

REVIEW

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A review of the biological and clinical implications of RAS-MAPK pathway alterations in neuroblastoma

Vid Mlakar¹, Edouard Morel¹, Simona Jurkovic Mlakar¹, Marc Ansari^{1,2} and Fabienne Gumy-Pause^{1,2*} 

Abstract

Neuroblastoma is the most common extra-cranial solid tumor in children, representing approximately 8% of all malignant childhood tumors and 15% of pediatric cancer-related deaths. Recent sequencing and transcriptomics studies have demonstrated the RAS-MAPK pathway's contribution to the development and progression of neuroblastoma. This review compiles up-to-date evidence of this pathway's involvement in neuroblastoma. We discuss the RAS-MAPK pathway's general functioning, the clinical implications of its deregulation in neuroblastoma, and current promising therapeutics targeting proteins involved in signaling.

Keywords: Neuroblastoma, RAS-MAPK, ALK, RAS, MEK1/2, ERK1/2, RASopathy, Inhibitors

Background

Neuroblastoma (NB) is the most common extra-cranial solid tumor in children, representing approximately 8% of all malignant childhood tumors and 15% of pediatric cancer-related deaths [1, 2]. Most NB cases are sporadic (98%) and occur in very young children, with 90% of patients being younger than 5 years old at diagnosis and the median age being 19 months old [1–3]. This cancer originates in the sympathetic nervous system, most frequently in the adrenal medulla or the sympathetic nerve chain. Although the primary tumor is typically located in the abdomen, neck, chest, or pelvis, the most frequently observed metastatic locations are regional lymph nodes, bone marrow, bones, and subcutaneous tissues [1–6].

NB is a complex, highly heterogeneous disease with very significant variability in prognosis, ranging from spontaneous regression to highly aggressive and resistant disease despite multimodal therapy. Biologically, NB is not characterized by a high rate of mutations but rather by frequent recurrent chromosomal aberrations, some of which can be used as genetic markers providing prognostic information: whole-genome duplication is usually associated with a good outcome, whereas segmental chromosomal aberrations like *MYCN* amplification (MNA) and 11q deletion are commonly associated with a poor prognosis [6, 7]. Based on multiple prognosis factors, such as age at diagnosis, the stage of the disease, histology, grade of differentiation, *MYCN* status, genomic profile, and ploidy, the International Neuroblastoma Risk Group (INRG) classification system [6, 8] determines four main categories of patients: very-low-risk, low-risk, intermediate-risk, and high-risk NB patients have estimated 5-year event-free survival rates of > 85%, > 75% to < 85%, > 50% to < 75%, and < 50%, respectively [8]. High-risk NBs are the most frequently observed, representing approximately 40% of NBs [8]. Although the majority of high-risk NB patients respond

* Correspondence: fabienne.gumypause@hcuge.ch

¹CANSEARCH Research Platform for Pediatric Oncology and Hematology, Department of Pediatrics, Gynecology and Obstetrics, Faculty of Medicine, University of Geneva, Avenue de la Roseraie 64, 1205 Geneva, Switzerland
²Division of Pediatric Oncology and Hematology, Department of Women, Child and Adolescent, University Hospital of Geneva, Rue Willy-Donzé 6, 1205 Geneva, Switzerland



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positively to an initial intensive multimodal therapy including surgery, high-dose chemotherapy with autologous bone marrow transplantation, radiotherapy, and immunotherapy, half of them will relapse. Relapse usually occurs within 2 years of the end of the initial treatment and is rarely curable. This very poor outcome clearly requires new therapeutics (see the recent review on NB by Matthay et al. [6]).

The *ALK* gene came to prominence in 2008 after its mutation was detected in the majority of cases of familial NB [9, 10]. It was found to be the most common somatically mutated gene in primary NB [11–13], particularly in the high-risk category [9, 14, 15]. Very recently, kinases in the RAS mitogen-activated protein kinase (RAS-MAPK) pathway, one of the downstream signal transduction pathways activated by ALK, were also reported to be frequently mutated in NB. This was especially the case in relapsed samples, in which activating mutations of this pathway were detected in almost 80% of these highly therapy-resistant tumors [16]. These results provide a rationale for using RAS-MAPK pathway alterations as biomarkers for novel targeted treatment approaches for NB.

This review describes the latest knowledge about RAS-MAPK pathway alterations in NB and provides a better understanding of its contribution to tumor development and progression; it also addresses current knowledge about using RAS-MAPK pathway inhibitors in NB treatment.

Methodology

Selection of RAS-MAPK genes and the analysis of genes associated with NB and the RAS-MAPK pathway

QIAGEN's Ingenuity Pathway Analysis software (IPA, QIAGEN, Redwood City, USA) was used to plot the established RAS-MAPK pathway and retrieve the references describing its interactions. The genes associated with NB were extracted from publications by Tolbert et al. [17] and Tonini et al. [18]. Next, IPA software (QIAGEN, Redwood City, USA) was used again to screen for references describing the associations between those genes and the RAS-MAPK pathway (Table 1). Figures 1 and 2 were created using BioRender.com.

