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REVIEW

The ALK receptor in sympathetic neuron development and neuroblastoma

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Abstract

The *ALK* gene encodes a tyrosine kinase receptor characterized by an expression pattern mainly restricted to the developing central and peripheral nervous systems. In 2008, the discovery of ALK activating mutations in neuroblastoma, a tumor of the sympathetic nervous system, represented a breakthrough in the understanding of the pathogenesis of this pediatric cancer and established mutated ALK as a tractable therapeutic target for precision medicine. Subsequent studies addressed the identity of ALK ligands, as well as its physiological function in the sympathoadrenal lineage, its role in neuroblastoma development and the signaling pathways triggered by mutated ALK. This review focuses on these different aspects of the ALK biology and summarizes the various therapeutic strategies relying on ALK inhibition in neuroblastoma, either as monotherapies or combinatory treatments.

Keywords ALK · Sympathetic ganglia · Neuroblastoma · Signaling · Targeted therapy

Introduction

The *ALK* (anaplastic lymphoma kinase) gene encodes a tyrosine kinase receptor that, together with the closely related leukocyte tyrosine kinase (LTK) receptor and the ROS receptor, constitutes a subfamily of the insulin receptor subfamily (Iwahara et al. 1997; Morris et al. 1997). The *ALK* gene was initially identified through the characterization of a t(2;5) translocation in anaplastic large-cell non-Hodgkin's lymphoma (ALCL). This translocation results in a fusion protein containing the N-terminal part of the nucleophosmin protein and the C-terminal part of the ALK receptor, leading to the constitutive activation of the ALK tyrosine kinase domain. Many different fusion proteins involving ALK have been described in a large variety of cancers (Hallberg and Palmer 2013). In 2008, mutations of the full-length ALK receptor were identified in neuroblastoma (Chen et al. 2008; George et al. 2008; Janoueix-Lerosey et al. 2008; Mosse et al. 2008), an embryonic tumor of the sympathetic nervous system, which accounts for 8–10% of pediatric cancers. Interestingly, the analysis of the ALK expression pattern in mammals highlighted that it is mostly expressed during the development of the central and peripheral nervous systems (see below). Somatic activating mutations were reported in a subset of sporadic neuroblastoma cases. In addition, germline ALK mutations were also described in some familial forms of the disease (Janoueix-Lerosey et al. 2008; Mosse et al. 2008) and, a few years later, in syndromic forms (de Pontual et al. 2011). Three main hotspots of mutations have been identified in sporadic cases at positions R1275, F1174 and F1245. ALK-mutated neuroblastoma belongs to the ALKoma entity that may benefit from tumor-targeted therapies with ALK tyrosine kinase inhibitors.

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ALK structure and expression

The ALK gene encodes a tyrosine kinase receptor that displays in its extracellular domain a glycine-rich region and two

additional MAM (meprin/A5/protein tyrosine phosphatase Mu) domains, which flank an LDLa (low-density lipoprotein class A) domain in the mammalian ALK receptor. In lower vertebrates, i.e., zebrafish and chick, the N-terminus of Alk is considerably shorter as compared to their human counterpart (Yao et al. 2013). Phylogenetic considerations implicate a common Alk/Ltk ancestor with a MAM domain that is retained in chick and zebrafish Ltk but lost in mammalian Ltk (Lopes et al. 2008).

The expression of Alk in mouse, rat and chick is mainly restricted to the developing central and peripheral nervous system, including thalamic nuclei, spinal cord motoneurons and sympathetic and enteric ganglia (Morris et al. 1997; Hurley et al. 2006; Vernersson et al. 2006; Degoutin et al. 2009). Alk expression has also been documented in several motor nuclei of the brainstem involved in the control of sucking and swallowing (Lopez-Delisle et al. 2014). Recent single cell RNA-seq analysis of the developing sympathoadrenal lineage demonstrated Alk expression in sympathetic neuroblasts at E12.5 and E13.5 (Furlan et al. 2017). This is in line with the increased number of sympathetic neuroblasts in the Knock-in Alk^{F1178} mouse at E14.5 (Cazes et al. 2014). Notably, E12.5/13.5 chromaffin cells show much lower Alk expression levels (Furlan et al. 2017).

In the adult mouse brain, ALK is expressed in the dentate gyrus as well as the CA1 and CA3 regions of the hippocampus (Weiss et al. 2012). In zebrafish, Alk expression is only observed in the CNS (Yao et al. 2013).

LTK, in spite of its structural similarity to ALK, shows only a minor overlap in its expression, which is restricted to pre-B lymphocytes, B lymphocytes and other hematopoietic cells, mature brain (including hippocampus) and placenta (Bernards and de la Monte 1990; Weiss et al. 2012). Notably, Ltk expression has not been described in the peripheral nervous system including sympathetic ganglia (Bernards and de la Monte 1990; Lopes et al. 2008). Recent RNA-seq analysis and transcriptomic profiling confirmed that Ltk as compared to Alk is expressed at much lower levels in embryonic and postnatal mouse sympathetic ganglia (Furlan et al. 2017; Cazes et al. 2014; Chan and Anderson, unpublished). Ros1 is not expressed in nervous tissue and has been detected only in epithelial compartments of lung, kidney, intestine and testis (Matsushime and Shibuya, 1990; Tessarollo et al. 1992).

