Anticancer Ther, 2017. **17**(4): p. 369-386.

[20] Chin, L.S., et al., *SNIP, a novel SNAP-25-interacting protein implicated in regulated exocytosis.* J Biol Chem, 2000. **275**(2): p. 1191-200.

[21] Di Stefano, P., et al., *P130Cas-associated protein (p140Cap) as a new tyrosine-phosphorylated protein involved in cell spreading.* Mol Biol Cell, 2004. **15**(2): p. 787-800.

[22] Grasso, S., et al., *The scaffold protein p140Cap limits ERBB2-mediated breast cancer progression interfering with Rac GTPase-controlled circuitries.* Nat Commun, 2017. **8**: p. 14797.

[23] Di Stefano, P., et al., *p140Cap protein suppresses tumour cell properties, regulating Csk and Src kinase activity.* EMBO J, 2007. **26**(12): p. 2843-55.

[24] Di Stefano, P., et al., *The adaptor proteins p140CAP and p130CAS as molecular hubs in cell migration and invasion of cancer cells.* Am J Cancer Res, 2011. **1**(5): p. 663-73.

[25] Lamy, P.J., et al., *Quantification and clinical relevance of gene amplification at chromosome 17q12-q21 in human epidermal growth factor receptor 2-amplified breast cancers.* Breast Cancer Res, 2011. **13**(1): p. R15.

[26] Combaret, V., et al., *Determination of 17q gain in patients with neuroblastoma by analysis of circulating DNA.* Pediatr Blood Cancer, 2011. **56**(5): p. 757-61.

[27] Bown, N., et al., *Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma.* N Engl J Med, 1999. **340**(25): p. 1954-61.

[28] Vandesompele, J., et al., *Unequivocal delineation of clinicogenetic subgroups and development of a new model for improved outcome prediction in neuroblastoma.* J Clin Oncol, 2005. **23**(10): p. 2280-99.

[29] Lastowska, M., et al., *Comprehensive genetic and histopathologic study reveals three types of neuroblastoma tumors.* J Clin Oncol, 2001. **19**(12): p. 3080-90.

[30] Salemme, V., et al., *The p140Cap adaptor protein as a molecular hub to block cancer aggressiveness.* Cell Mol Life Sci, 2020.

[31] Wright, P.E. and H.J. Dyson, *Intrinsically disordered proteins in cellular signalling and regulation.* Nat Rev Mol Cell Biol, 2015. **16**(1): p. 18-29.

[32] Repetto, D., et al., *Mapping of p140Cap phosphorylation sites: the EPLYA and EGLYA motifs have a key role in tyrosine phosphorylation and Csk binding, and are substrates of the Abl kinase.* PLoS One, 2013. **8**(1): p. e54931.

[33] Jaworski, J., et al., *Dynamic microtubules regulate dendritic spine morphology and synaptic plasticity.* Neuron, 2009. **61**(1): p. 85-100.

[34] Damiano, L., et al., *p140Cap suppresses the invasive properties of highly metastatic MTLn3-EGFR cells via impaired cortactin phosphorylation.* Oncogene, 2012. **31**(5): p. 624-33.

[35] Ito, H., et al., *Characterization of a multidomain adaptor protein, p140Cap, as part of a pre-synaptic complex.* J Neurochem, 2008. **107**(1): p. 61-72.

[36] Russo, I., et al., *p140Cap Regulates GABAergic Synaptogenesis and Development of Hippocampal Inhibitory Circuits.* Cereb Cortex, 2019. **29**(1): p. 91-105.

[37] Tomasoni, R., et al., *SNAP-25 regulates spine formation through postsynaptic binding to p140Cap.* Nat Commun, 2013. **4**: p. 2136.

[38] Yang, Y., et al., *Endophilin A1 regulates dendritic spine morphogenesis and stability through interaction with p140Cap.* Cell Res, 2015. **25**(4): p. 496-516.

