

REVIEW ARTICLE

**Anaplastic lymphoma kinase: signalling in development and disease**

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RTKs (receptor tyrosine kinases) play important roles in cellular proliferation and differentiation. In addition, RTKs reveal oncogenic potential when their kinase activities are constitutively enhanced by point mutation, amplification or rearrangement of the corresponding genes. The ALK (anaplastic lymphoma kinase) RTK was originally identified as a member of the insulin receptor subfamily of RTKs that acquires transforming capability when truncated and fused to NPM (nucleophosmin) in the t(2;5) chromosomal rearrangement associated with ALCL (anaplastic large cell lymphoma). To date, many chromosomal rearrangements leading to enhanced ALK activity have been described and are implicated in a number of cancer types. Recent

reports of the EML4 (echinoderm microtubule-associated protein like 4)–ALK oncoprotein in NSCLC (non-small cell lung cancer), together with the identification of activating point mutations in neuroblastoma, have highlighted ALK as a significant player and target for drug development in cancer. In the present review we address the role of ALK in development and disease and discuss implications for the future.

**Key words:** anaplastic lymphoma kinase (ALK), anaplastic large cell lymphoma (ALCL), extracellular-signal-regulated kinase (ERK), inflammatory myofibroblastic tumour (IMT), midkine, neuroblastoma, non-small cell lung cancer (NSCLC), pleiotrophin.

**DISCOVERY OF THE ALK (ANAPLASTIC LYMPHOMA KINASE) RTK (RECEPTOR TYROSINE KINASE)**

ALK was originally described as a novel tyrosine phosphoprotein in ALCL (anaplastic large cell lymphoma) cell lines in 1994 [1,2]. The identity of this protein was revealed as a chimaeric protein created via a translocation event between chromosomes (2;5)(p23;q35), generating a previously uncharacterized fusion protein, NPM (nucleophosmin)–ALK. NPM–ALK contains the N-terminal portion of the NPM protein and the kinase domain of a then novel tyrosine kinase protein that was named ALK after the disease [1].

**STRUCTURAL FEATURES OF ALK**

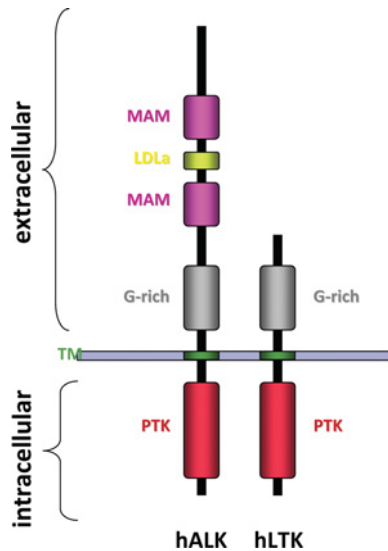
The intriguing characteristics of the full-length ALK RTK were not revealed until 1997, when they were simultaneously reported by two groups [3,4]. ALK displays the classical structural features of a RTK, with an extracellular ligand-binding domain, a transmembrane-spanning region and an intracellular tyrosine kinase domain. Based on overall homology, ALK is grouped with LTK (leucocyte tyrosine kinase), and together they form their own subgroup within the IR (insulin receptor) superfamily [3,4]. The human ALK gene encodes a protein of 1620 amino acids giving rise to a protein of approx. 180 kDa. However, as a result

of post-translational modifications such as N-linked glycosylations, ALK migrates at approx. 220 kDa on SDS/PAGE [3,4]. The ALK extracellular region contains a unique combination of domains among the RTKs, exhibiting an N-terminal signal peptide, followed by two MAM (meprin, A5 protein and receptor protein tyrosine phosphatase mu) domains, an LDLa (low-density lipoprotein class A) motif and a large glycine-rich region proximal to the membrane [3–6] (Figure 1).