ALK ligands and function

Studies on receptor activation The heparin-binding growth factors Midkine (MK) and Pleiotrophin (PTN) were initially described as activating ligands for mammalian ALK (Stoica et al. 2001; Stoica et al. 2002). For PTN, an indirect activation of ALK signaling through the stimulation of receptor protein tyrosine phosphatase-β (RPTPβ) and RPTBζ has been described (Perez-Pinera et al. 2007). ALK activation by MK has also been observed for cultured chick sympathetic neuroblasts and human glioblastoma cells (Lorente et al. 2011; Reiff et al. 2011). However, the function of PTN as an ALK ligand remains controversial, since several groups were unable to confirm ALK activation by PTN (Miyake et al. 2002; Moog-Lutz et al. 2005; Mathivet et al. 2007). Interestingly, heparin but not PTN and MK has been shown to induce ALK dimerization, auto-phosphorylation and downstream signaling in a neuroblastoma cell line (Murray et al. 2015). This suggests that ALK activation may involve heparin or sulfated proteoglycans as co-ligands similar to the activation of fibroblast growth factor receptor (FGFR). Very recently, two highly basic proteins, FAM150B and FAM150A, were discovered as ALK and LTK ligands, acting independently of heparin by binding to the glycine-rich region of the receptors (Guan et al. 2015; Reshetnyak et al. 2015). Although FAM150A was initially discovered as a LTK ligand, it is also able to bind to and activate ALK (Zhang et al. 2014; Guan et al. 2015). A potential role of FAM ligands in tumor initiation is suggested by the finding that FAM150B stimulates transformation of ALK-expressing NIH/3T3 cells and induces IL-3-independent growth of Ba/F3 cells expressing ALK (Reshetnyak et al. 2015).

Physiological function of ALK and ALK ligands in the sympathoadrenal lineage

Loss-of-function approaches to identify endogenous ALK function The important role of ALK mutations in familial and sporadic neuroblastoma (Chen et al. 2008; George et al. 2008; Janoueix-Lerosey et al. 2008; Mosse et al. 2008), together with the observation that activated ALK cooperates with MYCN to elicit neuroblastoma development in genetically engineered murine and zebrafish models (Berry et al. 2012; Zhu et al. 2012; Cazes et al. 2014), raised the question as to the endogenous function of ALK in sympathoadrenal development. The knockout of Alk in mice is viable and displays reduced hippocampal neurogenesis, behavioral defects and effects on the hypothalamic–pituitary–gonadal axis in male mice (Bilsland et al. 2008; Lasek et al. 2011; Weiss et al. 2012; Witek et al. 2015) (Fig. 1). Sympathetic ganglia and adrenal chromaffin cells have not been investigated. A massive loss of sympathoadrenal cells during early development can be excluded as norepinephrine deficiency results in embryonic death (Pattyn et al. 1999; Tsarovina et al. 2004; Morikawa et al. 2009). It would, however, still be of interest to investigate the Alk knockout for transiently reduced proliferation or delayed development observed upon elimination of Ascl1 and Sox11, as Alk-knockdown in chick sympathetic ganglia resulted in reduced neuroblast proliferation (Pattyn et al. 2006; Potzner et al. 2010; Reiff et al. 2011).

The chick embryo is a classical model system to study vertebrate sympathetic nervous system development both