Analysis of publicly available data

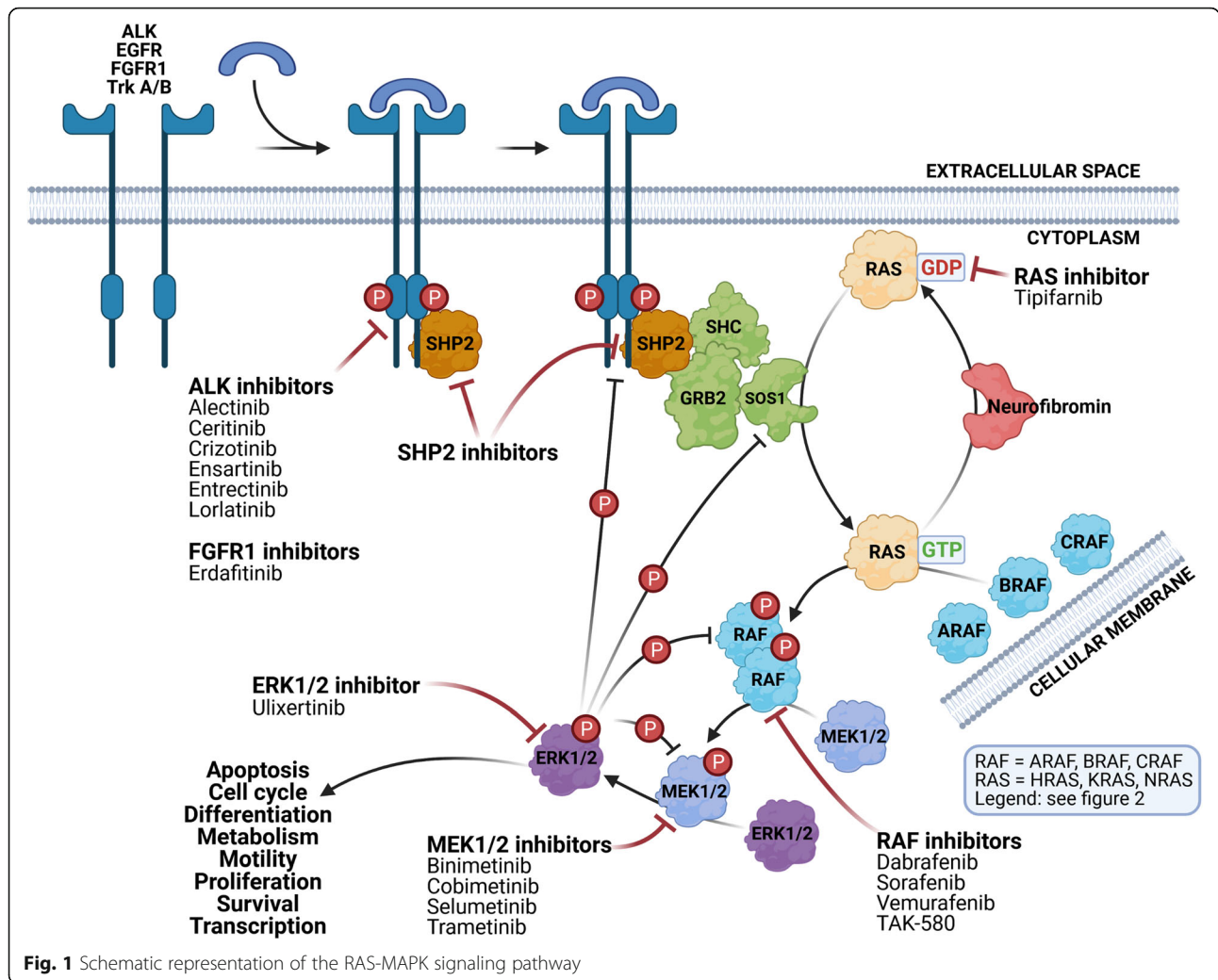
The COSMIC database was used to collate the mutation data on seven different tumor types: blood, brain, breast, colorectal, lung, neuroblastoma, and skin cancers. Data were extracted for thoracic NB and all NB, and then they were pooled by adding the number of analyses and identified mutations.

Analysis of RAS-MAPK inhibitors in clinical trials

The clinicaltrials.gov database was used to screen for all active, recruiting, or completed phase I to III clinical trials using molecules targeting the RAS-MAPK pathway in NB patients (Table 1, Figs. 1 and 2).

Table 1 Frequency of mutations in the RAS-MAPK genes of different adult and childhood cancers (source: COSMIC database, April 6th, 2020)

Level	Gene	Mutations	Neuroblastoma (%)	Brain (%)	Breast (%)	Colorectal (%)	Blood (%)	Lung (%)	Skin (%)
Receptor	<i>ALK</i>	F1174L, R1275Q, R1245V	6.3	0.8	7.3	5.7	5.6	4.4	9.1
Protein adaptor	<i>SHC1</i>	E343D	0.1	0.1	0.6	2.0	0.2	0.7	1.0
	<i>GRB2</i>			0.4	2.2	0.6	1.0	0.3	0.9
	<i>PTPN11</i>	Mutations in SH2 and PTP domains	1.0	1.7	1.3	1.7	4.7	0.9	2.4
SOS	<i>SOS1</i>	N993Sfs*5	0.1	0.7	3.3	3.5	1.3	2.1	3.7
RAS	<i>N-RAS</i>	Q61K/E/L, G13R, A59T	0.7	1.0	0.5	3.7	9.5	0.8	15.0
	<i>K-RAS</i>	G12V		0.8	1.4	32.4	4.9	14.8	2.8
	<i>H-RAS</i>	Q61K	0.1	0.0	0.6	0.9	0.2	0.5	10.6
RAF	<i>BRAF</i>	V600E, F595L, R719P	0.1	4.7	2.1	12.3	8.2	2.2	41.0
	<i>RAF1</i>	L397V	0.1	0.3	1.6	2.3	0.6	1.0	3.1
	<i>ARAF</i>			0.2	0.6	2.0	0.3	1.4	1.9
MEK1/2	<i>MAP2K1</i>	K57N		0.3	1.8	1.9	2.2	0.7	4.7
	<i>MAP2K2</i>	c.920-66G > T	0.1	0.1	0.8	1.3	0.5	0.6	2.1
ERK1/2	<i>MAPK3</i>	E367D	0.1	0.1	0.4	1.0	0.2	0.3	1.4
	<i>MAPK1</i>			0.4	1.6	1.3	1.1	0.3	1.5
NF	<i>NF1</i>	Inactivating nonsense mutations	1.2	7.2	6.1	9.5	3.5	7.2	16.8



The clinical implications of ALK and RAS-MAPK pathway alterations in NB

The implications of ALK and the RAS-MAPK pathway in primary NB tumors

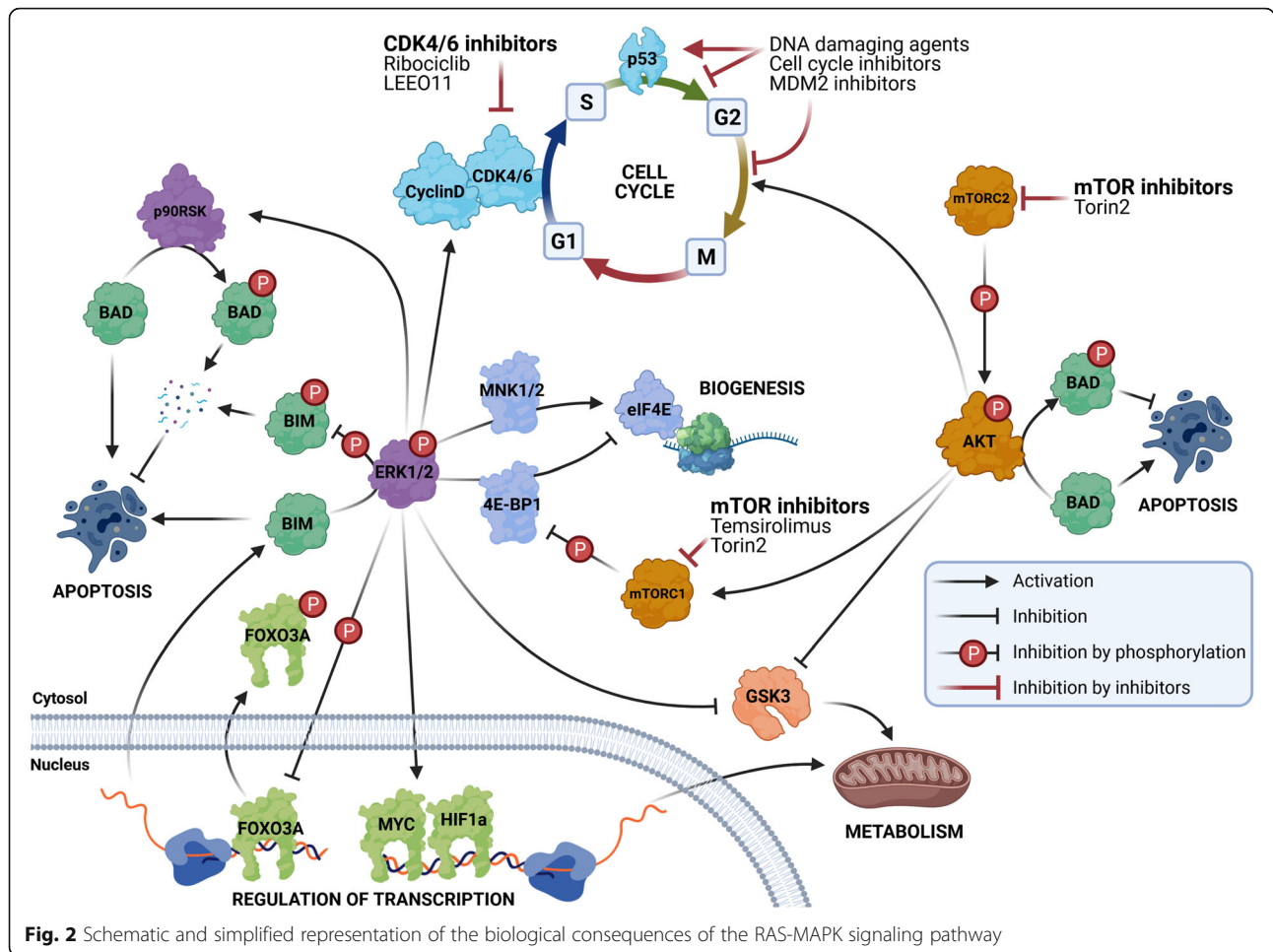
Many different studies have reported *ALK* mutations in approximately 6–10% of sporadic primary NB tumors and around 12–14% of the high-risk category [9–11, 15]. To understand the relative importance of *ALK* in the development of NB we compared the frequency of *ALK* mutations in NB with the frequencies of *ALK* mutations in other tumor types. *ALK* is found to be mutated more frequently in NB than in brain, colorectal, lung, and blood cancers. Only breast and skin cancers have higher *ALK* mutation rates than NB, suggesting that *ALK* mutations play a more significant role in NB development than in other common cancer types (Table 1).

