Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology

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Abstract | The burgeoning field of anaplastic lymphoma kinase (ALK) in cancer encompasses many cancer types, from very rare cancers to the more prevalent non-small-cell lung cancer (NSCLC). The common activation of ALK has led to the use of the ALK tyrosine kinase inhibitor (TKI) crizotinib in a range of patient populations and to the rapid development of second-generation drugs targeting ALK. In this Review, we discuss our current understanding of ALK function in human cancer and the implications for tumour treatment.

Anaplastic lymphoma kinase (*ALK*) was discovered through the identification of a 2;5 chromosomal translocation in anaplastic large-cell non-Hodgkin's lymphoma (ALCL)[1]. The nucleophosmin (NPM)–ALK fusion protein produced by this rearrangement[1,2] results in constitutive activation of the ALK kinase, which is normally regulated by the extracellular ligand-binding domain in the full-length receptor[1,3,24]. We now know that ALK activation in cancer can also arise through overexpression and mutation of full-length ALK[4–6] (FIG. 1). Just when and where does ALK activation become a 'troublemaker' in the context of human tumours? ALK alterations have been described in several human cancers, but it is not fully understood whether these reflect a true tumorigenic role of ALK. In the case of *ALK* translocations, the ALK fusion proteins that have been examined seem to have an important role in driving tumorigenesis. In tumours in which full-length ALK is mutated, such as in neuroblastoma and thyroid cancer, the picture is less clear, although the overriding genetic evidence in neuroblastoma implicates mutant ALK as an important oncoprotein.

ALK function

ALK function in mice and humans. The single *ALK* locus in mice and humans encodes a classical receptor tyrosine kinase (RTK) that comprises an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (FIG. 2). Together with the related leukocyte tyrosine kinase (LTK) receptor, ALK constitutes a subfamily within the insulin receptor superfamily on the basis of kinase domain similarities. The extracellular domains of ALK and LTK are unique among RTKs, as they contain a glycine-rich region and, in the case of ALK, additional LDLa and MAM domains[6]. Pleiotrophin (PTN) and midkine (MK) have been reported as activating ligands for mammalian ALK[7,8]. These small heparin-binding growth factors have a role in neural development, survival and tumorigenesis[9,10]. MK and PTN are also able to activate other receptors, including receptor protein tyrosine phosphatase-β (RPTPβ) and RPTPζ, N-syndecan, low-density lipoprotein receptor-related protein (LRP) and integrins[6]. PTN can also function via the RPTPβ and RPTPζ phosphatases to activate ALK signalling[11]. Currently, the stimulation of ALK by PTN and MK remains controversial, as some groups have shown PTN- and MK-mediated ALK activation, whereas others have reported contradictory results[12–15]. Therefore, in contrast to non-vertebrate model organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, the natural ligand for mammalian ALK remains enigmatic.

Similarly, the precise physiological role of ALK in mammals is unclear. Some interesting observations have been made in patients treated with ALK inhibitors. The ALK tyrosine kinase inhibitor (TKI) crizotinib[16,17] is remarkably well tolerated, but reports from human clinical trials using crizotinib describe unexplained side effects, such as reduced heart rate[18], suppression of testosterone levels in men[19] and visual disturbances — effects that are reversible on discontinuation of treatment[20,19]. Whether these observations represent ALK-specific effects or off-target effects of crizotinib will require more thorough exploration, especially as a recent Phase I/II clinical trial examining the effect of the CH5424802 ALK inhibitor reported lower levels of visual symptoms than those observed with crizotinib treatment[21]. Overall, ALK expression patterns in

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Key points

- Anaplastic lymphoma kinase (ALK) is involved in the initiation and progression of many different cancer types, including lymphomas, neuroblastoma and non-small-cell lung cancer. It is clear that ALK can be activated by translocation, as well as by mutation. The *ALK* locus is a hotspot for activating translocation events, with 22 different translocation partners identified. The resulting ALK fusion proteins are found in a wide range of cancer types. An alternative mechanism for ALK activation is through point mutation of the *ALK* locus, most commonly within the kinase domain, as reported in patients with neuroblastoma and thyroid cancer.
- The physiological function of ALK in mammals is enigmatic, although it is clear that ALK is not required for viability, as *Alk*$^{-/-}$ mice are viable. The role of ALK in model systems, such as *Drosophila melanogaster*, *Caenorhabditis elegans* and *Danio rerio*, is more clearly defined in development, with ALK signalling used repeatedly in a spatially and temporally regulated manner. In both *D. melanogaster* and *C. elegans*, ALK also has genetically defined ligands.
- The spatial and temporal expression pattern of the different oncogenic ALK fusion proteins is determined by the fusion partners. Furthermore, although not well studied, comparisons of the different ALK fusion proteins suggest that they display differences in signalling and in transforming and tumorigenic potential.
- The first clinically approved drug to target ALK — crizotinib — is a tyrosine kinase inhibitor (TKI) that was approved by the US Food and Drug Administration (FDA) for use in ALK-positive non-small-cell lung cancer. Recent reports suggest that ALK TKIs will be useful in the treatment of other less frequently occurring ALK-positive cancer types. A number of second-generation ALK TKIs are currently in clinical trials and are able to inhibit secondary 'resistance' mutations that are found in patients treated with crizotinib.
- Several important issues remain to be addressed, such as cooperativity between ALK and other oncogenes and tumour suppressors, the differences in signalling output between different ALK oncogenes, the streamlined identification of ALK-positive patients in multiple cancer types, putative combinatorial drug strategies for patients and an explanation for why the *ALK* locus is a hotspot for translocation.

chickens, mice, rats and humans suggest key roles in the development of the nervous system[3,22–27]. It should be noted that ALK mRNA and protein levels seem to diminish in all tissues after birth — they reach their minimum levels at 3 weeks of age and are maintained at low levels in adult animals[3]. Only mild behavioural phenotypes have been described in *Alk*-knockout mice[28–30], which is in keeping with the good tolerance of patients to ALK inhibitors. Double knockout *Alk*$^{-/-}$ *Ltk*$^{-/-}$ mice have also been reported to be viable[30], suggesting that pharmacological inhibition of ALK before adulthood, for example in children with ALK^{+} tumours, may offer a tolerable clinical option.

Insight into ALK function from model systems. An increasing amount of knowledge regarding the function of ALK is being accumulated from model systems, such as *D. melanogaster*, *C. elegans* and the zebrafish *Danio rerio*. In contrast to mice and humans, there is only one ALK family member in *C. elegans* (called suppressor of constitutive dauer formation 2 (SCD-2)) and in *D. melanogaster* (called Alk)[31–33]. In zebrafish there are two members of the ALK family, which are encoded by *ltk* and *alk*[34,35] (TABLE 1). ALK-activating ligands are Jelly belly (Jeb) in *D. melanogaster*[36–39] and hesitation behaviour 1 (HEN-1) in *C. elegans*[32,40]. These are rather unusual secreted ligands that both contain a low-density lipoprotein-receptor class A (LDLRA) domain. To date, no such ligand has been reported to activate ALK signalling in vertebrate systems.

In *D. melanogaster*, Jeb–Alk signalling is required for survival and is highly spatially and temporally regulated. It is involved in the specification of founder cells in embryonic visceral muscle[36–39], the development of neuronal circuitry in the visual system[41], the development of synapses of the neuromuscular junction[42] and is involved in the regulation of body size, learning and memory[43]. Other functions of ALK signalling in *D. melanogaster* include the protection of neuroblasts during nutrient deprivation[44] and a role in alcohol resistance[29].

In *C. elegans*, HEN-1–SCD-2 signalling is non-essential for nematode development, but it seems to be required for the integration of sensory inputs. SCD-2 was originally identified in a screen for mutants that suppress constitutive dauer formation[45], implicating ALK signalling in the regulation of the transforming growth factor-β (TGFβ) dauer formation pathway in *C. elegans*. Crosstalk between ALK and TGFβ pathways is not unique to nematodes, as *D. melanogaster* Alk has been reported to induce expression of the TGFβ homologue, Decapentaplegic (Dpp), in the embryonic visceral mesoderm[46]. Further parallels between ALK signalling in *C. elegans* and *D. melanogaster* can be drawn at the neuromuscular junction, where SCD-2 signalling is important for the regulation of presynaptic differentiation in *C. elegans*[31].