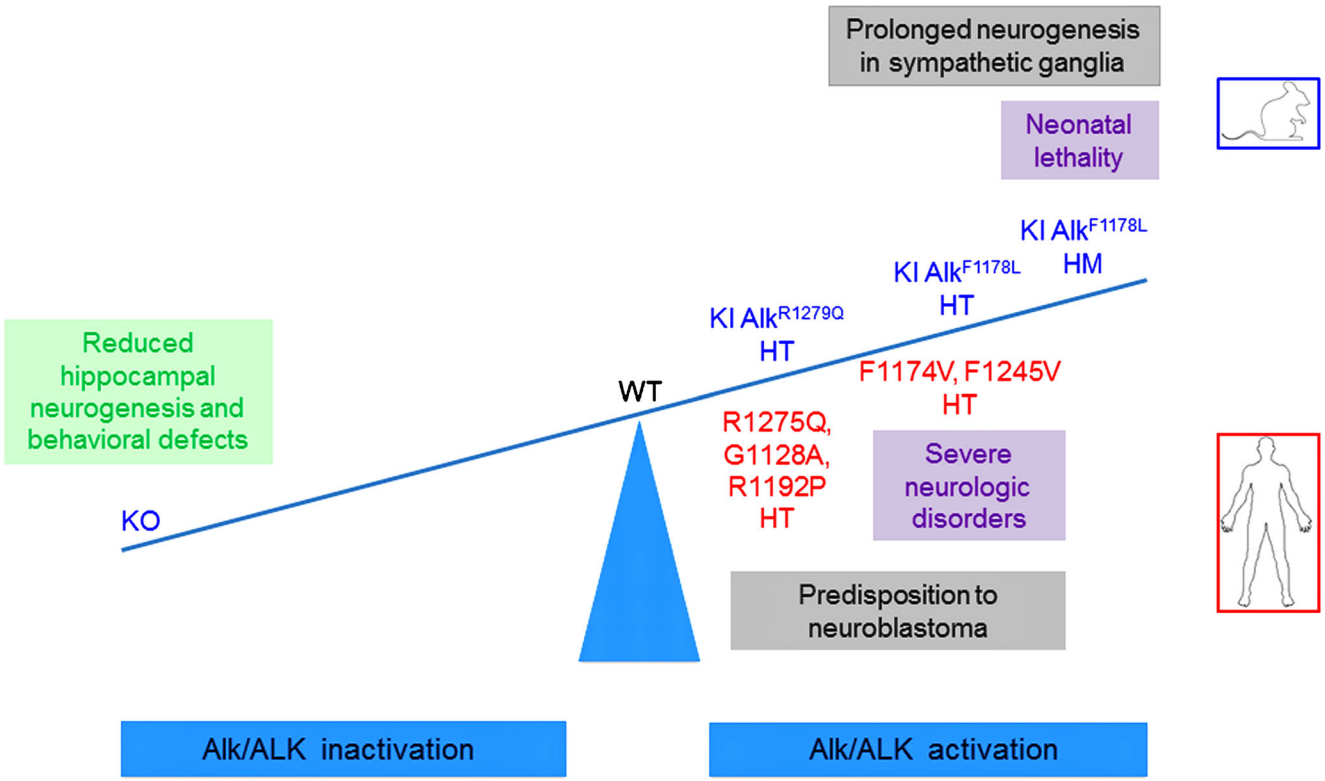

Fig. 1 Phenotypes of Alk/ALK activation in mice and humans. In mouse, the phenoptypes of both the KO and KI of the most frequent ALK mutations (R1275Q and F1174L) have been investigated. In neuroblastoma patients, germline *ALK* mutations occur in familial or syndromic forms. *KO* knock-out; *WT* wild-type; *KI* knock-in; *HT* heterozygous; *HM* homozygous

in vitro and in vivo. Gain- and loss-of-function approaches in the embryo and in cultures of sympathetic neuroblasts and neurons have been used to elucidate the role of fate-specifying differentiation factors (BMPs) and lineage-specific transcription factors (Phox2b, Gata2/3, Hand2), as well as of proliferation and survival factors (IGFI/II; NGF) during sympathetic neuron development (reviewed in Rohrer 2011). Chick sympathetic neuroblast proliferation depends on Alk signaling as shown by in vitro and in vivo knockdown of Alk and Mk and by pharmacological inhibition by TAE-684 (Reiff et al. 2011). These results so far represent the only direct evidence for a physiological role of Alk signaling in sympathetic neurogenesis. It is not known whether Alk-deficient mice display reduced neurogenesis in sympathetic ganglia as shown for the adult hippocampus (Weiss et al. 2012). Notably, compensatory effects by signaling pathways known to affect sympathetic neurogenesis, e.g., IGFI/II and Wnt, may be more effective in the Alk knockout as compared to acute Alk-knockdown in the chick embryo (Zackenfels et al. 1995; Armstrong et al. 2011).

Concerning the potential Alk ligand during neurogenesis, high expression and functional effects have been described for Mk in chick sympathetic ganglia (Reiff et al. 2011). During neurogenesis in embryonic mouse sympathetic ganglia, Mk is also expressed at about 30-fold higher levels than Ptn, while Fam150B could only be detected at background levels (Furlan et al. 2017; Chan and Anderson, personal communication). Also, Fam150A was not detected in these single-cell RNA-seq studies, excluding an autocrine function of Fam150A/B in sympathetic neuroblasts. Thus, Fam150B is an unlikely candidate ligand for Alk in normal sympathetic ganglia. As Alk activation is modulated by heparin and/or sulfated proteoglycans in a cell type-specific manner, a function for Mk in mammalian sympathetic neuroblasts is well possible, in spite of its inability to activate ALK in NC1 neuroblastoma cells (Murray et al. 2015; Reshetnyak et al. 2015). In this context, it may also be mentioned that MK expression has been observed in 79% of neuroblastoma tumors, as compared to 38% of tumors with FAM150B expression (Reshetnyak et al. 2015; Regairaz et al. 2016).

Taken together, these data strongly imply that neurogenesis in sympathetic ganglia during normal development depends on Alk signaling. These results, together with the important roles of activating *ALK* mutations in familial and sporadic neuroblastoma, suggest that neuroblastoma development may be initiated by aberrant ALK signaling. In the following, we discuss the in vitro and in vivo approaches to identify the regulatory mechanisms involved.