ALK mutations in NB are mainly single amino acid substitutions; in 90% of cases they are located in the kinase domain, with hot spots at amino acids F1174, R1275, and F1245 [9–11, 15]. Interestingly, in

approximately 50% of NB cases, *ALK* mutation is found together with *MYCN* amplification and correlated to poor prognosis [11].

Interestingly, in an additional 2–3% of NB samples, *ALK* was found to be activated by gene amplification, increasing both protein expression and activity [19–21]. *ALK* amplification, which is mutually exclusive of point mutation, is also almost always associated with *MYCN* amplification, with both genes located in the same locus (2p23–24, Table 2) and associated with poor prognosis [11, 13, 15, 22].

Although *ALK* mutations have been observed in all clinical risk groups, a recent study analyzing a large cohort of more than 1500 NB patients demonstrated that the presence of an *ALK* mutation was independently correlated with survival: patients with an *ALK* mutation showed a 1.4-fold greater risk of an event within 5 years than patients without one. Statistically significant correlations were independently observed for all the groups of *ALK* mutations: *ALK* aberration, *ALK* copy number gain,



and *ALK* amplification [15]. For a more general review of *ALK* and NB, we recommend the recent review by Trigg et al. [23].

Different studies have also reported somatic RAS-MAPK pathway gene alterations (mutation, amplification, or deletion) leading to the activation of this pathway in large cohorts of sporadic NB patients, with a frequency of around 4–10% [21, 24, 25]; the most frequently mutated genes were *PTPN11* (2.9%), *NRAS* (0.8%), and *NF1* [14, 24]. Typically, the first mutations observed in *NRAS* were the known activators Q61K and C181A, which were initially discovered in SK-N-SH and SK-N-AS cell lines, respectively [16, 26–28]. Also, *HRAS* and *KRAS* can be mutated, notably by G12D and Q61K substitution, for instance [16, 29].

Most recently, Ackerman et al. sequenced the genomes of 418 pre-treated NB. In line with a previous study, they found that 9% had *ALK* mutations, but also that 6% had alterations in the RAS-MAPK pathway genes (*NF1*: 1.2%; *HRAS*: 1%; *PTPN11*: 1%; *NRAS*: 0.5%; *BRAF*: 0.5% *KRAS*: 0.2%; and *FGFR1*: 0.2%). *ALK*-RAS

pathway alterations were detected in all NB risk categories and were found to be strongly correlated to poor outcomes, even in the high-risk category [25]. Interestingly, patients harboring RAS pathway mutations were found to have a worse prognosis than those with *ALK* mutations. Furthermore, Ackermann et al. also investigated the relationship between *ALK*-RAS alterations and telomere maintenance mechanisms (defined by *MYCN*, *TERT*, and *APB* alterations). Although the presence of that telomere maintenance mechanism was associated with worse outcomes compared to those without those mechanisms, an additional mutation in the *ALK*-RAS pathway increased NB aggressiveness, resulting in a higher mortality rate. In contrast, in NB patients without a telomere maintenance mechanism, the presence of an *ALK*-RAS alteration did not affect patient outcomes [25]. Eleveld et al. were recently able to define an mRNA signature, consisting of six genes (*ETV4*, *ETV5*, *DUSP6*, *MAFF*, *ETV1*, and *DUSP4*) that was correlated with an increase in RAS-MAPK pathway activity. A high expression of these signature genes was associated with poor survival in primary NB but also with *MEK1/2* and

Table 2 Chromosomal locations of the RAS-MAPK pathway genes and most common chromosomal anomalies in NB

Gene	Chromosome location	Most common chromosomal anomalies in NB			
		Nature	Location	References	
<i>ALK</i>	2p23-2p23.1	Loss	1p36.1 - 1p36.3	7553646	
<i>SHC1</i>	1q21.3		1p34	1551108	
<i>GRB2</i>	17q25.1		1pter - 1p32	7519871	
<i>PTPN11</i>	12q24.13		11q13 - 11q23	2068183	
<i>SOS1</i>	2p22.1		14q23-14q32	11464899	
<i>N-RAS</i>	1p13.2		3p25.3 - 3p14.3	9667647	
<i>K-RAS</i>	12p12.1		4p15 - 4p11	20516441	
<i>H-RAS</i>	11p15.5		14q31-14qter	9739017	
<i>BRAF</i>	7q34		Gain	18q11.2 - 18q12.3	9739017
<i>RAF1</i>	3p25.2			2p23-2ter	9739017
<i>ARAF</i>	Xp11.3			17q21-17qter	10379019
<i>MAP2K1</i>	15q22.31			1q22 - 1qter	18923191
<i>MAP2K2</i>	19p13.3			7p11.2 - 7p22	18923191
<i>MAPK3</i>	16p11.2				
<i>MAPK1</i>	22q11.22				
<i>NFI</i>	17q11.2				

References: PMID

Genes in color are located at chromosomal anomalies

ERK1/2 phosphorylation and sensitivity to different MEK1/2 inhibitors in cell lines [30].