Within ALK, the LDLa domain has an unknown function. However, this module mediates the binding between the LDL-receptor and LDL [7,8], suggesting a potential role in ligand binding for this domain of ALK. MAM domains are thought to participate in cell–cell interactions [9], but their significance for ALK function is unclear. The importance of the MAM domain is nevertheless emphasized in studies from *Drosophila* in which a point mutation altering a highly conserved aspartic acid residue in the MAM domain to arginine renders dALK (*Drosophila* ALK) inactive [10]. The functional significance of the glycine-rich domain has also been reported in *Drosophila*, where several loss-of-function dALK mutants display point mutations that convert a single glycine residue within the glycine-rich region into an acidic amino acid [10]. The domain organization of ALK is conserved throughout evolution, with the highest conservation found in the kinase domains. In fact,

Abbreviations used: ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; ALO17, ALK lymphoma oligomerization partner on chromosome 17; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase; BCR-Abl, breakpoint cluster region-Abl; CARS, cysteinyl-tRNA synthetase; Cdc42, cell division cycle 42; C/EBPβ, CCAAT/enhancer-binding protein β; CLTC, clathrin heavy chain; CML, chronic myeloid leukaemia; CNS, central nervous system; dALK, *Drosophila* ALK; DLBCL, diffuse large B-cell lymphoma; Dpp, decapentaplegic; DRG, dorsal root ganglia; EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein like 4; ERK, extracellular-signal-regulated kinase; FOXO3a, forkhead box O 3a; FRS2, fibroblast growth factor receptor substrate 2; GIST, gastrointestinal stromal tumour; Grb2, growth-factor-receptor-bound protein 2; HEK, human embryonic kidney; Hen-1, hesitation-1; IL-3, interleukin-3; IMT, inflammatory myofibroblastic tumour; IR, insulin receptor; IRS-1, IR substrate-1; JAK, Janus kinase; Jeb, jelly belly; JNK, c-Jun N-terminal kinase; LDLa, low-density lipoprotein class A; LTK, leucocyte tyrosine kinase; MAM, meprin, A5 protein and receptor; EML4, echinoderm microtubule-associated protein like 4; ERK, extracellular-signal-regulated kinase; MEK, MAPK/ERK kinase; MK, midkine; MSN, moesin; mTOR, mammalian target of rapamycin; MUC-1, mucin-1; MYH9, non-muscle myosin heavy chain; NF-κB, nuclear factor κB; NIPA, nuclear interacting partner of ALK; NPM, nucleophosmin; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PLCγ, phospholipase Cγ; PTN, pleiotrophin; RANBP2, Ran-binding protein 2; RPTP, receptor protein tyrosine phosphatase; RTK, receptor tyrosine kinase; SCC, squamous cell carcinoma; SCD-2, suppressor of constitutive dauer 2; SEC31L1, SEC31 homologue A; SH2, Src homology 2; Shc, Src homology and collagen homology; SHH, sonic hedgehog; Shp1, SH2 domain-containing phosphatase 1; STAT, signal transducer and activator of transcription; TFG, TRK-fused gene; TGFβ, transforming growth factor β; TPM, tropomyosin; UCN-01, unco-ordinated 1.

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**Figure 1** Domain structure of human ALK and human LTK

The N-terminal region of human ALK (hALK) comprises two MAM domains (amino acids 264–427 and 480–626), one LDLa domain (amino acids 453–471) and a glycine rich (G-rich) region (amino acids 816–940). A transmembrane (TM)-spanning segment, connects the extracellular region with the protein tyrosine kinase (PTK), domain (amino acids 1116–1383)-containing intracellular region. The closest family member, LTK [hLTK (human LTK)], is depicted with the corresponding regions denoted. The signal peptide (amino acids 1–16), the glycine rich, G-rich, domain (amino acids 63–334) and the kinase domain (amino acids 510–777) located in the intracellular C-terminal region of the protein.

mouse and human ALK show 87% overall homology at the protein level, and within the kinase domain these differ at only four amino acids. Although mouse and human ALK are highly similar, it should be noted that human ALK contains one extra tyrosine residue, Tyr<sup>604</sup>, which has been implicated in tumour progression [11] (Figure 2). Within the activation loop of the kinase domain, ALK contains a YxxxYY motif in common with the IR. It has been reported that phosphorylation of the first tyrosine residue (Tyr<sup>1278</sup>) of this YxxxYY motif is predominant in the autoactivation of the ALK kinase domain. Phosphorylation of Tyr<sup>1278</sup> appears to be determined in part by the intervening RAS amino acid triplet in the activation loop (Y'RAS'YY), immediately following Tyr<sup>1278</sup> of ALK, which differs from the activation loop of the IR RTK [12,13].