[39] Fossati, G., et al., *Reduced SNAP-25 increases PSD-95 mobility and impairs spine morphogenesis.* Cell Death Differ, 2015. **22**(9): p. 1425-36.

[40] Repetto, D., et al., *p140Cap regulates memory and synaptic plasticity through Src-mediated and citron-N-mediated actin reorganization.* J Neurosci, 2014. **34**(4): p. 1542-53.

[41] Alfieri, A., et al., *Synaptic Interactome Mining Reveals p140Cap as a New Hub for PSD Proteins Involved in Psychiatric and Neurological Disorders.* Front Mol Neurosci, 2017. **10**: p. 212.

[42] Blanchoin, L., T.D. Pollard, and R.D. Mullins, *Interactions of ADF/cofilin, Arp2/3 complex, capping protein and profilin in remodeling of branched actin filament networks.* Curr Biol, 2000. **10**(20): p. 1273-82.

[43] Grasso, S., et al., *The SRCIN1/p140Cap adaptor protein negatively regulates the aggressiveness of neuroblastoma.* Cell Death Differ, 2020. **27**(2): p. 790-807.

[44] Harenza, J.L., et al., *Transcriptomic profiling of 39 commonly-used neuroblastoma cell lines.* Sci Data, 2017. **4**: p. 170033.

[45] Gross, N., et al., *New anti-GD2 monoclonal antibodies produced from gamma-interferon-treated neuroblastoma cells.* Int J Cancer, 1989. **43**(4): p. 665-71.

[46] Fernald, K. and M. Kurokawa, *Evading apoptosis in cancer.* Trends Cell Biol, 2013. **23**(12): p. 620-33.

[47] Kamili, A., et al., *Mouse models of high-risk neuroblastoma.* Cancer Metastasis Rev, 2020. **39**(1): p. 261-274.

[48] Corallo, D., et al., *The zebrafish as a model for studying neuroblastoma.* Cancer Cell Int, 2016. **16**: p. 82.

[49] Ribatti, D. and R. Tamma, *The chick embryo chorioallantoic membrane as an in vivo experimental model to study human neuroblastoma.* J Cell Physiol, 2018. **234**(1): p. 152-157.

[50] Shultz, L.D., et al., *Human cancer growth and therapy in immunodeficient mouse models.* Cold Spring Harb Protoc, 2014. **2014**(7): p. 694-708.

[51] Meitar, D., et al., *Tumor angiogenesis correlates with metastatic disease, N-myc amplification, and poor outcome in human neuroblastoma.* J Clin Oncol, 1996. **14**(2): p. 405-14.

[52] Yuan, X.L., et al., *miR-373 promotes neuroblastoma cell proliferation, migration, and invasion by targeting SRCIN1.* Onco Targets Ther, 2019. **12**: p. 4927-4936.

[53] Johnsen, J.I., et al., *Molecular mechanisms and therapeutic targets in neuroblastoma.* Pharmacol Res, 2018. **131**: p. 164-176.

[54] Weiss, W.A., et al., *Targeted expression of MYCN causes neuroblastoma in transgenic mice.* EMBO J, 1997. **16**(11): p. 2985-95.

[55] Heukamp, L.C., et al., *Targeted expression of mutated ALK induces neuroblastoma in transgenic mice.* Sci Transl Med, 2012. **4**(141): p. 141ra91.

[56] Goto, S., et al., *Histopathology (International Neuroblastoma Pathology Classification) and MYCN status in patients with peripheral neuroblastic tumors: a report from the Children's Cancer Group.* Cancer, 2001. **92**(10): p. 2699-708.

[57] Guo, J., et al., *Efficacy, pharmacokinetics, tisssue distribution, and metabolism of the Myc-Max disruptor, 10058-F4 [Z,E]-5-[4-ethylbenzylidine]-2-thioxothiazolidin-4-one, in mice.* Cancer Chemother Pharmacol, 2009. **63**(4): p. 615-25.