In zebrafish, both ALK family members have been functionally described. *ltk* was originally described as the *shady* locus in a screen for neural crest development mutants[47]. The shady phenotype refers to the loss of the neural crest-derived iridophore pigment cell pattern, reflecting the lack of specification of these cells in the *ltk* (previously known as *shady*) mutant[35]. Unusually, zebrafish Ltk contains a MAM domain and is in this respect — together with its expression pattern in the neural crest — more similar to ALK than to other LTK homologues. ALK has recently been reported to have a role in neurogenesis in the developing zebrafish central nervous system (CNS)[34]. Although ligands for ALK and LTK have yet to be described in zebrafish, their roles in the developing CNS and neural crest suggest that this model system may provide important information regarding mammalian ALK signalling. Insight into the role of ALK family RTKs in neural crest development may be of particular importance in the understanding of neuroblastoma, which is a tumour of neural crest origin, in which activating ALK mutations have been described (discussed below)[48–52].

In parallel to the advances being made in our understanding of the role of ALK signalling during development in model systems, these models are being used to understand the role of ALK in cancer. For example, a number of human neuroblastoma ALK mutants have been assayed for ligand-independent activity and sensitivity to ALK inhibitors by ectopic expression in the *D. melanogaster* eye[53,54]. Transgenic zebrafish have also been developed to examine the role of ALK in neuroblastoma development[55], and the iridophore phenotype of the *ltk* mutant is phenocopied by ALK TKIs, exploiting an *in vivo* zebrafish assay for examining combinatorial strategies for ALK inhibition[56].

Dauer
In *Caenorhabditis elegans*, dauer refers to the entry of the animal into an arrested state when environmental conditions are not favourable for further growth. During the dauer state, feeding is indefinitely arrested and locomotion is markedly reduced. The dauer state ends when the animal experiences favourable conditions.

Neural crest
The neural crest originates from the neural tube and migrates outwards as a transient, multipotent migratory cell population that is unique to vertebrates. The neural crest gives rise to diverse cell lineages, including melanocytes, neuroendocrine cells, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons, and glia.

Iridophore
A pigment cell derived from the neural crest and found in many animals. Iridophores contain crystals that reflect different wavelengths of light, which give them an apparent colour, although no true pigment is present.

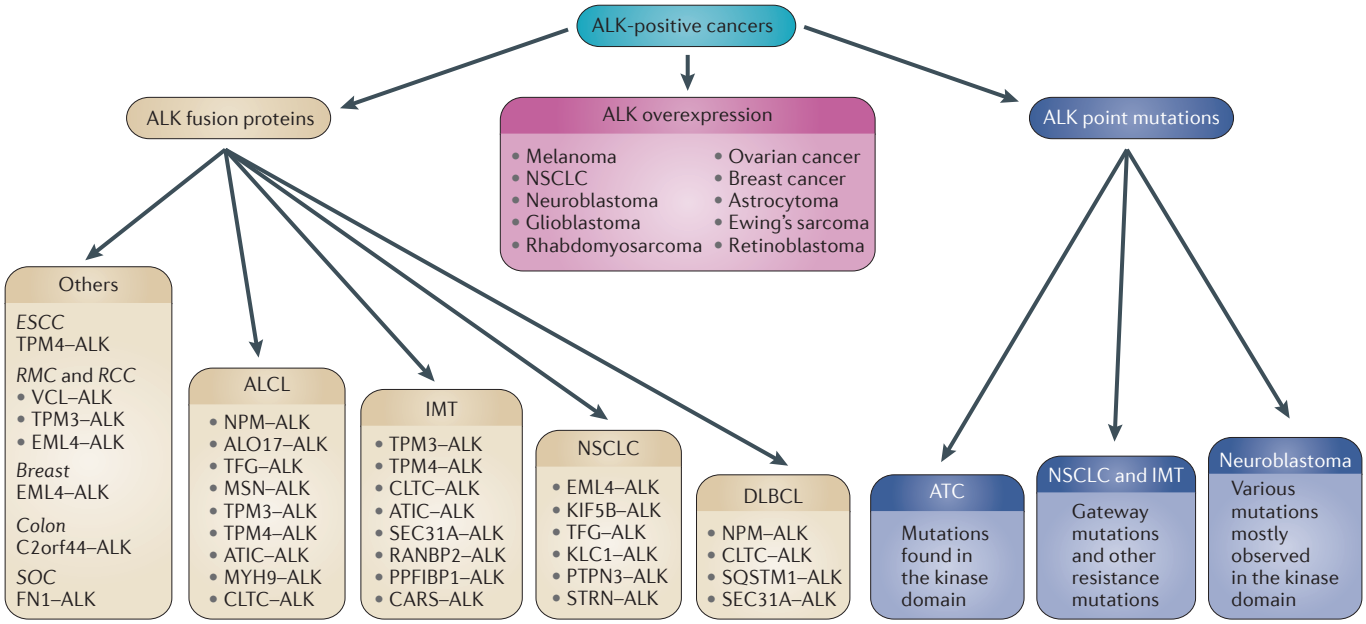


Figure 1 | **ALK in cancer — an overview.** Schematic summary of the different categories of anaplastic lymphoma kinase (ALK)-positive cancers. ALK fusion proteins (shown in beige), in which the kinase domain of ALK is fused to the amino-terminal portion of various proteins, have been described in numerous cancers, such as anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumour (IMT), diffuse large B cell lymphoma (DLBCL), non-small-cell lung cancer (NSCLC), renal medulla carcinoma (RMC), renal cell carcinoma (RCC), breast cancer, colon carcinoma, serous ovarian carcinoma (SOC) and oesophageal squamous cell carcinoma (ESCC). ALK overexpression, although mechanistically not understood, has been reported in various cancer types and cell lines (pink). ALK mutations comprise a third category (blue), and include both primary tumour-associated mutations and secondary mutations that are observed in crizotinib-resistant patients. Secondary mutations in the context of ALK fusions have been described in NSCLC, IMT and anaplastic thyroid cancer (ATC). ALK point mutations have been found mainly in neuroblastoma, as well as in NSCLC and ATC; most of the mutations are situated within the kinase domain of ALK. ALO17, ALK lymphoma oligomerization partner on chromosome 17; CARS, cysteine-tRNA synthetase; CLTC, clathrin heavy chain; EML4, echinoderm microtubule-associated protein-like 4; FN1, fibronectin 1; KIF5B, kinesin family member 5B; KLC1, kinesin light chain 1; MSN, moesin; MYH9, myosin heavy chain 9; NPM, nucleophosmin; PPFIBP1, PTPRF-interacting protein-binding protein 1; PTPN3, protein tyrosine phosphatase non-receptor type 3; RANBP2, RAN-binding protein 2; SQSTM1, sequestosome 1; STRN, striatin; TFG, TRK-fused gene; TPM, tropomyosin; VCL, vinculin.

Investigation of ALK signalling in model systems has led to many insights into the function of this RTK in developmental processes. There are a number of remaining issues, such as that of a Jeb- or HEN-1-like ligand for vertebrate ALKs — whether one exists at all, and if so what role it has in the development of tissues such as the neural crest in vertebrates. With their controlled genetic backgrounds, model systems have the potential to provide useful information on ALK mutants found in human cancer and on the sensitivity of these mutants to various inhibitors.

ALK signalling. A complete picture of ALK signalling is challenging to piece together, as current information is gleaned from the study of multiple forms of activated ALK (fusion proteins, cancer-associated mutants and amplifications), and we should therefore be cautious in how we apply this knowledge. Most information regarding ALK signalling comes from studies of oncogenic ALK fusion proteins such as NPM–ALK and echinoderm microtubule-associated protein-like 4 (EML4)–ALK, and has been complemented in recent years by studies of oncogenic full-length ALK mutants, such as those identified in neuroblastoma. Targets of the ALK fusion proteins are likely to include both 'legal' and 'illegal' targets and pathways, given that ALK activity has lost its normal spatial and temporal restraints, which are determined by the respective fusion partners. The different fusion partners affect ALK homodimerization, as well as ALK signalling potential[57]. Indeed, the comparison of a number of ALK fusion proteins suggests differences in transforming and tumorigenic potential[58].