Mutations in the genes coding for the RAF family of proteins are very rare in NB. *BRAF* aberrations have been observed in 1% of NB cases, whereas the study by Shukla et al. reported no alterations in *ARAF* and *CRAF* [26]. However, there was a greater expression of all the RAF proteins in the high-risk NB category [31]. The main mutation found in *BRAF* is the V600E substitution [26]. This mutation is located in *BRAF*'s kinase domain and leads to a constitutively active form of *BRAF* [32]. Interestingly, a tandem duplication in the *BRAF* gene was also detected in a relapse NB. This duplication resulted in the expression of a *BRAF* transcript that encodes a protein with two kinase domains. Accordingly, the protein was twice as active, which made the RAS-MAPK signaling pathway more active [16].

A *MEK1/2* mutation is a very rare event in NB, with a frequency of 0.5% [26]. In one NB sample, a *MEK1* somatic activating mutation (K57N) was observed, located between the protein's nuclear export signal and its kinase domain. This genetic lesion leads to constitutive activation of the RAS-MAPK pathway in vitro [33]. On the contrary, no reports of *ERK* mutations have been found in NB.

Comparisons of NB with six other major cancer types showed significant differences in the frequency of mutations in the major RAS-MAPK pathway genes. NB, brain and breast tumors do not demonstrate high mutation frequencies along this pathway (Table 1), while colorectal, blood, lung, and skin cancers have high frequencies in at least one of the RAS-MAPK pathway genes. Interestingly, two genes, *RAF1* (3p25.2) which encodes for the *CRAF* protein, and *GRB2* (2p23-2p23.1), were found to be located in regions that sustain frequent chromosomal abnormalities in NB (Table 2).

Finally, different studies have also reported alterations in genes associated with the RAS-MAPK pathway, such as *CDK4*, *LIN28B*, *CCND1*, *SMO*, *SOS1*, *CIC*, *DMD*, and *DUSP5* [24, 25, 30, 34, 35].

ALK and RAS-MAPK mutations at relapse

Different studies have reported that *ALK* mutations were more frequently detected in NB relapse samples, with a frequency of 15–40% [16, 24, 36]. In 2015, Eleveld et al. analyzed 23 paired diagnostic and relapsed NB samples. They were able to show not only a clonal evolution from diagnosis to relapse sample but also that 78% (18/23) of the relapsed NB samples harbored a genetic aberration predicted to activate the RAS-MAPK pathway (*ALK* (10/

23); *NFI* (2/23); *PTPN11* (1/23); *FGFR1* (1/23); *NRAS* (1/23); *KRAS* (1/23); *HRAS* (1/23); and *BRAF* (1/23) [16]. In line with these results, Padovan-Merhar et al. reported that suspected *ALK* driver mutations were present in 7% (3/43) of samples at diagnosis, 17% (7/41) of the post-treatment samples, and 20% (11/54) of the samples at relapse. Furthermore, they observed more suspected oncogenic *ALK* mutations in relapsed disease than at diagnosis, as well as enrichment of RAS-MAPK pathway mutations at relapse [24]. Also, single-nucleotide variants (SNVs) in RAS proteins are more frequent in relapsed NB, notably, for instance, in *HRAS* with the somatic Q61K mutation [16, 24, 26], resulting in the constitutively active form of RAS due to inactivation of the GTPase domain [37].

Familial NB, sporadic NB germline susceptibility genes, and *RASopathies*

Familial cases of NB are very rare, representing 1–2% of all NB cases, and they are inherited in an autosomal-dominant manner. In recent years, many advances have been made in our genetic comprehension of these cases. *PHOX2B* (4p12), a gene known to be an essential regulator of the development of a normal autonomic nervous system and frequently associated with neurocristopathies, was the first to be identified in this setting in 2004 [38, 39]. However, it was only detected in a small subset of familial NB (approximately 10%). In 2008, two different, independent studies reported *ALK* (2p23) as a gene involved in more than half of familial NB cases. Additional studies finally reported *ALK* germline mutations in almost 80% of these families [15, 17, 40]. The majority of these mutations are located in the *ALK* tyrosine kinase domain, resulting in constitutive activation of the kinase [9, 10]. The penetrance is variable, highly related to the activating effect of the mutation [15], but estimated at around 50% overall [41]. As yet, no other genes have been identified to explain the 10% of familial cases without the *PHOX2B* or *ALK* germline mutations.

Interestingly, NB susceptibility genes were recently identified in the germline DNA of a patient with sporadic NB, suggesting that common germline polymorphisms with low penetrance (i.e., *NBAT-1*, *CASC15*, *BARD1*, *LMO1*, *HSD17B12*, *DUSP12*, *LIN28B*, *HACE1*, *SPAG16*, *NEFL*, *MLF1/RSRC1*, *CPZ*, *CDKN1B*, *SLC16A1*, *MSX1*, *MMP20*, *KIF15*) or more rare variants with higher penetrance (i.e., *ALK*, *BARD1*, *CHEK2*, *AXIN2*, *TP53*, *APC*, *BRCA2*, *SDHB*, *SMARCA4*, *LZTR1*, *BRCA1*) may also have a relevant role in NB carcinogenesis [17, 18]. Remarkably, when investigating the associations between these genes and the RAS-MAPK pathway, many of them were reported to interact with this pathway either in NB or other cancers (Table 3). This further supports the

view that the RAS-MAPK pathway is one of the central targets in the process of tumorigenesis.

Additionally, NB has been reported to be associated with diseases well-known to predispose cancer, such as Li Fraumeni syndrome (*TP53* mutations) [42], familial paraganglioma/pheochromocytoma (*SDHB* mutations) [43], and Beckwith–Wiedemann syndrome (*CDKN1C* mutations or loss of expression) [44]. It has also been associated with other rare syndromic diseases, more particularly with those characterized by the presence of germline mutations located along the RAS-MAPK pathway. This group of diseases, also known as *RASopathies*, includes neurofibromatosis 1 (*NFI* mutations), Costello syndrome (*HRAS* mutations), Noonan syndrome, and such Noonan-like syndromes as Noonan syndrome-like disorder with loose anagen hair and the LEOPARD syndrome (mutations in *PTPN11*, *SOS1*, *KRAS*, *NRAS*, *RAF1*, *BRAF*, *MEK1*, *SHOC2*, *MEK2*, *RIT1*, and *CBL*) [41, 45–47].

Biology of the RAS-MAPK pathway

The RAS-MAPK pathway's importance is not only underpinned by its clinical relevance as displayed through its frequent dysregulation in cancerous cells. The pathway is firmly embedded in normal cell function as one of the central signaling axes that affect many different cellular functions (Figs. 1 and 2).