From normal neurogenesis to neuroblastoma development

In vitro approaches

To study the effects of increased Alk signaling on neuroblastoma initiation, Alk has to be activated in the cells of origin. There is considerable evidence to suggest that neuroblastoma arises from sympathoadrenal neuroblasts. Neuroblastoma is located in sympathetic ganglia and the adrenal, can be induced by forced expression of tumor driver genes in noradrenergic neuroblasts and neuroblastomas show similar gene expression profiles as embryonic neuroblasts (Weiss et al. 1997; Molenaar et al. 2012; Althoff et al. 2015; de Preter et al. 2006). An alternative possibility would be that initial aberrations already occur in neural crest progenitor cells that secondarily acquire adrenergic properties. Indeed, mouse neural crest cells transfected with MYCN generate neuroblastoma-like tumors upon subcutaneous injection into nude mice (Olsen et al. 2017). It should be noted, however, that the penetrance of neuroblastoma formation was low and that other neural crest-derived tumors (osteosarcoma) were also generated. Similar findings were observed upon forced expression of wild-type and activated ALK variants (ALK R1275Q and ALK F1174L) in murine neural crest progenitor cells (Schulte et al. 2013; Montavon et al. 2014). MONC-1 or JoMa-1 cell lines were used, generated by v-Myc- or Myc-ERT-mediated immortalization of neural crest cells, respectively (Maurer et al. 2007; Rao and Anderson, 1997). ALK-expressing cells, in contrast to the parental cell lines, maintained proliferation and survival even in the absence of Myc signaling (Schulte et al. 2013; Montavon et al. 2014). Upon orthotopic transplantation into the adrenal, undifferentiated tumors but no neuroblastoma were generated, although in a previous study a low proportion of tumors with neuroblastoma-like properties had been observed upon subcutaneous grafting of ALK-F1174L JoMa1 cells (Schulte et al. 2013; Montavon et al. 2014).

In contrast to neural crest progenitor cells, cultured neuroblasts respond to increased Alk signaling by a transient increase in proliferation, followed by neuron differentiation and long-term survival of postmitotic sympathetic neurons (Reiff et al. 2011; Kramer et al. 2016). Similar differentiation effects, reflected by neurite outgrowth and acquisition of neuron-like morphology, were described upon Alk activation in PC12 cells (Gouzi et al. 2005; Guan et al. 2015) (Table 1). The most informative results on the role of activated Alk signaling in neuroblastoma development were obtained by the analysis of Alk^{F1178L} knock-in mice, since activated Alk is expressed under the control of its own endogenous promoter (Cazes et al. 2014). Notably, enhanced proliferation in sympathetic ganglia and an extended neurogenesis period but no tumor formation, in the absence of MYCN co-expression have been described in these mice (Cazes et al. 2014) (Fig. 1).

Together, these results demonstrate that, depending on the stage of development, different effects are elicited by increased Alk signaling. As neuroblastomas with activated/mutated ALK show differentiated characteristics, a function of ALK in neuroblasts rather that neural crest progenitors is implicated in neuroblastoma development.

In vivo approaches

Various mouse models have been developed to explore the function of mutated ALK. Activated ALK is sufficient to elicit neuroblastoma formation when expressed transgenically under the strong β-actin promoter in mouse sympathetic neuroblasts (Heukamp et al. 2012). However, tumors are not induced in mice when mutated ALK is expressed under the control of the weaker Th-promoter or of the endogenous promoter (Berry et al. 2012; Cazes et al. 2014). Also, in the zebrafish, ALK^{F1174L} does not induce neuroblastoma (Zhu et al. 2012). Neuroblastoma is induced, however, by the combination of ALK^{F1174L} (or Alk^{F1178L}) and MYCN overexpression (Berry et al. 2012; Heukamp et al. 2012; Cazes et al. 2014). Tumor generation in response to activated ALK and MYCN is rapid, fully penetrant and without additional genomic alterations, demonstrating that combined mutated ALK and MYCN signaling is sufficient to induce neuroblastoma from sympathoadrenal progenitors. What are the regulatory mechanisms downstream of MYCN/$ALK^{mutated}$ signaling that may be essential for tumor generation? Increased *MYCN* transcription, stabilization of MYCN protein and reduced apoptosis have been observed by comparing ALK^{F1174L}/MYCN with MYCN tumors (Berry et al. 2012; Heukamp et al. 2012; Zhu et al. 2012). Interestingly, the tyrosine kinase receptor Ret is induced in MYCN/Alk^{R1279Q} or Alk^{F1178L} tumors, generating an additional target for tumor treatment (Cazes et al. 2014). It should be noted, however, that these mechanisms are deduced from the comparison of fully-grown tumors and thus may not reflect the situation at initial stages of tumor development, which is still unclear. A recent study, addressing the cellular and molecular effects of MYCN/ALK by co-expressing ALK^{F1174L} and MYCN in cultured sympathetic neuroblasts, demonstrated increased survival and differentiation as compared to MYCN-treated cultures, as well as an upregulation of the ubiquitin ligase SKP2, which targets p27 for degradation (Kramer et al. 2016). However, detailed analysis of the molecular and cellular changes in sympathetic ganglia of $ALK^{mutated}$/MYCN mice at precancerous stages is urgently needed to shed more light on the role of activated ALK in neuroblastoma tumor initiation. Open questions include (1) the mode of neuroblast expansion (global or clonal), (2) subtype-identity of proliferating cells, (3) differences between sympathetic and chromaffin progenitors and (4) molecular signatures of precancerous cells.