In terms of general signalling output, ALK activates multiple pathways, including phospholipase Cγ (PLCγ), Janus kinase (JAK)–signal transducer and activator of transcription (STAT), PI3K–AKT, mTOR, sonic hedgehog (SHH), JUNB, CRKL–C3G (also known as RAPGEF1)–RAP1 GTPase and MAPK signalling cascades, which affect cell growth, transformation and anti-apoptotic signalling[5,6,59,60] (FIG. 3). ALK signalling involves adaptors such as Suc1-associated neurotrophic factor target 2 (SNT2; also known as FRS3)–fibroblast growth factor receptor substrate 2 (FRS2), insulin receptor substrate 2 (IRS2), SHC and growth factor receptor-bound protein 2 (GRB2), and it also affects more downstream targets, such as BCL-2-interacting mediator of cell

death (BIM; also known as BCL2L11), p27 (also known as KIP1 and CDKN1B) and cyclin D2, which are vital components for cell survival and growth[5,6,59,60]. Other interesting but less well characterized targets of ALK fusion protein signalling include nuclear interacting partner of ALK (NIPA; also known as ZC3HC1)[61,62], the small GTPases RAC1 and cell division control protein 42 (CDC42)[63,64], p130CAS (also known as BCAR1)[65], the cytoplasmic protein tyrosine phosphatases SHP1 (also known as PTPN6) and SHP2 (also known as PTPN11), SRC[66–68], and the lipid kinase FYVE finger-containing phosphoinositide kinase (PIKFYVE)[69]. Novel ALK targets have been identified by proteomics efforts, leading to a substantial list of potential players in ALK signalling[67,70–78]. This list is complemented by transcriptome-based approaches that have identified several ALK-regulated genes[79]. Work in the neuroblastoma field has identified *MYCN* as a transcriptional target of activated, full-length ALK[80]. A number of additional genes transactivated in this case by NPM–ALK activity have also been reported, some of which have been validated by small interfering RNA (siRNA) analyses, such as *JUNB,* Y-box transcription factor (*YBX1*), *BCL2A1,* matrix metalloproteinase 9 (*MMP9*), *CDKN2A* and hypoxia-inducible factor 1α (*HIF1A*)[79–83]. There are also reports of microRNAs involved in ALK signalling, with miR-135b, miR-29a and miR-16 being downstream of NPM–ALK, as well as miR-96-mediated regulation of ALK expression[84–87].

The signalling pathways used by the different ALK fusion proteins and the full-length activated receptor share common components, but given the variable cellular contexts, a number of differences can be expected on the basis of tumour type and, when applicable, on the identity of the ALK fusion partner. It is clear that many questions about activated ALK signalling remain to be answered, not only for the numerous ALK fusion proteins but also for the wild-type and mutant versions of ALK. Issues that are important to address for future therapeutic treatments include identifying the ALK

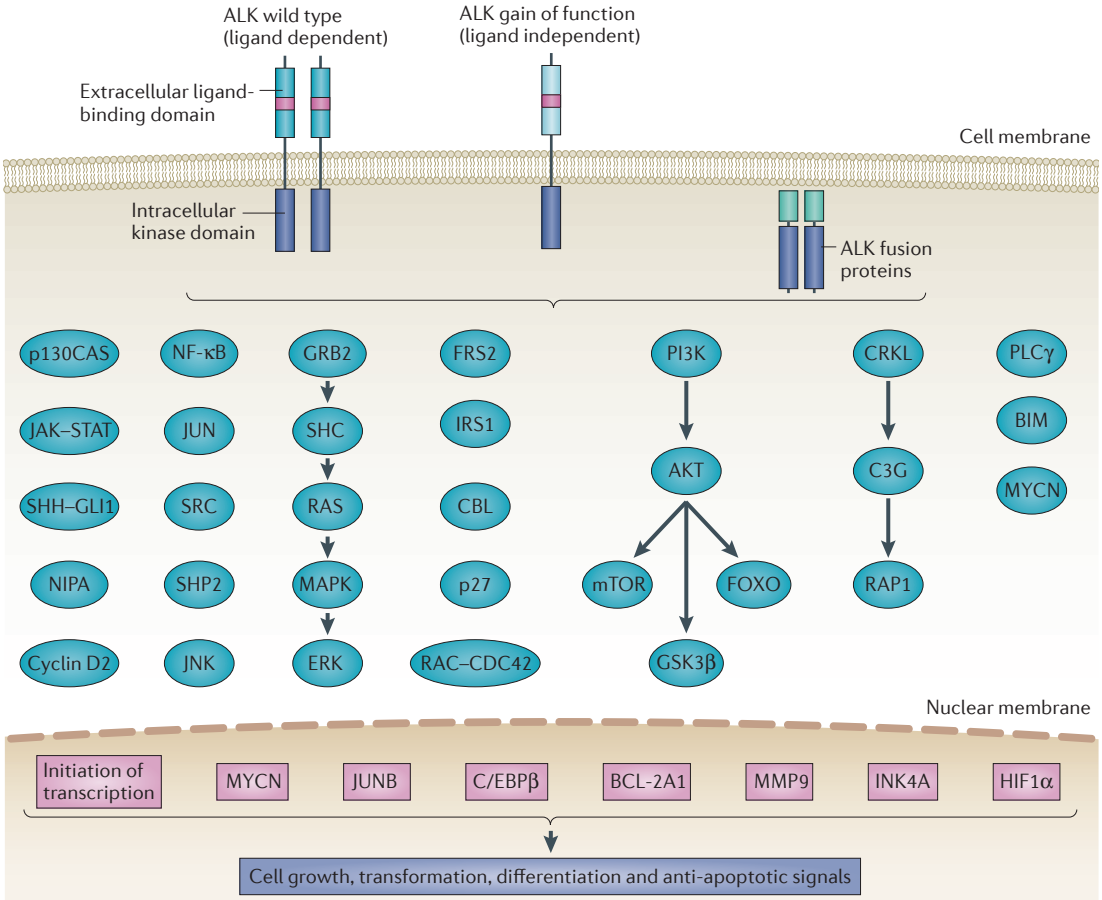
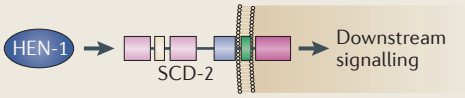
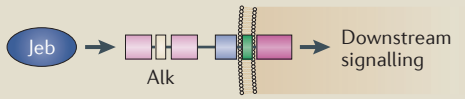
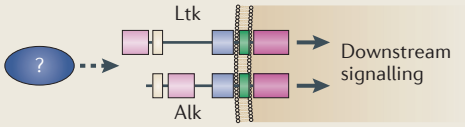


Figure 2 | **Signalling downstream of ALK.** Anaplastic lymphoma kinase (ALK) mediates signalling via the RAS–MAPK, PI3K–mTOR, phospholipase Cγ (PLCγ), RAP1, Janus kinase (JAK)–signal transducer and activator of transcription (STAT) and JUN pathways. Proteins such as insulin receptor substrate 1 (IRS1), SHC, growth factor receptor-bound protein 2 (GRB2), SHP2, C3G, CBL, CRKL and fibroblast growth factor receptor substrate 2 (FRS2) interact with and are phosphorylated by ALK when it is activated. ALK also regulates a number of genes at the transcriptional level, some of which have been validated, such as those encoding MYCN, JUNB, CCAAT/enhancer-binding protein-β (C/EBPβ) and BCL-2A1. BIM, BCL-2-interacting mediator of cell death; CDC42, cell division control protein 42; FOXO, forkhead box O; GSK3β, glycogen synthase kinase 3β; JNK, JUN N-terminal kinase; HIF1α, hypoxia-inducible factor 1α; MMP9, matrix metalloproteinase 9; NF-κB, nuclear factor-κB; NIPA, nuclear interacting partner of ALK; SHH, sonic hedgehog.

Table 1 | **Overview of ALK signalling pathways in model organisms**

Species	Developmental process	Refs
Caenorhabditis elegans (ligand = HEN-1; receptor = SCD-2) 	Synapse formation	31
	Ageing	210
	Sensory behavioural response	40,211–213
	Dauer formation	32,45
Drosophila melanogaster (ligand = Jeb; receptor = Alk) 	Embryonic visceral muscle	33,36–39,214,215
	Neuromuscular junction	42,216
	Central nervous system	29,41,43,44
Danio rerio (ligand = unknown; receptors = Ltk (shady) and Alk) 	Ltk: neural crest iridophore development	35
	Alk: neurogenesis	34

ALK, anaplastic lymphoma kinase; Jeb, Jelly belly; HEN-1, hesitation behaviour 1; Ltk, leukocyte tyrosine kinase; SCD-2, suppressor of constitutive dauer formation 2.

ligand in mammalian systems, which has the potential to improve our understanding of ALK overexpression in cancer.