For example, the RAS-MAPK's ERK1/2 end kinase is known to regulate the transcription of several hundred genes through its activation of a vast array of transcription factors. Through ERK1/2 regulation of transcription, RAS-MAPK regulates the progression of cells from the G1 to the S phase by activating expression of Cyclin D [48, 49]. Interestingly, Cyclin D1 is also a well-established NB oncogene that is associated with 11q13.3 gains [50]. Another prominent example of the importance of transcription regulation is the inhibition of apoptosis through ERK1/2-mediated repression of BIM, which is one of the most potent members of the BH3-only proteins responsible for the inhibition of antiapoptotic proteins such as BCL-2, BCL-xL, MCL1, BCL-W, and A1 [51]. Inhibition of BIM happens not only through direct phosphorylation but also through the inhibition of transcription by ERK1/2 phosphorylation of the FOXO3A transcription factor [52]. Phosphorylation of FOXO3A promotes its expulsion from nucleus and subsequent proteasomal degradation [52]. Metabolic switching, increased production of lactate, and a decrease in oxidative phosphorylation are other consequences of an activated RAS-MAPK pathway [53, 54]. The key targets responsible for metabolic adaptation are the phosphorylation of MYC and HIF1 α [55–57], which are known to regulate the transcription of genes involved in glucose metabolism and respiration [53, 58].

Table 3 Interactions of the RAS-MAPK pathway with NB associated genes

	Candidate genes	Mutation	Location	RAS-MAPK involvement	References	
Low penetrance genes	CASC14/NBAT-1	Loss of function	6p22	MAPK1/3 inhibition	[1, 2]	
	CASC15		6p22			
	BARD1	Gain of function	2q35	MAPK1 ^a	[3–6]	
	LMO1	Gain of function	11p15.4	MAPK1/3 activation	[7, 8]	
	HSD17B12		11p11.2			
	DUSP12	NA	1q23.3	MAPK1, HRAS inhibition	[9–12]	
	LIN28B	Gain of function	6q16	MAPK1/3 activation	[13, 14]	
	HACE1	Loss of function	6q16	MAPK1/3 inhibition	[13, 15, 16]	
	SPAG16		2q34			
	NEFL		8p21			
	MLF1/RSRC1	NA	3q25	ARAF ^a	[17]	
	CPZ		4p16			
	CDKN1B	Inhibited by GRB2 through p27Kip1		12p13.1	MAPK1 GRB2	[16, 18–22]
	SLC16A1	NA		1p13.2	KRAS ^a NRAS ^a HRAS ^a	[23–27]
High penetrance genes	MSX1	Gain of function	4p16.2	ARAF activation	[28, 29]	
	MMP20	NA	11q22.2	Activation of pathway	[30]	
	KIF15	NA	3p21.31	Activation of pathway	[31–33]	
	CHEK2	Loss of function	22q12.1	MAPK1/3 inhibition	[5, 34, 35]	
	AXIN2		17q24.1			
	BRCA2	Loss of function	13q13.1	GRB2	[36–39]	
	SDHB		1p36.13			
	SMARCA4	Loss of function	19p13.2	NF1 ^a MAPK1 ^a	[40–42]	
	LZTR1	Loss of function	22q11.21	NF1 NRAS inhibition	[23, 25, 26, 43, 44]	
	BRCA1	Loss of function	17q21.31	NF1 MAPK3 MAPK1	[45–48]	
	NBPF23		1q21.1			
	SEZ6L2/PRRT2		16p11.2			
	APC	Loss of function	5q22.2	MAP2K1 inhibition	[39, 49]	
	TP53	Loss of function	17p13.1	MAPK1 HRAS GRB2 KRAS BRAF SHC1 PTPN11 MAP2K1 MAP2K2] ARAF	[50–83]	

For references 1 to 83 in this table, please see Supplementary Material list 1

NA Information not available

^aPutative interaction with RAS-MAPK pathway modeled by IPA

Recently, it has been demonstrated that ERK1/2 is involved in cell differentiation through the transcriptional repression of key pluripotency transcription factors, such as NANOG, KLF2, and KLF4 [59], and through crosstalk with LIF and BMP signaling through STAT3 [60, 61].

The regulation of gene expression, however, is exerted not only at the level of transcription but also at the level of translation. ERK1/2, for example, is known to interact with MNK1/2 [62], which in turn activates eIF4E, a known activator of translation [63]. Interestingly, this process is regulated by a feedback loop, through MSK1/2, which activates an eIF4E inhibitor, 4E-BP1 [64].

Although the regulation of transcription is the major target of RAS-MAPK pathway signaling, RAS-MAPK also regulates cellular functions through the direct phosphorylation of its targets. ERK1/2 is also known to inhibit apoptosis by direct phosphorylation of the proapoptotic factor BIM, mentioned above [65]. Through ERK1/2, RAS-MAPK is also involved in the response to oxidative stress and, together with phosphorylated Hsp27, it is necessary for the proteasome degradation of BIM and the inhibition of apoptosis [66]. p90RSK, which controls apoptosis activation through interaction with BAD, is another important modulator of apoptosis that is a target of ERK1/2 [67]. Finally, cellular mobility is also directly affected by ERK1/2, which is known to regulate the rate and polarity of actin polymerization [68, 69] and to promote the turnover of focal adhesion [70–72]. For more information on RAS-MAPK signaling's effects on cellular biology, we suggest a recent review by Lavoie et al. [64].

ALK and other RTKs involved in RAS-MAPK activation

The RAS-MAPK pathway is usually activated by receptor tyrosine kinase (RTK) (Fig. 1). As discussed above, ALK is the most studied RTK in NB. However, other RTKs are known to activate the RAS-MAPK pathway and have sometimes been found to be mutated in relapsed NB. These include FGFR1 (fibroblast growth factor receptor 1), EGFR (epidermal growth factor receptor) [24], and Trk A and B [73]. The main aberration found in FGFR1 [50] is the somatic N546K mutation [16], which is located in the kinase domain and alters auto-phosphorylation, leading to an increase in its kinase activity [74]. Because the present review focuses on the RAS-MAPK pathway, we refer the reader to other excellent general reviews of RTKs [75] and their involvement in NB [23, 76].

RAS activation and RAS associated proteins

Activated RTKs' first targets are the three RAS proteins—KRAS, NRAS, and HRAS (Fig. 1). The activation is a complex sequence of events, involving the ultimately

inactive RAS being converted into its active form by the addition of GTP. At least three adaptor proteins—SHP2 (PTPase encoded by *PTPN11*), Shc, and Grb2—are required for the first step in the signalization reaction [77]. This complex forms after RTK's phosphorylation by the recruitment of PTPases which contain the SH2 motifs responsible for specific binding between PTPase and RTK. Next, PTPase becomes tyrosine-phosphorylated, which induces a significant change in its conformation and increases phosphatase activity [78, 79]. The change in conformation appears to be essential for the recruitment of the other two factors, Shc and Grb2 [80]. Nevertheless, SHP2 also appears to serve other functions, as some studies suggest that it dephosphorylates STAT3, an important transcription factor activated by terminal ERK1/2 kinase. After being recruited, Shc is activated by phosphorylation at Tyr317 [81]. Adaptor proteins are assembled on RTKs' intracellular sites, and many known mutations lead to the constitutive association of both Shc and Grb2 proteins with RTK [77]. As already mentioned, mutations in the *PTPN11* gene are found in 3% of high-risk NBs [82]. The major mutation is the heterozygous A72T substitution located in the first SH2 domain of SHP2, converting SHP2 into a constitutively active phosphatase. After the assembly of Shc and Grb2 adaptor proteins, Sos1 protein is recruited to catalyze the addition of GTP to RAS [83]. Sos1 is a mammalian nucleotide exchange factor that, once recruited to the Shc/Grb2 complex by translocation to the plasma membrane, activates RAS through the exchange of GDP for GTP [84].