Table 1 Summary of assays evaluating the activity of ALK mutants

Mutation	Domain	Colony assay (NIH3T3)	Tumorigenicity (nude mice)	IL-3 independent growth Ba/F3	Neurite formation (PC12)	Rough eye in *Drosophila*	Kinase assay	K_M, ATP	k_{cat}
WT		-[1,3,4,5]	0[1]+[5]	-[2,3]	-[3]	-[3]	+[1,5]	+	0
Δ1-5	extraC				+++[3]				
Δ2-3	extraC	+++[1]	+++[1]				+++[1]		
Δ4-11	extraC	+++[5]	+++[5]		+++[3]		+++[5]		
R1060H	Jxtm	-[4]							
K1062M	Jxtm	++[1]	+++[1]				++[1]		
T1087I	Jxtm	-[3]		-[3]	+[3]				
D1091N	Jxtm	-[3]		-[3]	+[3]				
A1099T	Jxtm	-[3]		-[3]	+[3]				
G1128A	P-loop	++[3,4]		+[3]	+++[3]			+	+
T1151M	N-lobe	-[3,4]		-[2,3]	+[3]			++	+
M1166R	αC/A-loop	+[3,4]		-[3]	+++[3]			+	++
I1170N	αC/A-loop	++[4]						++	++
I1170S	αC/A-loop	++[4]						++	++
I1171N	αC/A-loop	++[3,4]		-[3]	+++[3]			++	++
F1174I	Phe-core	+++[3]		+[3]	+++[3]				
F1174L	Phe-core	+++[1,3,4]	+++[1]	+[2,3]	+++[3]	+++[3]	+++[1]	+	+++
F1174S	Phe-core	+++[3]			+++[3]	+++[3]			
I1183T	N-lobe	-[4]						+	0
R1192P	N-lobe	-[3]++[4]		+[3]	+++[3]			+	++
L1196M	Active site	++[4]						++	+
A1200V	Active site	-[4]						+	0
L1204F	C-lobe	-[4]						+	0
R1231Q	C-lobe	-[4]						+	0
A1234T		-[3]		-[2,3]	+[3]	-[3]			
L1240V	Phe-core	++[4]							
F1245C	Phe-core	+++[3,4]		+[3]	+++[3]			+	+++
F1245V	Phe-core	+++[4]						+	+++
I1250T	C-lobe	-[3,4]			-[3]	-[3]		+	0
D1270G	Active site	-[4]						+	0
R1275Q	αC/A-loop	++[3,4]		+[2]-[3]	+++[3]		+++[5]	++	++
Y1278S	αC/A-loop	++[4]						+	++
G1286R	A-loop	-[4]						+	0
T1343I	C-lobe	-[4]						+	0
D1349H	C-lobe	-[4]						+	0
R1464*		-[3]		-[3]	+[3]	-[3]			

The three hotspots are highlighted. References are: [1] studies performed by Ogawa's group: Chen et al. 2008 and Okubo et al. 2012; [2] results of George et al. 2008; [3] results of Hallberg's group: Schönherr et al. 2011a, 2011b and Chand et al. 2013; [4] results of Mossé's group: Bresler et al. 2011, 2014; and [5] results of Cazes et al. 2013. The K_M, ATP and k_{cat} values for nonphosphorylated ALK tyrosine kinase domain variants are from Mossé's group

ALK mutations in neuroblastoma

Somatic mutations of the *ALK* gene were initially described in around 8% of sporadic neuroblastoma cases at diagnosis (Chen et al. 2008; George et al. 2008; Janoueix-Lerosey et al. 2008; Mosse et al. 2008). In 2014, the analysis of almost 1600 neuroblastoma primary tumors confirmed this mutation rate and showed that mutations at positions 1174, 1245 and

1275 account for 85% of all mutations (Bresler et al. 2014). The F1174 substitutions occur preferentially in cases exhibiting *MYCN* amplification. ALK amplifications are also observed in a few percent of primary tumors. In univariate analysis, ALK activation or amplification is associated with a poorer prognosis, both for event-free survival and overall survival (Bresler et al. 2014). Subsequent analysis of neuroblastoma cases revealed that ALK mutations could be present at the relapse stage although they were not detected by Sanger sequencing at diagnosis (Martinsson et al. 2011; Schleiermacher et al. 2014). Further analysis by NGS approaches showed that ALK mutations may occur at subclonal levels both at diagnosis and at relapse (Bellini et al., 2015; Eleveld et al. 2015). These observations, in the context of targeted therapies, highlight the importance of evaluating the ALK mutational status in tumors at relapse.

In addition to the missense point mutations, genomic rearrangements leading to variants, which lack parts of the extracellular domain, have been identified in neuroblastoma cell lines or tumor samples. In these cases, the rearranged *ALK* allele was also amplified. The described variants include the following forms: Δ2–3 (Okubo et al. 2012), Δ4–11 (Cazes et al. 2013) and Δ1–5 (Fransson et al. 2015). The transforming potential of the Δ2–3 and Δ4–11 forms has been documented (see below), showing that such rearrangements constitute an alternative mechanism to point mutations resulting in ALK activation.