ALK alterations in human cancer

Translocations involving the ALK locus. For reasons that are not well understood, ALK seems to be a 'hotspot' for translocation to a wide variety of loci. The list of translocations involving ALK has lengthened considerably since the original discovery of NPM–ALK in ALCL[1]. The various translocations lead to the production of ALK fusion proteins that dimerize to constitutively activate ALK. Such ALK fusions function as oncogenic drivers in the progression of both haematopoietic malignancies and solid tumours[6,59] (FIG. 1). ALK fusions have been reported in inflammatory myofibroblastic tumour (IMT), non-small-cell lung cancer (NSCLC), diffuse large B cell lymphoma (DLBCL), squamous cell carcinoma (SCC) and renal cell carcinoma (RCC) (FIG. 1).

So far, 22 different genes have been described as being translocated with *ALK* and, to add to the complexity, within the different ALK fusions there are examples of several breakpoint variants, as illustrated by the *EML4–ALK* translocations observed in NSCLC, by which multiple *EML4* exon breakpoints fuse in-frame with exon 20 of *ALK*[88]. In ALCL, which mostly affects children and young adults, the most well-studied *ALK* translocation product is NPM–ALK, which occurs in up to 80% of cases[1,89–91]. However, ALK has many translocation partners in ALCL, such as ring finger protein 213 (*RNF213*; also known as *ALO17*)[92], *ATIC*[92–94], TRK-fused gene (*TFG*)[95,96], moesin (*MSN*)[97], tropomyosin 3 (*TPM3*) and *TPM4* (REFS 98–100), myosin heavy chain 9 (*MYH9*)[101], and clathrin heavy chain (*CLTC*)[102] (FIG. 1).

IMT, which mostly occurs in young patients, was the first solid tumour to be associated with *ALK* translocation[103]. As with ALCL, ALK fusions in IMT involve a number of different partners, such as *TPM3*, *TPM4* (REF. 104), *CLTC*[105,106], cysteinyl-tRNA synthetase (*CARS*)[107], *ATIC*[108], *SEC31A*[109], PTPRF-interacting protein-binding protein 1 (*PPFIBP1*)[110] and RAN-binding protein 2 (*RANBP2*)[111] (FIG. 1). Indeed, up to 50% of IMT cases contain an *ALK* rearrangement. Of note, in ALCL and IMT, the presence of *ALK* translocations is associated with a better prognosis[91,112–116].

By contrast, *ALK*-translocation-positive DLBCL, which is a rare B cell lymphoma, is correlated with aggressive disease progression[117–121], and this patient group might benefit from individually tailored therapeutic approaches that target ALK. The most frequent chromosomal rearrangement of *ALK* in DLBCL is the t(2;17)(p23;q23) translocation, which generates CLTC–ALK[122]. Other ALK fusions reported in DLBCL are NPM–ALK, sequestosome 1 (SQSTM1)–ALK and SEC31A–ALK[123–126].

Although the proportion of patients with NSCLC harbouring ALK fusions is smaller than occurs in, for example, ALCL or IMT, the higher worldwide incidence of lung cancer, with 1.4 million deaths per year, means that NSCLC contributes by far the largest ALK-fusion-positive patient population[127]. The US National Cancer Institute's Lung Cancer Mutation Consortium, among others, reports *ALK* translocations in around 4% of NSCLC cases[128,129]. The EML4–ALK fusion is the most common translocation in NSCLC and was simultaneously identified in two very different experimental approaches[130,131]. Rikova *et al.*[130] used one of the first global phosphotyrosine proteomic analyses of NSCLC cells and identified two ALK fusion proteins: EML4–ALK and TFG–ALK. In a more classical approach, Soda *et al.*[131] used a retroviral cDNA expression library from a lung adenocarcinoma specimen. Other ALK translocations have also been described in NSCLC that arise much less frequently than *EML4–ALK* — namely, kinesin family member 5B (*KIF5B*)–*ALK*, kinesin light chain 1 (*KLC1*)–*ALK*, and protein tyrosine phosphatase non-receptor type 3 (*PTPN3*)–*ALK* and striatin (STRN)–ALK[132–135].