## ALK FUNCTION IN MODEL ORGANISMS

### dALK

The *in vivo* function of ALK has been most thoroughly studied in *Drosophila melanogaster* where ALK was initially shown to drive ERK (extracellular-signal-regulated kinase) activation *in vivo* [5]. During embryonic development of the fruitfly, dALK plays a vital role in the formation of the visceral musculature of the gut [10]. In the absence of dALK, *Drosophila* embryos hatch into gut-less larvae, which consequently die. This phenotype is due to a lack of specification of a particular cell type, the founder cell, in the developing gut musculature of *Drosophila* embryos deficient in dALK signalling [14–16]. Activation of the dALK signal transduction pathway in wild-type flies is initiated by binding of the Jeb (jelly belly) ligand to a specific set of cells in the embryonic visceral mesoderm, which thereby are specified as founder cells. Founder cells then fuse with fusion competent myoblasts to give rise to the multinucleated visceral musculature of the gut [14–18]. Since dALK signalling specifies founder cells,

loss of dALK results in an absence of founder cells, as well as muscle cell fusion, which as a consequence leads to defective assembly of a functional gut musculature in dALK mutant flies.

The Jeb protein is now well-established as an activating ligand for dALK, and as such is also required for founder-cell specification [14–16]. Jeb is a secreted protein of approx. 61 kDa containing a secretory signal and an LDLa domain [19]. The interaction of Jeb and dALK appears to be mediated via the LDLa domain in Jeb, since a Jeb mutant protein lacking the LDLa domain is unable to bind dALK [15]. Activation of the Jeb/dALK signalling pathway leads to ERK activation and further downstream transcription of target molecules such as Duf (dumb-founded)/Kirre (kin of irregular chiasm) [14–16], Org [15], Hand [20] and Dpp (decapentaplegic) [21] in the fruitfly (Figure 2).

The Jeb/dALK signalling pathway is also critical for development of the *Drosophila* embryonic endoderm in an indirect manner, since dALK activity in the visceral mesoderm is required for Dpp [TGF $\beta$  (transforming growth factor  $\beta$ )] transcription and subsequent signalling in the adjacent endoderm [21]. Moreover, dALK and Jeb play a central role acting as an anterograde signalling pathway mediating neuronal circuit assembly in the *Drosophila* visual system. In the developing *Drosophila* eye, dALK is expressed in, and required for, the targeting of neurons in the optic lobe, whereas Jeb is primarily produced by photoreceptor axons, functioning to control target selection of R1–R6 axons in the lamina and R8 axons in the medulla. Lack of either protein results in mistargeting of the R8 axons during later maturation of the optic lobe neuropile [22].