Various assays have been used to decipher the consequences of ALK mutations on the receptor activity (Table 1). These include the evaluation of neurite outgrowth upon overexpression of the variants in rat adrenal pheochromocytoma PC12 cells, the evaluation of their oncogenic potential in NIH/3 T3 cells or the measuring of their capacity to grow in the absence of Il-3 in Ba/F3 cells. Activation of various ALK human mutants has also been measured using the *Drosophila* model in which a specific mutant is ectopically expressed in the eye. Since the DAlk ligand is unable to bind and activate the human ALK receptor, no phenotype is observed for ALK ligand-dependent forms. In contrast, overexpression of ligand-independent and activated forms leads to a rough eye phenotype. The biochemical properties of a number of mutants have also been determined for purified tyrosine kinase domains. Table 1 provides a summary of the results of these assays performed for ALK bearing different point mutations as well as for the rearranged Δ2–3, Δ4–11 and Δ1–5 forms. The three hotspot mutations at F1174, F1245 and F1275 are clearly ligand-independent oncogenic mutations. Among the other identified mutations, some of them do not show evidence of activation. The study of knock-in mice bearing the F1178L and R1279Q mutations in the mouse Alk receptor (corresponding to the F1174L and R1275Q in the human ALK receptor) was able to demonstrate in vivo that the oncogenic potential of the F1178L mutation was higher compared to that of the R1279Q mutation (Cazes et al. 2014).

Interestingly, the spectrum of ALK germline mutations observed in familial cases is different from that of somatic mutations observed in sporadic cases. Indeed, the R1275 residue is the main hotspot observed in neuroblastoma familial cases, whereas no mutations at positions F1174 and F1245 have been reported in such forms (Fig. 1). However, germline F1174V and F1245V de novo mutations have been observed in patients presenting a syndromic form associating congenital neuroblastoma and severe neurological disorders, including major feeding and breathing difficulties (de Pontual et al. 2011). Homozygous mice for the Alk^{F1178L} mutation are characterized by a dramatic reduced milk intake and show a high neonatal lethality (Lopez-Delisle et al. 2014). These observations are consistent with a role of the ALK receptor not only in the peripheral nervous system but also in the central nervous system, in agreement with its expression pattern.

Full-length ALK signaling

Whereas the signaling pathways downstream of ALK fusion proteins have been extensively characterized, the signaling pathways triggered by activated full-length ALK remain less defined, in particular due to the lack of well identified ALK ligands. Several strategies have been developed to bypass this limitation and define full-length ALK downstream pathways. These include the generation of chimeras between ALK and various receptor domains (Souttou et al. 2001; Piccinini et al. 2002; Gouzi et al. 2005), as well as the use of monoclonal ALK agonist antibodies inducing the dimerization of the receptor (Motegi et al. 2004; Moog-Lutz et al. 2005; Martinsson et al. 2011). A number of studies have relied on overexpression of the normal or modified ALK receptor in heterologous systems, with the limitation of a cellular context not fully physiological. Investigations of ALK signaling pathways have also been performed in neuroblastoma cell lines treated with agonist or antagonist antibodies, small-molecule inhibitors or by RNA interference.

The induction of the Ras-MAPK(ERK) pathway, revealed through the ERK1/2 phosphorylation and that of the PI3K/AKT pathway, revealed by AKT phosphorylation, are almost unanimous in all studied models and are observed in neuroblastoma cell lines (Stoica et al. 2002; Mathivet et al. 2007; Janoueix-Lerosey et al. 2008; George et al. 2008; Bresler et al. 2011; Duijkers et al. 2011; Schönherr et al. 2012; Heukamp et al. 2012; Sattu et al. 2013; Moore et al. 2014; Umapathy et al. 2014). ERK activation has been shown to be involved in DNA synthesis in the SK-N-SH neuroblastoma cell line (Motegi et al. 2004). Several lines of evidence indicate that ShcC acts as a major docking protein in the ALK–ERK axis. A direct interaction between ALK and ShcC has been

demonstrated in ALK-amplified neuroblastoma cells and an in vitro kinase assay revealed that ShcC was a potent substrate of activated ALK (Miyake et al. 2002). In the SK-N-SH cell line, which does not exhibit ALK amplification, ALK also interacts with ShcC (Motegi et al. 2004). Other docking proteins such as FRS2, IRS-1 and c-Cbl have been reported to be phosphorylated on tyrosine residues upon ALK activation (Piccinini et al. 2002; Motegi et al. 2004**;** Degoutin et al. 2007).

STAT3 has been identified as a downstream ALK target mostly in heterologous systems exhibiting ALK overexpression (Schönherr et al. 2011a, b; Chand et al. 2013; Fransson et al. 2015). Only one study has reported STAT3 phosphorylation by activated ALK in neuroblastoma cells (Sattu et al. 2013). However, STAT3 phosphorylation was delayed compared to ERK and AKT phosphorylation, suggesting that STAT3 may be an indirect target of ALK activated pathways rather than a direct one.

Several studies have reported that activated ALK is able to activate *MYCN* transcription (Reiff et al. 2011; Berry et al. 2012; Schönherr et al. 2012; Sattu et al. 2013; Umapathy et al. 2014) and various pathways involved in this regulation have been described. Two studies pointed out the role of PI3K, since its inhibitor, the LY294002 compound induced a decreased *MYCN* transcription (Schönherr et al. 2012; Umapathy et al. 2014). Umapathy and colleagues further deciphered the mechanism from ALK activation to *MYCN* transcription, suggesting that PI3K/AKT activates MEK5, which then phosphorylates ERK5/MAPK7 leading to *MYCN* transcription. The involvement of MEK1/2 and STAT3 in this regulation has also been proposed (Schönherr et al. 2012; Sattu et al. 2013).