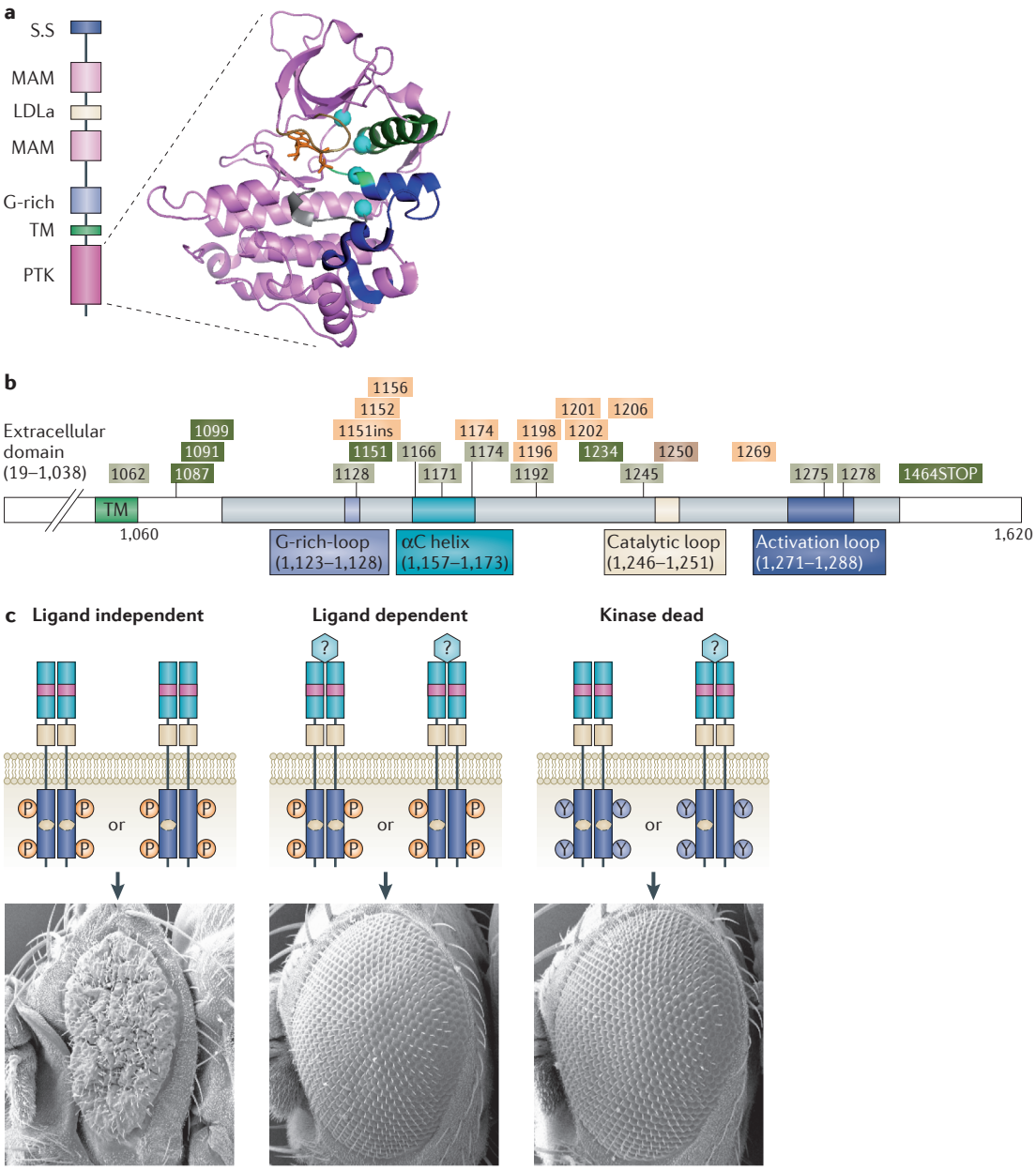


Figure 3 | **Understanding activating ALK mutations at the molecular level.** **a** | The anaplastic lymphoma kinase (ALK) domain structure is shown. The extracellular domain of ALK contains two MAM domains (shown in pink), one LDLa domain (beige) and a glycine-rich extracellular domain (G-rich; blue). A single transmembrane (TM) domain is situated between the extracellular and intracellular portions (green), and the intracellular protein tyrosine kinase (PTK) domain is shown in purple. S.S is the signal sequence. The ribbon diagram of the kinase domain of ALK shows the G-rich loop (1,123–1,128; brown), the αC helix (1,157–1,173; dark green), the catalytic loop (1,246–1,251; grey), DFG motif (lime green) and the activation loop (1,271–1,288; blue). The structure shown is of residues 1,093–1,400 of the ALK intracellular domain bound to ADP (orange)[200,201]. The regulatory spine of ALK, including I1171, C1182, F1271 and H1247, is shown in cyan[200,207]. Structural data are not available for residues 1,062, 1,087 and 1,091. The figure was generated with PyMol using published coordinates (Protein Data Bank code: 3LCS). **b** | Schematic diagram indicating protein structure and boundaries for certain domains of ALK, such as the extracellular domain (19–1,038), the transmembrane domain (1,039–1,060; green), the G-rich loop (1,123–1,128; blue), the αC helix (1,157–1,173; turquoise), the catalytic loop (1,246–1,251; beige) and the activation loop (1,271–1,288; dark blue). Numbers above the schematic diagram indicate verified ligand-independent mutations (pale green), ligand-dependent mutations (dark green) and kinase-dead mutations (brown) in neuroblastomas. Mutations discovered or acquired owing to crizotinib treatment found in patients with non-small-cell lung cancer and inflammatory myofibroblastic tumours are depicted in yellow. **c** | The three classes of ALK mutations in neuroblastoma are shown. Biochemical analysis, cell culture and *in vivo* genetic model systems have been used to classify the different ALK mutations found in patients into three classes: gain-of-function ligand-independent mutations, ligand-dependent mutations and kinase-dead mutations. Scanning electron microscope images of eyes from *Drosophila melanogaster* expressing different ALK mutations are shown.

Retrospective studies suggest that *ALK* rearrangement pre-crizotinib treatment was not a favourable prognostic factor in NSCLC[136]. Currently, patients with NSCLC are examined for a number of well-defined molecular subtypes; patients harbouring the EML4–ALK fusion are regarded as one adenocarcinoma subgroup, and this population is typically younger than the average patient with lung cancer, is never- or non-smoking and is wild type for epidermal growth factor receptor (EGFR) and KRAS[137,138]. Since the initial reports of ALK inhibitors as an effective treatment option[20], the latest report of ongoing Phase III trials indicates a better performance of crizotinib over standard chemotherapy in patients with previously treated, advanced *ALK*-translocation-positive NSCLC[139].

Other *ALK*-translocation-positive malignancies include RCC, colon carcinoma, breast cancer, ovarian cancer and oesophageal squamous cell carcinoma (ESCC). *ALK* translocations with *TPM3*, vinculin (*VCL*) and *EML4* have been identified in RCCs[140,141]. However, in a recent investigation of 534 adult patients with RCC, only two *ALK* rearrangements were identified, both with a poor outcome[142]. Thus, *ALK* rearrangements seem to be uncommon events in RCC. *ALK* rearrangements with *EML4* and *C2orf44* have also been observed in colon carcinomas, and *EML4–ALK* has been reported in breast cancer[143,144]. Recent characterization of primary ovarian tumour tissues identified a novel ALK fusion: fibronectin 1 (FN1)–ALK in patients with serous ovarian carcinoma[145]. Similarly, a low frequency of *ALK* translocation, in this case *TPM4–ALK*, has been described in ESCC[146,147].

Despite the variety of ALK fusion partners, some general features can be noted. First, the initiation of the transcription of ALK fusion proteins is driven by the regulatory regions of the partner gene. Second, the subcellular localization of the fusion protein is determined by the partner protein, which means that ALK activity can occur in the nucleus and/or in the cytoplasm. Finally, the dimerization of ALK fusions occurs through the ALK partner protein and involves trans-autophosphorylation, and thus activation of the ALK kinase domain (FIG. 2).

It is likely that more *ALK*-translocation-positive subpopulations of other cancer types will be identified in the future. This suggests a scenario in which ALK fusions will be expressed in many cancers, although only in a small proportion of patients of any given cancer type. Whether all ALK fusion proteins are equally potent is unclear. Expression levels, the tissues in which the *ALK* translocations occur and the unique signalling properties of the individual ALK fusions all contribute to the final oncogenic outcome. Although not well understood, differences have been reported between a number of ALK fusion proteins with regard to proliferation rate, colony formation, invasion and tumorigenicity owing to activation of various signalling pathways[58]. Moreover, how do we relate the differences we observe in cells growing *in vitro* to the context of the patient tumour and its surrounding stroma?

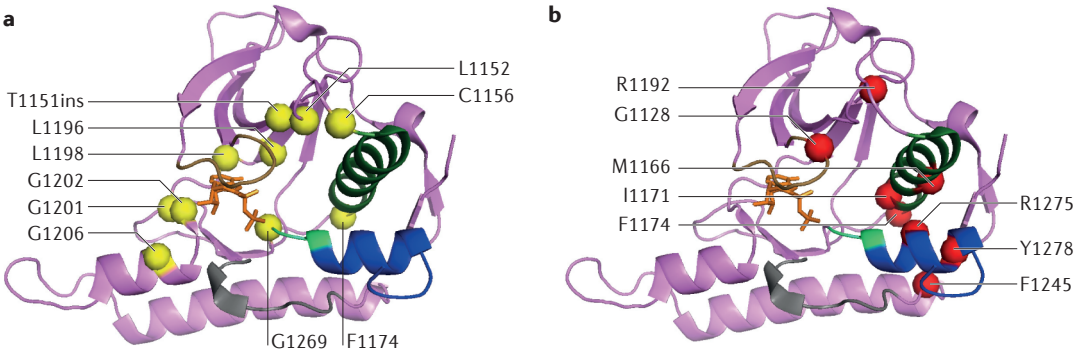
Overexpression of ALK. Amplification of the *ALK* locus and overexpression of ALK protein has been reported in many different types of cancer cell lines and in human tumour samples[148–155] (FIG. 1), but there is little information addressing the importance of these events in the initiation or progression of disease. However, in neuroblastoma, amplification of chromosome 2 — which encodes both *MYCN* and *ALK* — is of particular note given the documented role of ALK and MYCN in driving neuroblastoma and recent results suggesting that they may cooperate in neuroblastoma progression[156,157]. In some cases of neuroblastoma, amplification is accompanied by mutation (discussed below), correlating with poor prognosis[156].

Mutations of ALK. With the approval of crizotinib it has become more important to accurately define the functional effect of ALK mutants in the hope that appropriate treatment protocols can be used for tumours in which ALK is activated by mutation, as well as for those that arise in *ALK* translocations. In addition, a better understanding of the various ALK mutations should aid in revealing the mechanisms by which ALK is activated. Activating point mutations and small deletions in *ALK* have been described in neuroblastoma, thyroid cancer and NSCLC, as well as in crizotinib-resistant *ALK* translocations in NSCLC and IMT[158–163]. Genetic investigations underscore the role of ALK in tumorigenesis, with several groups identifying point mutations in ALK in both familial and sporadic neuroblastoma[48–52]. We focus below on activating ALK mutations in neuroblastoma, a childhood cancer that is considered to be a disease of the developing sympathetic nervous system because it originates from precursor cells of neural crest tissue that are normally only active during embryonic development[164]. Investigation of full-length *ALK* as an oncogene in neuroblastoma should allow us to begin to understand the role of this receptor in the subset of tumours expressing full-length, mutated ALK, as well as during developmental processes.