### *Caenorhabditis elegans* ALK [SCD-2 (suppressor of constitutive dauer-2)]

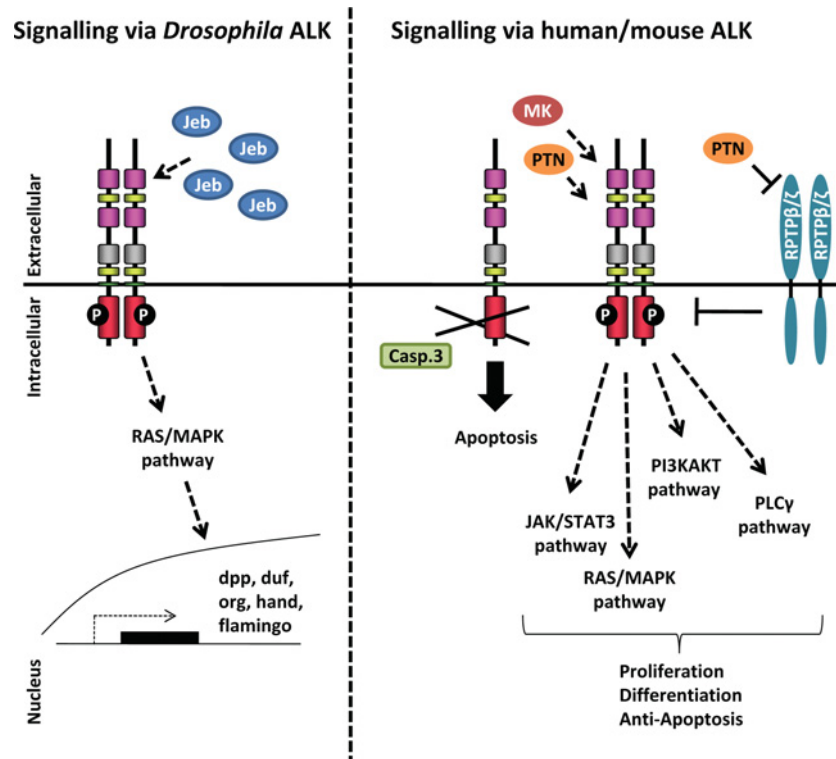
In *C. elegans*, ALK has been implicated in neuronal control of entry into dauer and synapse stabilization [23,24]. *C. elegans* ALK (T10H9.2), now formally known as SCD-2, was identified as an effector through which the F-box protein Fsn-1 (F-box protein at the synapse-1) stabilizes synapse formation in GABAergic neuromuscular junctions [23]. More recently, SCD-2 mutants were characterized which display a temperature-sensitive adaptation to dauer entry [24]. Epistatic analysis in *C. elegans* suggests that SCD-2 modulates the TGF $\beta$  pathway upstream of Daf3 (abnormal dauer formation 3; Smad)/Daf5 (Sno/Ski) [24]. The SCD-2 ligand has been identified as Hen-1 (hesitation-1) [25], which like *Drosophila* Jeb is a secreted ligand containing an LDLa domain, and the genetically mapped signal transduction pathway employs the adaptor SOC-1 (suppressor of Clr-1) and the MAPK (mitogen-activated protein kinase) SMA-5 (small body size-5) [24].

### Zebrafish LTK/ALK

Elegant studies in the zebrafish *Danio rerio* have recently demonstrated an *in vivo* role for LTK. During zebrafish embryogenesis LTK signalling leads to specification of iridophores from the neural crest lineage, and mutants in LTK (known as *shady*) display defects in pigmentation patterns [26]. The LTK/ALK family in zebrafish comprises two genes: LTK and ALK, both of which contain MAM domains [26]. This is in contrast with mouse and human LTK which both lack MAM domains. Although zebrafish LTK has no reported ligand to date, its expression in the developing neural crest is of particular interest given the recent reports of human ALK activating mutations in neuroblastoma (see below).

### MAMMALIAN ALK

Mammalian ALK has been postulated to play a role in the normal development and function of the nervous system, a



**Figure 2** Signalling via ALK

Signalling via dALK occurs via binding of the ligand Jeb, downstream activation of ERK and transcription of downstream target genes. Signalling via mammalian ALK is thought to occur via ligand-mediated dimerization in response to the MK and PTN ligands. ALK mediates signalling via the JAK/STAT, RAS/MAPK, PI3K and PLC $\gamma$  pathways. Activation of ALK via RPTP $\beta/\zeta$ , independently of direct ALK–ligand interactions has also been proposed. Lastly, ALK is proposed to function as a dependency receptor which is cleaved by caspase 3 (Casp. 3) in the absence of ligand, thereby promoting apoptosis.

hypothesis arising from the extensive expression of ALK mRNA throughout the nervous system during mouse embryogenesis [3,4,27]. Furthermore, this pattern of ALK mRNA expression is recapitulated in the developing CNS (central nervous system) of the chicken where ALK localizes to a subset of spinal motor neurons, the sympathetic ganglia and DRG (dorsal root ganglia) [28]. A similar pattern of expression in a subtype of DRG neurons during DRG development has also been reported in the rat [29]. In mice, the intensity of the ALK transcript and protein diminishes after birth, reaching minimum levels at 3 weeks of age, and is thereafter maintained at low levels in the adult animal [3]. Immunohistochemical studies of adult human tissue reveal a pattern consistent with that previously reported for mouse ALK, with a weak ALK signal observed in the CNS [30]. ALK mRNA transcripts of different sizes have also been reported in the testis, small intestine, colon, prostate and brain of adult human material, suggesting that differential splicing of ALK may occur [1].