[58] Schramm, A. and H. Lode, *MYCN-targeting vaccines and*

immunotherapeutics. Hum Vaccin Immunother, 2016. **12**(9): p. 2257-8.

[59] Trigg, R.M. and S.D. Turner, *ALK in Neuroblastoma: Biological and Therapeutic Implications.* Cancers (Basel), 2018. **10**(4).

[60] Sattu, K., et al., *Phosphoproteomic analysis of anaplastic lymphoma kinase (ALK) downstream signaling pathways identifies signal transducer and activator of transcription 3 as a functional target of activated ALK in neuroblastoma cells.* FEBS J, 2013. **280**(21): p. 5269-82.

[61] Janoueix-Lerosey, I., et al., *Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma.* Nature, 2008. **455**(7215): p. 967-70.

[62] Schwartzseid, E.E., *Ethics as an important determinant of success of orthopaedic dental care for debilitated and elderly patients.* Gerodontology, 1989. **8**(3): p. 83-8.

[63] Tanaka, T., et al., *MEK inhibitors as a novel therapy for neuroblastoma: Their in vitro effects and predicting their efficacy.* J Pediatr Surg, 2016. **51**(12): p. 2074-2079.

[64] Hart, L.S., et al., *Preclinical Therapeutic Synergy of MEK1/2 and CDK4/6 Inhibition in Neuroblastoma.* Clin Cancer Res, 2017. **23**(7): p. 1785-1796.

[65] Fodor Becsky, A., J. Gonzalez Santander, and I. Schneider Keller, *[Statistical study of 1,010 cases of dento-alveolar injuries].* Odontol Chil, 1978. **26**(120): p. 87-91.

[66] Liu, X., et al., *Deregulated Wnt/beta-catenin program in high-risk neuroblastomas without MYCN amplification.* Oncogene, 2008. **27**(10): p. 1478-88.

[67] Tian, X.H., et al., *XAV939, a tankyrase 1 inhibition, promotes cell apoptosis in neuroblastoma cell lines by inhibiting Wnt/beta-catenin signaling pathway.* J Exp Clin Cancer Res, 2013. **32**: p. 100.

[68] Wickstrom, M., et al., *Wnt/beta-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signalling prevents chemoresistance.* Nat Commun, 2015. **6**: p. 8904.

[69] Beierle, E.A., et al., *Inhibition of focal adhesion kinase and src increases detachment and apoptosis in human neuroblastoma cell lines.* Mol Carcinog, 2010. **49**(3): p. 224-34.

[70] Kratimenos, P., et al., *Multi-targeted molecular therapeutic approach in aggressive neuroblastoma: the effect of Focal Adhesion Kinase-Src-Paxillin system.* Expert Opin Ther Targets, 2014. **18**(12): p. 1395-406.

[71] Bjelfman, C., et al., *Expression of the neuronal form of pp60c-src in neuroblastoma in relation to clinical stage and prognosis.* Cancer Res, 1990. **50**(21): p. 6908-14.

[72] Navarra, M., et al., *Antiproliferative and pro-apoptotic effects afforded by novel Src-kinase inhibitors in human neuroblastoma cells.* BMC Cancer, 2010. **10**: p. 602.

[73] Radi, M., et al., *Identification of potent c-Src inhibitors strongly affecting the proliferation of human neuroblastoma cells.* Bioorg Med Chem Lett, 2011. **21**(19): p. 5928-33.

[74] Hishiki, T., et al., *Src kinase family inhibitor PP2 induces aggregation and detachment of neuroblastoma cells and inhibits cell growth in a PI3 kinase/Akt pathway-independent manner.* Pediatr Surg Int, 2011. **27**(2): p. 225-30.

[75] Vitali, R., et al., *Activity of tyrosine kinase inhibitor Dasatinib in neuroblastoma cells in vitro and in orthotopic mouse model.* Int J Cancer, 2009. **125**(11): p. 2547-55.