One of the most important aspects of RAS phosphorylation is its location. It has been demonstrated that the entire complex's location with respect to cellular membranes facilitates the rate of exchange. The diffusion of proteins was shown to be insufficient for the reaction to happen [85]. Active transport is necessary for the subsequent transmission of the signal towards its targets. Although the sequences of all three RAS proteins are almost identical, they are located at different cellular compartments. KRAS is more abundant in cytoplasmic membranes, whereas HRAS is more common in intracellular vesicular membranes. These differences are presumably due to differential processing in the Golgi apparatus. Nevertheless, it has been demonstrated that Grb2a associates with both KRAS and HRAS, irrespective of their location [86].

In a physiologically normal state, cells have several means of controlling the activation and deactivation of RAS proteins. The first negative regulation to be described was the activation of the hydrolysis of RAS-bound GTP to GDP by p120GAP, neurofibromin, and GAP1. Neurofibromin, coded by the *NFI* gene, exerts its inhibitory action by stimulating the hydrolysis of RAS-

GTP [87]. *NF1* seems particularly important in the development of NB as *NF1* aberrations have been found in primary NB and at an even higher frequency in relapsed NB samples [24, 88]. The *NF1* mutations observed in primary NB are typically SNVs, promoting the loss of neurofibromin's activity. In relapsed tumors, however, we mostly observe *NF1* deletion. Depending on the NB cell lines, we observe either homozygous deletion of various sizes or heterozygous deletion with a splice site mutation in one of the alleles [16, 88]. In all of these situations, loss of neurofibromin results in the constitutively active form of RAS and thus over-signalization of the RAS-MAPK pathway.

The second negative regulation to be described was the feedback loop through the phosphorylation of Sos1 and the subsequent inactivation of RTK/Shc/Grb2/Sos1 by ERK1. The phosphorylation of Sos1 does not activate the dissociation of Sos1 from Grb2 but rather the dissociation of the entire Grb2/Sos1 complex from phosphorylated RTK [89]. It is currently unknown how much the deregulation of this feedback contributes to the activation of the RAS-MAPK pathway and to the development of NB or other cancer types.

RAF activation

In normal cells, RAF proteins (ARAF, BRAF, and CRAF) represent the next step in the signaling cascade (Fig. 1). Although *BRAF* is one of the most frequently mutated genes in many cancers, it is interesting that BRAF does not directly interact with activated RAS. Signal transduction to the BRAF protein is accomplished by the heterodimerization between CRAF and BRAF that is dependent on the presence of RAS. That heterodimerization is also dependent on the phosphorylation of BRAF at the serine 621 and of the CRAF's C terminus, whereas CRAF's N terminus is not important for this interaction. On the other hand, CRAF's association with RAS is independent of RAS activity as assessed by the dominant-negative form of RAS (G12V) or by the truncated RAS mutant lacking the COOH-terminal serine residues necessary for membrane localization [90, 91]. Interestingly, MEK1/2 phosphorylation can be achieved by CRAF alone, but it is significantly stronger in the presence of the BRAF/CRAF heterodimer. In cells, this complex is located at the membrane, which is to be expected as RAS is a membrane-bound protein. Nevertheless, the evidence suggests that membrane localization is not responsible for the dimerization of BRAF/CRAF. 14-3-3 is an additional protein necessary for the interaction between BRAF and CRAF. It is responsible for maintaining the complex structure and RAS-dependent RAF activation through the binding of CRAF's S621 amino acid residue [92–94]. Although the three RAF isoforms are very similar, their ability to activate

downstream targets is markedly different. As discussed above, the heterodimerization of different RAF isoforms is important to their activity, with the most sensitive being CRAF's dependence on the presence of BRAF. Likewise, BRAF's activity is significantly lower in the absence of CRAF. ARAF, however, the least active isoform, is also the most dependent on BRAF but is independent of CRAF. It is of note that BRAF is capable of spontaneous homodimerization but that CRAF and ARAF are not. Mutations in *BRAF* and *CRAF* play significant roles by increasing homo and heterodimerization and catalytic activity [95]. Although ARAF is the least active isoform of RAF, it is very significantly activated by the oncogenic Src protein [96].

MEK1/2 and ERK1/2 regulation

The RAF protein kinase family is the major activator of the mitogen-activated protein kinases, MEK1 and MEK2 (MEK1/2) (Fig. 1). CRAF activates both MEK1 and MEK2, whereas ARAF and BRAF predominantly activate MEK1 [97]. To be fully active, MEK1/2 must be phosphorylated on two serine residues, at positions 218 and 222, located in the activation loop. Although RAF proteins are the predominant activators, MEK1/2 can also be phosphorylated at the same residues by MEK Kinase (MEKK), c-Mos, and growth factor-stimulated MEK1/2 activator [98–100]. Once activated, MEK1/2 can, in turn, activate its main substrates—ERK1 and ERK2—by phosphorylation. The ERK family is composed of several isoforms, from ERK1 to ERK8, but only ERK1 and ERK2 (also known as the p44 and p42 kinases, respectively) are involved in the RAS/MAPK signaling pathway. To learn more about other isoforms, see Bogoyevitch et al. [101]. In contrast to the activation of MEK1/2, no other ERK1/2 activators are currently known. MEK1/2 phosphorylates ERK1/2 at threonine 183 and tyrosine 185 in the Thr-Glu-Tyr motif [102]. ERK1/2 activation is facilitated by its spatial proximity to MEK1/2 within a protein complex including KSR and MP-1 [103]. The enzymatic reaction is enhanced by electrostatic interactions between the basic amino acids (lysine) present in the ERK2-docking site in the N-terminal region of MEK1 and the acidic amino acids (aspartate) located in the MEK1-docking site in the C-terminal portion of ERK2 [104, 105]. The specificity of the RAF-MEK1/2-ERK1/2 reactions is reinforced by the fact that MAPKs are proline-directed kinases, meaning that they strongly recognize their specific phosphorylation sites through the proline at position +1 of them. This proline is required for efficient activation of MEK1/2 from RAF and of ERK1/2 from MEK1/2 [97, 106].