*In vitro* studies also support a role for ALK in neuronal development. For instance, substitution of the extracellular region of ALK with the mouse IgG Fc domain, resulting in the creation of a constitutively active membrane-bound ALK–IgG Fc hybrid protein, has the capacity to induce neuronal differentiation of PC12 cells. Inhibition of MEK (MAPK/ERK kinase) abolishes ALK–IgG Fc-induced PC12 cell neurite outgrowth, implying that neurite outgrowth activity is mediated via the MAPK pathway [31]. Other groups have also reported a role for ALK in neurite outgrowth in cell culture [32–35]. Moreover, employing antibodies to activate ALK, it has been shown that Shc (Src homology and collagen homology) association with ALK is required for downstream ERK1/2 activation and neurite extension,

further strengthening the hypothesis that neuronal differentiation induced by ALK is mediated via the MAPK pathway [33,36]. FRS2 (fibroblast growth factor receptor substrate 2)/SNT has also been reported to bind both ALK and NPM-ALK [36,37]. Since prolonged activation of ERK is associated with differentiation of PC12 cells, FRS2 recruitment may contribute to the neuronal differentiation of PC12 cells stimulated by activated ALK [36]. Experiments replacing the extracellular domain of ALK with the extracellular region of the EGFR (epidermal growth factor receptor), leads to the phosphorylation of ALK and subsequent activation of PLC $\gamma$  (phospholipase C $\gamma$ ) and PI3K (phosphoinositide 3-kinase). Activation of chimaeric EGFR–ALK efficiently transforms NIH 3T3 cells, further illustrating the potential oncogenic properties of ALK when deregulated [38].

#### LIGANDS OF MAMMALIAN ALK

In mammals PTN (pleiotrophin) also known as HB-GAM (heparin-binding growth-associated molecule) [39], OSF-1 (osteoblast-specific factor-1) [40], HARP (heparin affinity regulatory peptide) [41] and HBNF (heparin-binding neurotrophic factor) [42]; and MK (midkine) [43], also known as RIHB (retinoic acid-inducible heparin-binding protein) [44], have been postulated to be the activating ligands for ALK [6,45]. MK and PTN are small, heparin-binding growth factors implicated in diverse processes such as neural development, cell migration and angiogenesis [46,47]. The observation that PTN could function as a ligand for ALK arises from the isolation of a small portion of the extracellular region of ALK which was identified upon

screening a human foetal brain phage display cDNA library for PTN-binding partners [6]. Subsequently, the PTN-related protein MK was identified as an ALK ligand. Furthermore, antibodies directed toward the ALK extracellular domain could inhibit the *in vitro* ligand–receptor interaction, suggesting that MK and PTN bind ALK [45].

MK and PTN are conserved throughout evolution and are found in species ranging from *Drosophila* to humans [46]. The subject of receptors for MK and PTN is a complex one since, in addition to ALK, a number of other proposed receptors exist. To date, MK and PTN have been shown to bind and signal via RPTP $\beta/\zeta$  (receptor protein tyrosine phosphatase  $\beta/\zeta$ ) [48,49] and N-syndecan [50,51], whereas MK can also bind LRP (LDL-related protein) [52] as well as the  $\alpha 4\beta 1$ - and  $\alpha 6\beta 1$ -integrins [53].

Enhanced proliferation has been reported upon ALK activation via PTN [6,54], a function that seems to be dependent on PKB (protein kinase B)/Akt activation, thus implicating the PI3K pathway as a target of PTN–ALK signalling [54,55]. Moreover, a role for ALK in opposing apoptosis via the MAPK pathway in NIH 3T3 cells has been suggested [54]. In agreement, ribozyme-mediated reduction of ALK in glioblastoma cell lines results in increased apoptosis [55]. MK–ALK signalling has also been reported to be important for proliferation [45,56]. MK-mediated activation of ALK leads to IRS-1 (IR substrate-1) and Shc interaction, stimulating downstream signalling and ultimately activation of NF- $\kappa$ B (nuclear factor  $\kappa$ B), thereby inducing cell growth, which is abrogated upon knockdown of the p65 subunit of NF- $\kappa$ B [56].