In order to decipher the pathways downstream of activated ALK, a phosphoproteomic approach has recently been used after treatment of three neuroblastoma cell lines exhibiting ALK activation (R1275Q, F1174L or amplification) with crizotonib (Chen et al. 2016). This analysis highlighted the RAS/JNK pathway as an important oncogenic signaling pathway in the different studied samples. ALK and JNK inhibitors inhibited cell growth by inducing cell apoptosis and cell cycle arrest.

Notably, the constitutive activity of the ALK mutated receptor has been shown to induce its retention in the reticulum/Golgi compartments, due to an impaired glycosylation (Mazot et al. 2011). However, the minor pool of the receptor addressed to the plasma membrane was much more phosphorylated. Interestingly, the maturation and cell-surface localization of the mutated receptor were restored upon inhibition of the kinase activity with small-molecule inhibitors (Mazot et al. 2011; Carpenter et al. 2012). The impact of the mislocalization of the mutated receptor on the activity of its downstream signaling pathways still remains to be determined.

ALK as a therapeutic target

Monotherapies

Several ALK inhibitors have now been investigated in vitro, in neuroblastoma pre-clinical models or during clinical trials, some of them being still ongoing (Table 2). These small-molecules are competitive inhibitors of the ATP binding within the kinase domain. Most of them may act on several tyrosine kinase receptors and are not fully specific for ALK. Their binding properties to the ATP pocket may be slightly different from one to another, as well as their capacity to inhibit the various mutated forms of the receptor. This is the case for crizotinib (Zou et al. 2007), which has been described to have a lower efficacy to inhibit the F1174 and F1245 mutated forms (Bresler et al. 2011). However, receptor inhibition of these forms may be achieved by increasing the dose, probably because the F1174L mutant exhibits a higher ATP affinity. A phase I/II clinical trial using crizotinib was set up in the United States, shortly after the identification of ALK mutations in neuroblastoma patients (Mosse et al. 2013). This trial, including a small number of patients at the relapse stage for which the ALK status was not systematically known, showed that a number of neuroblastoma patients may benefit from ALK-targeted therapies. In addition, inhibition of full-length ALK was more difficult to achieve compared to ALK fusion proteins. In adults, crizotinib was approved in 2011 for patients with non-small cell lung carcinoma carrying ALK fusions, since a high efficacy had been documented in such patients (Kwak et al. 2010). However, as is the case for other tyrosine kinase inhibitors, these targeted therapies are limited by the frequent appearance of resistance mechanisms. Indeed, in adult tumors with ALK fusions, secondary mutations have been observed following crizotinib treatment, in particular the F1174L mutation (Choi et al. 2010; Sasaki et al. 2010). Ceritinib is a second-generation ALK inhibitor (Marsije et al. 2013), derived from the TAE-684 molecule, which initially showed a good efficacy in neuroblastoma but could not be used in patients due to toxicity issues. Ceritinib is superior to crizotinib for patients with non-small cell lung carcinoma (Friboulet et al. 2014; Shaw et al. 2014). In neuroblastoma, a superior efficacy of ceritinib over crizotinib has been recently reported in several cell lines and in transgenic Th-ALK F1174L/MYCN mice after 7 days of treatment (Tucker et al. 2017). Ceritinib phase I clinical trials are ongoing and the efficacy of ceritinib in neuroblastoma patients remains to be determined. Whereas crizotinib has been identified through a systematic drug screening, lorlatinib has been designed based on the receptor structure, combined with physical-property-based optimization (Johnson et al. 2014). Very encouraging results have been obtained regarding the efficacy of lorlatinib, in vitro on purified ALK receptors, in terms of IC_{50} values on neuroblastoma cell lines (median value of 10 nM) and in various preclinical models, including xenografts and PDXs

Table 2 ALK inhibitors in neuroblastoma

Inhibitor	Targets	Pre-clinical	Clinical trials
Monotherapies			
Crizotinib (PF-02341066)	ALK, MET, ROS	Bresler et al. 2014	Phase I/II (Mossé et al. 2013)
			Phase II - NCT02034981 (AcSé Crizotinib)
			Phase II -NCT00939770
Ceritinib (LDK378)	ALK, IGF-1R, InsR, STK22D	Debruyne et al. 2016	Phase I - NCT01742286
			Phase I - NCT02780128
Lorlatinib (PF-06463922)	ALK, ROS1	Infarinato et al. 2016; Guan et al. 2016	Phase I/II- NANT (New Approaches to Neuroblastoma Therapy)
Alectinib (CH5424602)	ALK, GAK, LTK, RET	Sakamoto et al. 2011; Lu et al. 2017	–
Combinatory treatments			
Crizotinib + topotecan/cyclohosph.	ALK, MET, ROS + Topoisomerase/ADN	Krytska et al. 2015	Phase I-NCT01606878
			Phase I-NCT02559778
Crizotinib + temsirolimus	ALK, MET, ROS + mTOR	Berry et al. 2012; Moore et al. 2014	Phase I- NTR5584
Ceritinib + ribociclib	ALK, IGF-1R, InsR, STK22D + CDK4/6	Wood et al. 2016	Phase I - NCT02780128
Ceritinib + NVP-CGM097	ALK, IGF-1R, InsR, STK22D + MDM2	Wang et al. 2017	–

(Infarinato et al. 2016; Guan et al. 2016). In addition, lorlatinib is able to inhibit the wild-type form of the ALK receptor and several ALK-mutated forms including the F1174 L and F1245C previously shown to be resistant to crizotinib. The efficacy of lorlatinib appears to be 10 to 30 times higher compared to crizotinib in the same models.