Upon activation, ERK dimerizes to form either ERK1 or ERK2 homodimers, although the functional implication of this dimerization remains unclear. Khokhlatchev

et al. showed that this dimerization is involved in nuclear translocation, whereas most recently, Casar et al. demonstrated it to be essential for the activation of cytoplasmic targets [107, 108]. Via phosphorylation, active-ERK1/2 regulates a large number of cytoplasmic and nuclear targets which mainly regulate cell proliferation, differentiation, survival, and apoptosis (Figs. 1 and 2). Many of those targets contain a D domain and a DEF motif that improves the efficacy and specificity of the interaction with ERK1/2's CD/CRS (common docking motif/cytosolic retention sequence) domain [109]. In 2017, based on various phospho-proteomic analyses, Únal et al. reviewed 2507 ERK1/2-targets, of which 659 were direct and 1848 were indirect targets [110]. Direct targets are mainly involved in cell-cycle regulation and cell-signaling, for example, the well-known ERK1/2 targets, the Ets family of transcription factors—ELK-1, ELK-4, Ets-2—but also MYC, phospholipase A2, p90^{RSK}, paxillin, and calnexin [67, 111–113]. Under stress conditions, ERK1/2 can become involved in cell death by phosphorylating p53 and thus inducing apoptosis [114]. However, ERK1/2 is also available for the activation of substrates in a phosphorylation-independent way through protein–protein interaction, such as with topoisomerase II alpha [115].

Importantly, the signal duration must be finely regulated to avoid the loss of cell control which can promote the development of diseases such as cancer. Negative feedback and phosphatase activity regulate the signal induced by the ERK1/2 / MAPK pathway. ERK1/2 can rapidly inhibit the pathway's main components (e.g., MEK1/2, RAF, Sos1, RTK, KSR) through phosphorylation and regulate the expression of phosphatases such as dual-specificity MAPK phosphatase (MKPs) and Sprouty proteins, both of which inhibit the pathway. To learn more about this negative regulation, see the review by Lake et al. [116].

RAS-MAPK inhibitors in clinics

There is thus strong evidence that the RAS-MAPK pathway plays an important role in the early development of NB, and this provides a rationale for the use of small-molecule inhibitors that can target the activated ALK and/or RAS-MAPK pathways. Many ongoing clinical trials testing the efficacy of RAS-MAPK pathway inhibitors against NB further underpin its importance. They are summarized in the Table 4 and visualized in Figs. 1 and 2.

The first phase I-II clinical trial testing an ALK inhibitor for NB was initiated more than 10 years ago; it evaluated crizotinib, which was first developed as a MET inhibitor [117]. Unfortunately, it was rapidly demonstrated that crizotinib's ability to inhibit ALK activation was not constant and it was dependent on the type of

ALK mutation and the crizotinib dose. Investigators also observed the emergence of resistance to this compound, leading to the development of new generations of ALK inhibitors such as alectinib, ceritinib, and lorlatinib. Table 5 summarizes the available data on how well ALK inhibitors inhibit different mutated ALKs. As they have tried to circumvent the emergence of resistance, due to the occurrence of either ALK mutations or downstream mutations in *PTPN11* and *NRAS*, researchers have focused on the downstream targets of the RAS-MAPK pathway.

Tipifarnib is the only RAS inhibitor currently being tested in clinical trials for NB. It is a general inhibitor of RAS proteins, through the inhibition of the RAS farnesylation that prevents RAS localization to cellular membranes, thereby inhibiting signaling [122]. Activated RAS' first downstream target is BRAF. Kakodkar et al. demonstrated that BRAF inhibition using Sorafenib inhibited NB growth in vivo, attenuated ERK signaling, and arrested the cell cycle at the G1/G0 stage [123]. Interestingly, Sorafenib showed a transient anti-tumor effect and short temporal stabilization in four NB patients treated with it on a companionate use basis [124]; it is currently being investigated in five early-stage clinical trials. BRAF is also a target of TAK-580, a pan-RAF inhibitor, presently being tested in a phase I study.

More recently, MEK1/2 inhibitors such as trametinib, cobimetinib, binimetinib, and selumetinib were shown to inhibit the growth of different NB cell lines harboring RAS-MAPK mutations [125–127]. Interestingly, sensitivity to MEK1/2 inhibition was found to be correlated with the disruption of specific genes. Cell lines with *RAS/BRAF* mutations were found to be more sensitive than cells with *NFI* deletion, whereas cells with *ALK* mutations, which consistently showed some sensitivity to MEK1/2 inhibitors, were the least susceptible to MEK1/2 inhibitors [16]. Interestingly some studies even reported that MEK1/2 inhibition was not always effective against NB cell lines [125–127], particularly cell lines presenting *ALK* addiction. In these cell lines, trametinib was found to increase the activation of AKT and ERK5 kinase, leading to increased cell proliferation and survival [125].

Different studies have reported that the sensitivity of NB cells to MEK1/2 inhibitors was positively correlated with their endogenous levels of phosphorylated ERK1/2 (pERK1/2) [126, 128]. In response to binimetinib, sensitive cells demonstrated a complete reduction of pERK1/2, whereas the resistant ones demonstrated incomplete reduction or retention of pERK1/2. Furthermore, basal NF1 protein expression was also found to be correlated with binimetinib response, with reduced expression being associated with higher sensitivity to MEK inhibition.

Table 4 Active, recruiting, and completed phase I to III clinical trials for NB

Targeted protein	Inhibitor	Clinical Trial #	Phase	Combination
ALK	Crizotinib	NCT00939770	I/II	None
		NCT03126916	III	Carboplatin / Cisplatin / Cyclophosphamide / Dinutuximab / Doxorubicin / Etoposide / Isotretinoin / Thiotepa / Topotecan / Radiation Therapy
		NCT01606878	I	Cyclophosphamide / Topotecan or Doxorubicin / Vincristine
		NCT03194893	III	None
		NCT01121588	I	None
		NTR5584	I	Temsirolimus
	Lorlatinib	NCT03107988	I	Cyclophosphamide / Topotecan
	Ceritinib	NCT02559778	II	DFMO
		NCT02780128	I	Ribociclib (CDK4/CDK6 inhibitor)
		NCT01742286	I	None
	Entrectinib	NCT02650401 ^a	I/II	None
	Ensartinib	NCT03155620	II	None
		NCT03213652	II	None
	Alectinib	NCT03194893	III	None
	FGFR	Erdafitinib	NCT03210714	II
NCT03155620			II	None
RAS	Tipifarnib	NCT03155620	II	None
		NCT04284774	II	None
BRAF	Dabrafenib	NCT02124772	I/II	Trametinib
		Sorafenib	NCT02559778	II
		NCT01518413	I	Irinotecan
		NCT01683149	I	Topotecan
		NCT00665990	I	Bevacizumab / Cyclophosphamide
		NCT02298348	I	Topotecan / Cyclophosphamide
	Vemurafenib	NCT03220035	II	None
		NCT01596140	I	Everolimus / Temsirolimus
		NCT03155620	II	None
	TAK-580 ^b	NCT03429803	I	None
MEK 1/2	Trametinib	NCT03434262	I	Ribociclib
		NCT02124772	I/II	Dabrafenib
	Selumetinib	NCT03155620	II	None
		NCT03213691	II	None
	Cobimetinib	NCT02639546	I/II	None
Binimetinib	NCT02285439	I/II	None	
ERK 1/2	Ulixertinib	NCT03698994	II	None
		NCT03155620	II	None

^aDoebele RC, Drilon A, Paz-Ares L, Siena S, Shaw AT, Farago AF, Blakely CM, Seto T, Cho BC, Tosi D et al. Entrectinib in patients with advanced or metastatic NTRK fusion-positive solid tumours: integrated analysis of three phase 1–2 trials. *Lancet Oncol.* 2020; 21(2): 271–282

^bpan-RAF inhibitor

These results suggest that the expression of pERK1/2 and NF1 could be used as biomarkers helping to identify a patient who may benefit from treatment with binimetinib [126]. Unfortunately, recent results suggest that long-term exposure to MAPK pathway inhibition could

result in acquired resistance, by relieving the ERK1/2-dependent feedback inhibition of RAF and the reactivation of the MAPK pathway [128, 129].