Although several groups have shown that PTN and MK activate ALK [6,45,54–57] some questions remain, since a number of groups have reported contradictory findings [32,33,58–60]. Agonist monoclonal ALK antibodies mediate efficient ERK1/2 phosphorylation and differentiation of PC12 cells; however, this could not be reproduced with PTN which failed to induce phosphorylation of ALK [32]. Furthermore, treatment with independently developed ALK-activating antibodies stimulates neuronal differentiation of SK-N-SH cells, which can be blocked by the MEK inhibitor PD98059, thus implicating the MAPK pathway in this process. However, this activation of ALK was not reproduced when MK and PTN were employed [33].

Interestingly, two different species of PTN, PTN15 and PTN18, have been described. PTN15 has been reported to promote glioblastoma growth via ALK, whereas PTN18 promoted glioblastoma migration in an RPTP $\beta/\zeta$ -dependent manner [57]. The existence of two different PTN isoforms has been hypothesized to explain the discrepancy in reports concerning the ability of PTN to activate ALK. However, some investigators have been unable to reproduce the effects of either PTN15 or PTN18 on ALK [59]. Thus the physiological significance of ALK activation via PTN and MK is still a matter of debate and investigation within the field.

One hypothesis concerning ALK activation via PTN comes from the observation that PTN can indirectly lead to phosphorylation of ALK via binding to, and inactivation of, the phosphatase RPTP $\beta/\zeta$  [61]. In this scenario, phosphorylation of ALK is independent of the ALK extracellular region, since a membrane-bound construct containing the ALK intracellular region is phosphorylated as effectively as the full-length protein [61].

MK and PTN display no obvious homology toward the dALK ligand Jeb, or the *C. elegans* ligand Hen-1 [19,25]. Jeb harbours a signal peptide and an LDLa domain [19], whereas MK and PTN are composed of two domains, one N-terminal N-domain and one C-terminal C-domain [62,63], which contain heparin-binding

modules important for their activity [63,64]. In *Drosophila* the homologues for MK and PTN are the orphan ligands Miple1 and Miple2 (where Miple is *Midkine* and *Pleiotrophin*) [65]. Although the combined expression pattern of Miple1 and Miple2 complement the expression pattern of dALK, suggesting that a role as activating ligands for dALK is possible, this has yet to be tested at the gene level [65].

Finally, a novel dependence receptor function for ALK has recently been reported, suggesting that ALK may have an activation independent function [60]. In this study [60], cleavage of ALK by caspase 3 was found to expose a pro-apoptotic intracellular domain of ALK, resulting in increased apoptosis in Jurkat and 13.S.1.24 cells treated with apoptosis-inducing agents. Moreover, this pro-apoptotic function was counteracted by activation of ALK (Figure 2). The relevance of these intriguing results in an *in vivo* context remains to be explored.

## ALK IN THE MOUSE

Throughout the literature a number of comments have been made regarding ALK mutant mice generated by the Morris group, stating that they are viable without any gross alterations [66]. This is in agreement with observations from the Palmer and Hallberg groups, who have also generated ALK mutant mice (E. Vernersson, R.H. Palmer and B. Hallberg, unpublished work). A recent study describes a third independently generated mouse ALK knockout, which displays increased hippocampal progenitor proliferation, increased performance in hippocampal-associated tasks, as well as increased levels of dopamine within the basal cortex [67]. Interestingly, the authors observed an increase in the number of calretinin-positive cells within the hippocampus, a phenotype also noted in MK-knockout animals [67,68].