In the case of ALK fusion proteins, the resultant aberrant ALK activity is dictated by the fusion partner. This is in contrast to ALK mutations, which affect ALK-expressing tissues and presumably perturb physiological ALK signalling processes. Gain-of-function ALK mutations are observed in a substantial number of cases of familial neuroblastoma, as well as in sporadic cases, with two hotspot mutations occurring in the kinase domain: F1174 (mutated to L, S, I, C or V) and R1275 (mutated to Q or L and resulting in a loss of the basic side chain). These hotspot mutations are observed in around 85% of cases with mutant ALK[48–52,156]. Most of the ALK mutations described are located within the kinase domain and several — K1062M, F1174L and R1275Q — have been shown to behave in an oncogenic manner in mice and cell culture models[49,50] (BOX 1; FIG. 2; TABLE 2). For many of the ALK mutations described in neuroblastoma, the assumption of gain of function has been based on structural predictions[52] (BOX 1). Although biochemical examination of ALK mutants in neuroblastoma cell lines is complex owing to their variable

genetic backgrounds, such studies have been complemented by analyses in additional model systems. So far, the accumulated data from such analyses suggest that the different cancer-associated ALK mutations fall into three classes (FIG. 2c). The first class is gain-of-function ligand-independent mutations (such as F1174I, F1174S and F1174L[49,50,53,54,165]) and the second class is ligand-dependent mutations that are not constitutively active and require activation with agonist antibodies[53] (such as D1091N, T1151M and A1234T) (FIG. 2c; TABLE 2). The importance of these mutations, and whether they represent passenger or driver mutations in neuroblastoma, is

Box 1 | **Structural understanding of ALK mutations**



Comparison of the anaplastic lymphoma kinase (ALK) mutations found in neuroblastoma with the secondary crizotinib-resistant mutations reported in non-small-cell lung cancer (NSCLC) and inflammatory myofibroblastic tumour (IMT) highlights some interesting features (see the figure). Although the crizotinib-resistant ALK mutations generally cluster around the inhibitor and ATP-binding site, strongly activating neuroblastoma-associated ALK mutations occur nearby or around the putative residues that are important for initial activation of ALK (in the α-helix of the activation loop and in the αC helix), thereby aiding activation. One exception is the F1174 mutation, which is a hotspot mutation in neuroblastoma and has also been observed as a crizotinib-resistant mutation in a patient with IMT harbouring a RAN-binding protein 2 (*RANBP2*)–*ALK* translocation[52,163]. Neuroblastoma-associated mutations that confer strong ALK activation in *in vitro* assays include: G1128A, F1174L, F1174I, F1174S, R1192Q, F1245C and Y1278S, whereas the mutations M1166R, I1171N and R1275Q function as driver mutations *in vitro* but are unable to transform Ba/F3 cells[49,50,53,54,165,168]. From the crystal structure, residues M1166, R1275 and Y1278 cluster together in the interface between the αC helix and the DFG activation loop, which forms a short α-helix that packs against the αC helix in the inactivated form of ALK[200,201]. A recent crystal structure of the R1275Q mutation shows a destabilized DFG helix, which might indicate a conformational change from an inactive to an active form[205]. Biochemical analysis of the nucleophosmin (NPM)–ALK fusion has defined Y1278 as the first tyrosine to be phosphorylated in the YRASYY sequence that is present in the activation loop of the kinase domain[206]. Mutation of this residue in neuroblastoma is mechanistically intriguing, and it will be interesting to know whether the Y1278S mutant is phosphorylated. In the structure of the R1275Q mutation, although still unphosphorylated, the hydrogen bond between the hydroxyl-group of Y1278 and the backbone nitrogen of C1097 is lost, providing a structural rationale for the activating effect of the R1275Q mutation[200,201,205]. The activating mutations F1174L, F1174I, F1174S (in the αC helix) and F1245C (at the end of the αE helix), together with F1098 (next to the C1097-Y1278 hydrogen bond) and F1271 (in the DFG motif) form a hydrophobic cluster that is thought to regulate the activation process[200,201]. Such structural changes are thought to underlie the observed increased ATP-binding affinity of the ALK-F1174L mutation[168]. This hypothesis has yet to be confirmed, because a recent structure of the F1174L mutation did not show a structural alteration of this region compared with wild type[205]. Although no crystal structure exists of the F1245C mutation, it is thought that the hydrophobic interactions are disrupted, allowing the αC helix to rotate more freely.

A crystal structure for ALK in phosphorylated mode would substantially contribute to our understanding of the activation process and might highlight the importance of the I1171N mutation, which is located in the αC helix and is one of the four residues (I1171, C1182, F1271 and H1247) that make up the regulatory spine of ALK[200,207]. Currently, we can only speculate that the I1171N mutation might activate ALK by locking the regulatory spine in an active conformation.

Glycine-rich loops are present in all canonical protein kinases. During catalysis they function as a clamp covering the β- and γ-phosphates of ATP, and they have an important role in controlling nucleotide affinity and the phosphoryl transfer rate[208]. The G1128A neuroblastoma-associated mutation is situated in the glycine-rich loop (GxGxxG/A) and generates a gain-of-function kinase, the activity of which can be abrogated by ALK inhibitors[54]. Although the mechanism of activation is not clear, this mutation presumably alters the interaction of the kinase with ATP in a manner favouring increased phosphate transfer. Finally, R1192 normally connects to the αC helix with a salt bridge, thus the R1192Q mutation may offer less steric hindrance to the αC helix, which might assume a more kinase-active position.

The figure compares mutations observed in patients with neuroblastoma with those found in patients with crizotinib-resistant NSCLC and IMT. In part **a** of the figure, the crizotinib-resistant ALK mutations from patients with NSCLC and IMT are shown in the ALK kinase domain (yellow balls represent mutated residues; T1151ins indicates an insertion mutation). Part **b** of the figure shows verified gain-of-function mutations found in patients with neuroblastoma (red balls represent mutated residues). Also indicated are the glycine-rich loop (shown in brown), the αC helix (dark green), the catalytic loop (grey), the activation loop (blue) and the DFG motif (green). The figure was generated with PyMol using published coordinates (Protein Data Bank code: 3LCS).

currently unclear. The third class of mutation is kinase dead, but this so far only includes the I1250T mutation, which has been found in two patients[166]. Although kinase-dead ALK may be unable to signal itself, it is not clear whether this mutant is able to affect signalling through the wild-type copy of ALK in tumours. This is important to consider given our current understanding of kinase-dead BRAF, which displays cooperative tumorigenicity with oncogenic RAS[167].

The cataloguing of the activation status of the different ALK mutations is complex because certain mutants (in particular ALK-M1166R, ALK-I1171N and ALK-R1275Q) are able to differentiate PC12 cells, transform NIH3T3 cells and disrupt development in the *D. melanogaster* eye (causing a rough-eye phenotype), but are unable to transform Ba/F3 cells[53,54,168]. Such results suggest that we would be naive to rely on a single system to ascertain the oncogenic potential of ALK mutations and that using multiple systems should increase our understanding. How we then integrate this information into the complex genetic landscape of tumour development *in vivo* is an even greater challenge for the future. Although transgenic mouse models of ALK mutants have recently been reported to drive neuroblastoma formation with MYCN (discussed below), knock in of an activating mutant in the *ALK* locus has not yet been reported and may offer important insights *in vivo*.

Finally, it is important to remember that, in the different model systems, the activity of all neuroblastoma ALK mutations tested thus far can be abrogated by treatment with ALK small molecule inhibitors, such as NVP-TAE 684 and crizotinib, albeit with differing sensitivity[53,54,168,169].

ALK cooperation with other oncogenes: *MYCN*

Does ALK initiate tumour development? Although ALK fusion proteins seem to function as tumour initiators and are involved in the progression of many cancer types, the case for ALK activation as a driver in tumours lacking *ALK* translocations, of which neuroblastoma is currently the most well studied, is not clearcut. Neuroblastoma is heterogeneous and exhibits a number of chromosomal irregularities, such as deletions of regions of chromosomes 1p and 11q, gain of parts of 17q, triploidy and frequent amplification of *MYCN*, which is a prognostic indicator[156,170]. Because both *ALK* and *MYCN* are located in close proximity to each other on chromosome 2p, amplification of *MYCN* can also involve amplification of the *ALK* locus. For patients with neuroblastoma with *MYCN* amplification, *ALK* mutation has been reported to be associated with an unfavourable aggressive neuroblastoma phenotype[49–52,154,156,157,171–174]. When expressed together, ALK and MYCN promote NIH3T3 cell transformation and both wild-type and activated mutant forms of ALK stimulate transcription of *MYCN*[80]. RTKs, such as ALK, activate both ERK and PI3K on ligand stimulation or as a result of an activating mutation[175,176]. Such activity has been suggested to stabilize MYCN[177], which is normally degraded during mitosis in response to low levels of PI3K activity, and persistent high levels of MYCN drive neuroblastoma oncogenesis[178]. The connection between ALK and MYCN has been further explored in transgenic zebrafish and mouse models. In zebrafish, human ALK-F1174L alone does not lead to tumour development; however, the induction of interrenal gland tumours (similar to human neuroblastoma) was significantly increased in the presence of both ALK-F1174L and MYCN[55,179]. Similar results have been observed in mice, in which ALK-F1174L potentiates the oncogenic activity of MYCN in a neuroblastoma mouse model, inducing an earlier onset, higher penetrance and increased lethality[180]. Furthermore, expression of ALK-F1174L increases the endogenous expression of MYCN, owing to increased stabilization and upregulation of endogenous *MYCN* mRNA[180]. An independent transgenic mouse model in which ALK-F1174L is expressed in the neural crest results in tumour development, suggesting that activated ALK drives tumorigenesis[181]. In agreement with Berry *et al.*[180], expression of both ALK-F1174L and MYCN synergized to accelerate neuroblastoma development[181]. The current models have made important initial contributions to our understanding of tumour development in neuroblastoma, but can they be improved? Our understanding of neuroblastoma initiation and progression would be aided by the generation of knock-in ALK mutants or, as Heukamp and co-authors[181] suggest, by using a tamoxifen-dependent Cre model that allows ALK activation at different developmental time points.