Alectinib has been identified in 2011 as a potent and selective inhibitor of ALK (Sakamoto et al., 2011). Notably, in cell-free assays, the IC_{50} of CH5424802 for ALK activity were 1.9 nM, 1.0 and 3.5 nM for ALK WT, ALK F1174 L and ALK R1275Q, respectively. This study reported that two neuroblastoma cell lines with mutated or amplified ALK were sensitive to alectinib, whereas a non-mutated cell line was not. Alectinib has also been shown to strongly inhibit RET kinase activity (Kodama et al. 2014). Recently, it has been further reported that alectinib was able to suppress cell proliferation and induce apoptosis in neuroblastoma cell lines with either wild-type ALK or mutated ALK by blocking ALK-mediated PI3K/Akt/mTOR signaling (Lu et al. 2017). Furthermore, alectinib treatment of TH-MYCN transgenic mice resulted in decreased tumor growth and prolonged survival. In patients with ALK-positive NSCLC, a superior efficacy and lower toxicity has been reported for alectinib compared to crizotinib (Peters et al. 2017). Altogether, these data suggest that alectinib may be a promising therapeutic agent for the treatment of neuroblastoma.

Combinatory treatments

The observation of ALK mutations resistant to several ALK inhibitors in neuroblastoma patients before the use of ALK-targeted therapies, as well as the frequent occurrence of resistance mutations following such therapies, provide some rationale for the development of combinations of different molecules. These combinations, by targeting several actors of the pathways involving the ALK receptor, may allow better long-term effects during treatment of neuroblastoma patients. Several examples may be highlighted. The crizotinib/Torin2 combination has been shown to be more efficient compared to each molecule alone in a mouse model of neuroblastoma driven by the overexpression of ALK F1174L and MYCN (Berry et al. 2012). However, a more in-depth analysis of this combination in neuroblastoma cell lines with or without *MYCN* amplification revealed that, in the latter, an increase of the PI3K activity was observed (Moore et al. 2014). Therefore, an inhibitor targeting both mTOR and PI3K would be more appropriate to use in combination with an ALK inhibitor in neuroblastoma cases with mutated ALK and without *MYCN* amplification. The PI3K and mTOR inhibitor PF-05212384 has been shown to act in synergy with the ALK inhibitor crizotinib in several cell lines. At least one clinical trial is ongoing in children to evaluate the crizotinib and temsirolimus (mTOR inhibitor) combination (Table 2).

More recently, a systematic evaluation of 8 targeted-therapy molecules reported a synergistic effect of the CDK4/6 inhibitor ribociclib and the ALK inhibitor ceritinib (Wood et al. 2016). This effect was observed in vitro on ALK-mutated neuroblastoma cell lines as well as in preclinical models. Another study investigated ceritinib in combination with the MDM2 inhibitor CGM097 and reported that both drugs act synergistically to inhibit proliferation of ALK-

mutated and TP53 wild-type neuroblastoma in vitro and in xenograft models. However, in such models, tumor growth was observed after discontinuation of the treatment (Wang et al. 2017).

The association of ALK-targeted therapy with standard chemotherapeutic agents may also be considered. A recent study reported the higher anti-tumoral activity of crizotinib associated with the genotoxic agents, i.e., topotecan and cyclophosphamide, compared to each treatment alone in ALK-mutated cases with a p53 functional pathway (Krytska et al. 2016). The mechanism on which the synergy of the two types of agents relies remains to be determined.

Conclusion and perspectives

Our knowledge of the ALK biology has considerably increased during the last 10 years. Whereas ALK has remained for long an orphan receptor, several ligands have been proposed during recent years. Evidence obtained in different organisms converge on a key role of ALK in neurogenesis in sympathetic ganglia during normal development. The discovery of germline and somatic ALK mutations in neuroblastoma and the addiction of ALK-mutated neuroblastoma to the activated form of the receptor has highlighted *ALK* as a major neuroblastoma oncogene and an actionable therapeutic target in this pediatric cancer for which more efficient treatments are urgently needed. However, the precise role of activated ALK in neuroblastoma tumor initiation is still not fully understood. Clinical data obtained with ALK small-molecules inhibitors of the first generation for neuroblastoma patients underscored the importance of deciphering the downstream signaling pathways triggered by activated ALK to propose therapeutic strategies providing maximal clinical benefit. In addition to small-molecule inhibitors, immunotherapy approaches with specific antibodies may be considered, since ALK is present at the cell surface of tumor cells. A recent study evaluated such a possibility using a chimeric antigen receptor (CAR) targeting ALK (Walker et al. 2017). However, limited anti-tumor efficacy of ALK CAR T cells was observed in two xenograft models of human neuroblastoma, due in part to an insufficient target density on tumor cells. Since inhibition of the kinase activity of mutated ALK leads to upregulation of ALK surface expression (Mazot et al. 2011; Carpenter et al. 2012), combining a tyrosine kinase inhibitor with an ALK CAR strategy could represent an interesting co-therapy in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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