To overcome the development of resistance through the occurrence of downstream activation mutations, the

Table 5 ALK mutations and their impact on the efficiency of ALK inhibitors

ALK mutation	Poorly effective	Effective	Highly effective	Reference
G1123S	Ceritinib			[118]
G1128A		Crizotinib, Ceritinib	Brigatinib, Lorlatinib, Alectinib	[119, 120]
L1151Tins	Crizotinib			[118]
L1152R	Crizotinib			[118]
C1156Y	Crizotinib			[118]
I1171N	Crizotinib	Ceritinib	Brigatinib, Lorlatinib, Alectinib	[118–120]
I1171T	Crizotinib, Alectinib	Ceritinib	Brigatinib, Lorlatinib, Alectinib	[119, 120]
F1174C	Ceritinib			[121]
F1174L	Crizotinib (partial inhibition)	Ceritinib	Brigatinib, Lorlatinib, Alectinib	[118–121]
F1174V	Crizotinib, Ceritinib			[118]
R1192P		Crizotinib, Ceritinib	Brigatinib, Lorlatinib, Alectinib	[119, 120]
L1196M	Crizotinib			[118]
L1198F	Lorlatinib			[118]
G1202R	Crizotinib, Ceritinib, Alectinib			[118]
S1206Y	Crizotinib			[118]
F1245V		Crizotinib, Ceritinib	Brigatinib, Lorlatinib	[119, 121]
F1245C	Crizotinib		Alectinib	[118, 120]
G1269A	Crizotinib	Ceritinib	Brigatinib, Lorlatinib, Alectinib	[118–120]
R1275Q		Crizotinib, Ceritinib	Brigatinib, Lorlatinib, Alectinib	[117, 119, 120]
Y1278S		Crizotinib, Ceritinib	Brigatinib, Lorlatinib, Alectinib	[119, 120]

simultaneous inhibition of the RAS-MAPK pathway at several points has been proposed for NB. Valencia-Sama et al. investigated combinations of SHP2 inhibitors and inhibitors of RAF, MEK1/2, and ERK1/2. These researchers demonstrated the synergistic effects in vitro and in vivo of combining SHP2 with BRAF (vemurafenib), MEK1/2 (trametinib), or ERK1/2 (ulixertinib) inhibition in RAS-activating tumors (NRAS^{Q61K}) that were resistant to SHP2 inhibitors alone [130]. Concurrent inhibition using MEK1/2 (trametinib) and BRAF (dabrafenib) is currently being evaluated in a phase I/II clinical trial.

The RAS-MAPK pathway regulates many different cellular processes, as well as crosstalk with the PI3K/AKT/mTOR pathway (Fig. 2). It has been proposed that inhibition of the RAS-MAPK pathway should be supplemented with inhibition of other cellular functions, either to improve the efficacy of RAS-MAPK inhibitors or to help avoid the development of resistance.

PI3K/AKT/mTOR is one of the main signaling pathways that is frequently dysregulated in many different cancers, including NB [131]. This pathway shares many upstream activators and downstream targets with the RAS-MAPK pathway, and the crosstalk between them first attracted the attention of researchers in the early 1990s. It was demonstrated that pathways could interact either through mutual inhibition or activation and that

they were instrumental in the progressive adaptations to activating RAS-MAPK mutations, which cause significant oncogenic stress [131]. In addition, many well documented examples have demonstrated the importance of both pathways in the development of resistance to inhibitors of either pathway [132]. The simultaneous inhibition of RAS-MAPK and PI3K/AKT/mTOR in in vitro and in vivo NB models has demonstrated the synergistic effects between crizotinib and Torin2 (mTOR inhibitor) [133, 134] and has recently resulted in the opening of an early stage clinical trial to test the toxicity of a combined ALK and PI3K/AKT/mTOR inhibitors treatment.

As discussed above, Cyclin D, which is regulated by ERK1/2, is an important oncogene that promotes transition from the G1 phase to the S phase of the cell cycle. A preclinical study of Cyclin D inhibition, using a CDK4/6 inhibitor (LEE011) on a panel of NB cell lines, demonstrated its potential for application against NB [135]. In a recent study, Wood et al. demonstrated a synergy between ceritinib and ribociclib, another inhibitor of CDK4/6 [136]. Later, Hart et al. obtained similar results using the MEK1/2 inhibitors binimetinib and ribociclib [125, 127]. The NEPENTHE study is currently assessing the feasibility of using ceritinib and ribociclib in combination for the treatment of NB.

Other interesting combinations have included inhibition of ALK and MDM2, which demonstrated that it

was able to overcome the resistance to ceritinib conferred by MYCN upregulation [137]. Inhibition of YAP1 (hippo signaling) resulted in RAS-driven NB cells being sensitized to trametinib MEK1/2 and in enhanced G1/S cell-cycle arrest [138]. Regorafenib, which is known to inhibit the RAS/MAPK, PI3K/Akt/mTOR, and Fos/Jun pathways, has also shown good in vivo and in vitro efficacy against NB cells [139].

Conclusions

Even though NB is very well-known as a cancer with a low frequency of point mutations, the RAS-MAPK pathway shows a high degree of dysregulation. Since mutations in genes related to the RAS-MAPK pathway could lead to its dysregulation, looking at the frequency of directly activated mutations probably underestimates the actual frequency of dysfunctional RAS-MAPK in NB. Although this pathway seems to offer many targets for inhibition, many years of clinical investigation have proved that inhibition is a challenging task due to the frequent development of resistance, complex feedback-loop regulation, and a large number of interactions with other signaling pathways that can affect the functioning of the RAS-MAPK pathway. This last aspect is a particularly important task, and greater efforts should be invested in understanding how the RAS-MAPK pathway functions in the molecular background of NB cells. Current evidence suggests that inhibition of the RAS-MAPK pathway alone will not be sufficient to adequately target NB. Rather, it is becoming apparent that the inhibition of other pathways, such as PI3K/AKT/mTOR, or processes like the cell cycle, will be vital to achieving effective treatments for NB and avoiding the development of resistance.

Abbreviations

CD: Common docking; COSMIC: Catalogue of somatic mutations in cancer; CRS: Cytosolic retention sequence; INRG: International Neuroblastoma Risk Group; IPA: Ingenuity Pathway Analysis software; MNA: MYCN amplification; NB: Neuroblastoma; RTK: Receptor tyrosine kinase; SNV: Single nucleotide variants

Supplementary Information

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Additional file 1.

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Authors' contributions

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