ALK mutations in transgenic mouse models may provoke a cancer initiation event that requires a second hit, such as activation of MYCN or other genomic aberrations, which occur over time to promote transformation[182]. Indeed, ALK may cooperate with other oncogenes or in the background of loss of tumour suppressor genes. For example, there is evidence from studies in *D. melanogaster* for interaction between ALK and neurofibromin 1 (*NF1*), a known tumour suppressor in neuroblastoma[43,183]. In human neuroblastoma there are a number of commonly observed chromosome aberrations for which the importance for progression at the molecular level is unclear, such as those on chromosomes 1p, 11q and 17q[170]. The challenge for the future will be to decipher patterns and define key events in the development of tumours expressing non-translocated ALK mutants, perhaps in the manner of the 'Vogelgrams' in colon carcinoma[184,185].

Treatment of ALK-positive tumours

In 2011, crizotinib and the companion ALK break-apart fluorescence *in situ* hybridization (FISH) probe kit were approved by the US Food and Drug Administration (FDA)[16]. Break-apart FISH is the currently recommended methodology for the identification of ALK translocations in NSCLC; however, it is costly, experimentally demanding and not foolproof, and more robust screening methods for *ALK* aberrations, including PCR, immunohistochemistry (IHC) and next-generation sequencing, are being actively explored[186]. Further investigations and the development of antibodies with improved sensitivity and specificity are necessary for the effective identification of patients with ALK-positive tumours in cancer types in which ALK fusion proteins prove difficult to

Rough-eye phenotype
The disruption of the highly organized pattern of the approximately 800 hexagonal ommatidia in the adult *Drosophila melanogaster* eye. A rough-eye phenotype can result from the ectopic expression of activated signalling molecules such as anaplastic lymphoma kinase.

Interrenal gland
A structure in close proximity to or embedded in the kidney of fish. Interrenal glands are homologous to the cortical tissue of the mammalian adrenal gland.

Table 2 | **Reported cancer-associated mutations in human ALK**

Cancer type	Amino acid mutation	Domain in ALK*	Effect on ALK	Source	Refs
Primary ALK mutations					
Neuroblastoma	ALKΔ2–3	Δ224–318, extracellular	GOF, can occur in amplified *ALK*	Tumour and cell line	161
	ALKΔ4–11	Δ318–782, extracellular	GOF, can occur in amplified *ALK*	Tumour and cell line	158
	R1061Q	Juxtamembrane domain	Unknown	Tumour	156
	K1062M	Juxtamembrane domain	GOF	Cell line	49
	T1087I	Juxtamembrane domain	Ligand dependent	Tumour	49,53
	D1091N	β1 strand	Ligand dependent	Tumour	52,53
	A1099T	β2 strand	Ligand dependent	Tumour	53
	G1128A	P loop	GOF	Tumour	52,54
	T1151M	β3 strand	Ligand dependent	Tumour	50,53
	T1151R	β3 strand	Unknown	Tumour	217
	M1166R	αC helix	Ligand dependent or GOF	Tumour	52,53
	I1170T/S	αC helix	Unknown	Tumour	174
	I1171N	αC helix	GOF	Tumour	52,54
	F1174L/S	End of αC helix	GOF	Tumour and cell line	48–52,165
	F1174I	End of αC helix	GOF	Tumour	52,53
	F1174C/V	End of αC helix	Unknown	Tumour and cell line	49–52
	R1192Q	Between β4 and β5 strands	GOF	Tumour	52,54
	R1231Q	αE helix	Unknown	Tumour	156
	A1234T	αE helix	Ligand dependent	Tumour	50,53
	L1240V	αE helix	Unknown	Tumour	174
	F1245C	–2 to HRD	GOF	Tumour	50,52,54
	F1245I/L/V	–2 to HRD	Unknown	Tumour and cell line	48–52
	I1250T	+1 to HRD	Kinase dead	Tumour	52,166
	R1275Q	+2 to DFG	GOF	Tumour and cell line	48–52,54
	R1275L	+2 to DFG	Unknown	Tumour	51
	Y1278S	A loop	Unknown	Tumour	51
	1464STOP	C-terminal to kinase domain	Ligand dependent	Tumour	53
Lung adenocarcinoma	S413N	First MAM domain	Not a driver	Tumour	162
	V597A	Second MAM domain	GOF?	Tumour	162
	H694R	Between second MAM domain and G-rich domain	GOF	Tumour	162
	G881D	G-rich domain	GOF?	Tumour	162
	Y1239H	αE helix	Not a driver	Tumour	162
	D1311A	αF helix	Not a driver	Cell line	218
	E1384K	C-terminal of the kinase domain	GOF	Tumour	162
	K1518N	C-terminal of the kinase domain	Not a driver	Cell line	218
	K1525E	C-terminal of the kinase domain	Not a driver	Cell lines	218
Burkitt's lymphoma	R412C	First MAM domain	Not a driver	Cell line	218
Osteosarcoma	C1021Y	Extracellular, close to TM domain	Not a driver	Cell line	218
Uterine leiomyosarcoma	R1192Q	Between β4 and β5 strands	Not a driver	Cell line	218

Table 2 (cont.) | **Reported cancer-associated mutations in human ALK**

Cancer type	Amino acid mutation	Domain in ALK*	Effect on ALK	Source	Refs
ATC	L1198F	Between β5 strand and αD helix	GOF	Tumour	160
	G1201E	Between β5 strand and αD helix	GOF	Tumour	160
Carcinoma of the endometrium	A1252V	+3 to HRD	Not a driver	Cell line	218
Secondary mutations					
NSCLC (EML4–ALK fusion)	T1151ins	End of β3 strand	GOF	Tumour (CTP)	169
	L1152R	Between β3 strand and αC helix	GOF	Tumour (CTP)	198
	F1174L	End of αC helix	GOF	Tumour (CTP)	163
	L1196M	Gateway mutation	GOF	Tumour (CTP)	159,169,199
	G1202R	Between β5 strand and αD helix	GOF	Tumour (CTP)	169
	S1206Y	In αD helix	GOF	Tumour (CTP)	169
	G1269A	–1 to DFG	GOF	Tumour (CTP)	199
IMT (RANBP2–ALK fusion)	C1156Y	Between β3 strand and αC helix	GOF	Tumour (CTP)	159
Experimentally generated ALK mutations					
Experimentally generated (EML4–ALK fusion)	F1174L + L1198P	αC helix + between β5 strand and αD helix	GOF	PRM	219
	F1174L + G1123S/D	αC helix + between β1 and β2 strands	GOF	PRM	219
	L1198P	Between β5 strand and αD helix	GOF	SME	219
	G1269S	–1 to DFG	GOF	CME	219
	D1203N	Between β5 strand and αD helix	GOF	SME	219
	Y1278H/G1123S or D	1278-YRASYY-1283	Not determined	PRM	219

ALK, anaplastic lymphoma kinase; ATC, anaplastic thyroid cancer; CME, chemical mutagen (ENU) screen; CTP, crizotinib-treated patient; GOF, gain of function; IMT, inflammatory myofibroblastic tumour; NSCLC, non-small-cell lung cancer; PRM, PCR-based random mutagenesis screen; RANBP2, RAN-binding protein 2; SME, saturation mutagenesis screen; TM, transmembrane. *According to REF. 200.

detect. This can be compared with tumour types in which the expression of ALK fusion proteins is high, such as ALCL, for which screening of *ALK* translocation is already routine[187]. In addition to considering initial diagnostics, the importance of tumour re-biopsy and examination for ALK lesions during disease progression also needs to be considered. This is important given the emergence of resistance mutations in *ALK* fusion oncogenes in patients treated with crizotinib[159,188], as well as the emergence of activating ALK mutations later in the disease course of one patient with neuroblastoma that was wild-type for ALK on initial diagnosis[165].