[76] Foltz, L., et al., *PAG1 directs SRC-family kinase intracellular localization to mediate receptor tyrosine kinase-induced differentiation.* Mol Biol Cell, 2020. **31**(20): p. 2269-2282.

[77] Avalle, L., et al., *STAT3 in cancer: A double edged sword.* Cytokine, 2017. **98**: p. 42-50.

[78] Ara, T., et al., *Critical role of STAT3 in IL-6-mediated drug resistance in human neuroblastoma.* Cancer Res, 2013. **73**(13): p. 3852-64.

[79] Borriello, L., et al., *More than the genes, the tumor microenvironment in neuroblastoma.* Cancer Lett, 2016. **380**(1): p. 304-14.

[80] Rebbaa, A., P.M. Chou, and B.L. Mirkin, *Factors secreted by human neuroblastoma mediated doxorubicin resistance by activating STAT3 and inhibiting apoptosis.* Mol Med, 2001. **7**(6): p. 393-400.

[81] Yogev, O., et al., *In Vivo Modeling of Chemoresistant Neuroblastoma Provides New Insights into Chemorefractory Disease and Metastasis.* Cancer Res, 2019. **79**(20): p. 5382-5393.

[82] Cabodi, S., et al., *Integrin signalling adaptors: not only figurants in the cancer story.* Nat Rev Cancer, 2010. **10**(12): p. 858-70.

[83] Odate, S., et al., *Inhibition of STAT3 with the Generation 2.5 Antisense Oligonucleotide, AZD9150, Decreases Neuroblastoma Tumorigenicity and Increases Chemosensitivity.* Clin Cancer Res, 2017. **23**(7): p. 1771-1784.

[84] Damiano, L., et al., *p140Cap dual regulation of E-cadherin/EGFR cross-talk and Ras signalling in tumour cell scatter and proliferation.* Oncogene, 2010. **29**(25): p. 3677-90.

[85] Almstedt, E., et al., *Integrative discovery of treatments for high-risk neuroblastoma.* Nat Commun, 2020. **11**(1): p. 71.

[86] Baker, D.L., et al., *Outcome after reduced chemotherapy for intermediate-risk neuroblastoma.* N Engl J Med, 2010. **363**(14): p. 1313-23.

[87] Strother, D.R., et al., *Outcome after surgery alone or with restricted use of chemotherapy for patients with low-risk neuroblastoma: results of Children's Oncology Group study P9641.* J Clin Oncol, 2012. **30**(15): p. 1842-8.

[88] Smith, V. and J. Foster, *High-Risk Neuroblastoma Treatment Review.* Children (Basel), 2018. **5**(9).

[89] Turinetto, V. and C. Giachino, *Multiple facets of histone variant H2AX: a DNA double-strand-break marker with several biological functions.* Nucleic Acids Res, 2015. **43**(5): p. 2489-98.

[90] Musumeci, F., et al., *An update on dual Src/Abl inhibitors.* Future Med Chem, 2012. **4**(6): p. 799-822.

[91] Backman, U. and R. Christofferson, *The selective class III/V receptor tyrosine kinase inhibitor SU11657 inhibits tumor growth and angiogenesis in experimental neuroblastomas grown in mice.* Pediatr Res, 2005. **57**(5 Pt 1): p. 690-5.

[92] Chou, T.C., *Drug combination studies and their synergy quantification using the Chou-Talalay method.* Cancer Res, 2010. **70**(2): p. 440-6.

[93] Carmeliet, P. and R.K. Jain, *Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases.* Nat Rev Drug Discov, 2011. **10**(6): p. 417-27.

[94] Seeger, R.C., *Immunology and immunotherapy of neuroblastoma.* Semin Cancer Biol, 2011. **21**(4): p. 229-37.

[95] Maris, J.M., et al., *Neuroblastoma.* Lancet, 2007. **369**(9579): p. 2106-20.