It is now clear that the usefulness of long-term crizotinib treatment, as with other TKIs, is limited owing to the development of drug resistance (discussed below). Acquired mutations in ALK that confer crizotinib resistance are a serious complication in the treatment of cancer patients, which may be overcome by the development of more effective drugs, as well as by combination therapy strategies[189]. Future approaches should be aided by improved and ever-cheaper genome technologies, such as whole-exome, whole-genome and transcriptome sequencing[170,190]. Our current knowledge already paints a complex picture of individual cancers, highlighting not only potential initiating (driver) mutations but also progression mechanisms that may be exploited in treatment regimens. The challenge is to interpret this information and apply combination therapies that are tailored to the genomic landscape of the individual tumour. Currently, several ongoing clinical trials address combinatorial treatments for ALK-positive tumours, including one examining the use of heat shock protein 90 (HSP90) inhibitors together with either of the ALK TKIs crizotinib or LDK378 for NSCLC (ClinicalTrials.gov identifiers: NCT01712217, NCT01121575, NCT01579994 and NCT01772797). The use of HSP90 inhibitors is motivated by the knowledge that ALK is an HSP90 client and HSP90 antagonists have been reported to disrupt the ALK fusion protein–HSP90 complex, leading to the degradation of the ALK fusion protein[191–194]. Studies in patients with NSCLC suggest that HSP90 inhibitors have clinical activity in patients carrying *ALK* translocations in particular[195]. HSP90 inhibitors (in combination with ALK inhibition) have also been reported to block the activity of crizotinib-resistant ALK mutants[169]. Work in mice suggests that other combinations with ALK TKIs may be worthy of future exploration in patients; for example, mTOR inhibitors have been shown to be effective in a mouse model of ALK- and MYCN-driven neuroblastoma[180]. However, one important issue underlying the development of combination therapies is the fact that different drugs originate from different companies, therefore more focus on coordinating efforts rather than on profit margins is required for longer term

patient benefits. Other important considerations with combination strategies are toxicity and the difficulty in achieving therapeutic target inhibition when two drugs are given in combination.

Given that crizotinib has fairly modest adverse effects and that ALK does not seem to have an obvious role in mouse development, treatment of young patients with ALK-associated tumours may be viable. Indeed, the results of ongoing paediatric trials from the Children's Oncology Group show that crizotinib is well tolerated and may be an effective therapeutic treatment strategy for some children with certain categories of ALK-positive tumours, particularly those with ALK-positive ALCL, IMT and NSCLC[196]. The story becomes more complex in ALK-positive neuroblastoma, for which treatment with crizotinib shows variable responses, possibly reflecting the heterogeneous nature of neuroblastoma in terms of chromosomal irregularities[170]. In such cases, combination of ALK inhibitors with other inhibitors might be an option in the future, with the challenge being to distinguish relevant subsets of patients with neuroblastoma for such individualized therapeutic treatment[196].

ALK and resistance mechanisms. Initial reports of the promising therapeutic potential of ALK TKIs for the treatment of ALK-positive patients with NSCLC was accompanied by the identification of crizotinib-induced secondary mutations — C1156Y and L1196M — in the ALK kinase domain[159,188] (BOX 1). The L1196M mutation is equivalent to the gatekeeper mutations EGFR-T790M and ABL1-T315I, as ALK-L1196M interferes with crizotinib binding through steric hindrance[159] and, in comparison to EGFR-T790M, allows continued ALK activation in the presence of crizotinib[197]. The mechanism by which other secondary ALK mutations, such as C1156Y[159], L1152R and the insertion mutation T1151ins (REFS 169,198), lead to the activation of ALK in the presence of an ALK TKI is still unclear. Because no structure is currently available for active ALK, it is difficult to project the conformational disturbance that presumably results from these secondary mutations. Interestingly, ALK is also mutated in this region in neuroblastoma, and ALK-T1151M has been shown to behave as a ligand-dependent receptor in a manner that is comparable to wild-type ALK[53]. This suggests that similar mutations, in the context of *ALK* fusion oncogenes, do not activate the kinase per se, but rather subtly alter the affinity of the kinase domain for crizotinib and thus confer resistance.

The ALK-G1269A mutation has been reported in two patients with crizotinib-resistant NSCLC[199]. The location of the G1269A mutation (in the DFG -1 position of the activation segment of the kinase domain) is crucially situated for ATP or crizotinib binding and might reduce crizotinib binding owing to steric hindrance or by increasing the affinity for ATP at the expense of the affinity for crizotinib, in a similar manner to the L1196M mutation[197]. Indeed, G1269 is an important residue in ALK that contributes to the ATP-binding cavity, thereby reducing access to V1180 at the back of the ATP pocket[200,201].

Other ALK mutations that are relevant to crizotinib response include G1202R and S1206Y, which occur in the EML4–ALK fusion protein and which were identified in patients with NSCLC[169]. Interestingly, ALK-G1202R is analogous to the experimentally induced BCR–ABL mutation G340W and might represent a similar mechanism of activation[202]. The L1198F, G1201E, G1202R and S1206Y mutations are positioned in the ALK kinase domain adjacent to the crizotinib- and ATP-binding site and are thought to decrease the affinity of mutant ALK for crizotinib. Several of these mutations confer a high level of resistance to crizotinib[160,169]. Also important to note are two ALK mutations at functionally intriguing positions reported in anaplastic thyroid cancer: L1198F and G1201E (rather than the F1174 and R1275 hotspot mutations in neuroblastoma)[160] (BOX 1).

There are additional alternative methods of overcoming ALK inhibition. These include *ALK* amplification, aberrant amplification of *KIT*, and mutation of *KRAS* and *EGFR*, which have all been identified in patient samples[169,199]. These alterations bypass the requirement for ALK activity in the tumour cells. Thus, crizotinib resistance is a complex affair that includes ALK kinase domain mutations, copy number gain of *ALK* fusions and *ALK*, and other oncogenes.

Conclusions and perspectives

The evidence linking ALK to various human cancer types is now well accepted, and exciting developments in ALK research in recent years hold promise for the treatment of ALK-positive patients. However, the accumulated results of patients treated with crizotinib paint a picture of tumour resistance in response to this targeted therapy, leaving important issues for the research community to resolve. It remains to be seen whether the lessons learned from the study of tumours expressing ALK fusion oncoproteins are similar to those of tumours expressing point mutations. Indeed, in tumours in which full-length ALK is overexpressed, questions such as the identity and potential role of ALK ligands become important. Efforts in a number of model systems will hopefully address these issues in the future.

Although most available data about targeting ALK concern crizotinib, the new generation of drugs, including LDK378 (an ALK inhibitor), 3–39 (an ALK inhibitor), AP26113 (a dual EGFR and ALK inhibitor), CH5424802 (an ALK inhibitor), ASP3026 (an ALK inhibitor), X-396 (an ALK inhibitor) and IPI504 (an HSP90 inhibitor), all offer potential therapeutic options for ALK-positive tumours[136,189]. In the case of tumours in which activated ALK is mutated without truncating the protein, such as in neuroblastoma, the use of ALK inhibitory antibodies is also being actively explored as a therapeutic option[13,203,204]. An additional approach that is being explored for the treatment of ALK-positive tumours is vaccination with ALK oncoantigens[149]. Time will tell how well these promising approaches will be integrated into clinical reality. To do this effectively, skilled translational teams will need to process tumours and identify mutations, copy number changes, translocations and aberrant gene expression to allow effective individual cancer treatment.

Gatekeeper mutations
Mutations of crucial amino acid side chains in many kinases that determine the relative accessibility of the hydrophobic inhibitor-binding pocket, which is located adjacent to the ATP-binding site. The gatekeeper residue does not usually interact with ATP, thus its mutation does not affect the catalytic activity of the enzyme but most commonly interferes with the ability of the small-molecule inhibitor to bind effectively to the enzyme, thus conferring inhibitor resistance.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

DATABASES
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CORRIGENDUM

Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology

Bengt Hallberg & Ruth H. Palmer

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In the legend to Figure 3b, the labels for kinase-dead mutations and crizotinib-associated mutations were incorrect and should have read brown and yellow, respectively. This has now been corrected online.