MK and PTN exhibit similar expression patterns in rodents [69–72], and studies of MK mutant mice embryos, which display a strong up-regulation of PTN expression, suggest that PTN and MK are functionally redundant [73]. Indeed, the MK/PTN double mutant shows a more severe phenotype than either of the single knockouts. These phenotypes include female infertility [74] and hearing impairments [75]. The closest ALK relative, LTK, is expressed in pre-B-cells and adult neurons in the hippocampus and cerebral cortex [76]. Given that the ALK mutant phenotype is hippocampus related, the idea that LTK might compensate for ALK loss is intriguing and relevant [67]. It is known that LTK is able to promote neuronal differentiation of PC12 cells, underscoring the fact that ALK and LTK may potentially compensate for one another [77]. Thus far, however, mouse LTK appears only to be expressed in the adult and not during development [76], suggesting a clear distinction from ALK, which is extensively expressed during embryogenesis [3,4,27]. Furthermore, whereas LTK and ALK share a highly similar intracellular tyrosine kinase domain, there are notable differences in the extracellular region, with LTK lacking several domains found in ALK, such as the MAM and LDLa domains (Figure 1). To date, there is no published mouse LTK knockout to suggest a role for this RTK *in vivo*, and therefore the question of whether or not LTK might substitute for loss of ALK must await future investigation.

## ALK IN HUMANS

The human ALK appears to exist as a 140 kDa protein, as well as the 220 kDa full-length ALK species [3]. The 140 kDa ALK species is thought to be the result of a cleavage within the extracellular region of full-length ALK, generating an 80 kDa form of unknown function [32]. The 140 kDa ALK protein is



phosphorylated in response to activation of ALK [33,36,59]. In addition, the presence of an 85 kDa ALK protein which is phosphorylated in response to activating ALK antibodies has been observed in NIH 3T3 cells expressing full-length ALK [33]. Interestingly, a recent report suggests that an as yet unidentified factor secreted by Schwann cells can prevent ALK cleavage [29]. As yet no physiological function has been reported for these shorter ALK variants, and it is possible that the shorter forms of ALK are generated after activation of the receptor as part of down-regulation/degradation processes within the cell. However, future studies should lead to the definition of the cleavage sites, as well as the functional relevance of these forms of ALK *in vivo*.

## ALK IN DISEASE

Since the initial discovery of the NPM-ALK fusion protein in patients suffering from ALCL [1,2], ALK fusion proteins have also been described in IMTs (inflammatory myofibroblastic tumours) [78], NSCLC (non-small cell lung cancer) [79,80], DLBCLs (diffuse large B-cell lymphomas) [81], and SCC (squamous cell carcinoma) of the oesophagus [82,83]. Furthermore, full-length ALK has also been reported to be expressed in cell lines and tumours suggesting oncogenic progression via overexpression [30,55,58,84–92] or gain-of-function mutations, as has recently been reported in cases of neuroblastoma [93–97].

## ALCL

ALK has been most extensively studied in the context of ALCL, a disease first described in 1985 [98]. ALCL is a non-Hodgkin's lymphoma arising from T-cells, and is characterized by the expression of CD30. ALK-positive ALCL is most commonly observed in young adults and children, accounting for 3% of adult non-Hodgkin's lymphoma and 10–30% of all non-Hodgkin's lymphoma in children [99–101]. ALK expression is an important prognostic factor used to predict clinical outcome for patients presenting with ALCL, since ALK-positive patients have a significantly higher 5-year survival rate as compared with ALK-negative cases [101–105]. ALK-positive ALCL occurs to a higher extent in children and young adults [101]; however, ALK expression in ALCL is a positive prognostic factor independent of patient age [103]. Currently, combinatorial chemotherapy treatment, CHOP [cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine) and prednisone] is applied for treatment of ALCL patients as a first approach. In addition, radiation therapy can be employed as an important complement to CHOP therapy [103,105,106]. Currently, there is no treatment for ALK-positive ALCL aimed at directly targeting ALK.

Besides ALK, active caspase 3 expression is also used as a prognostic indicator for favourable outcome in ALCL. In fact, caspase 3 activity is strongly correlated with the expression of ALK [107]. The presence of STAT3 (signal transducer and activator of transcription 3) in both ALK-positive and ALK-negative ALCL has led to the suggestion that activated STAT3 may be a negative prognostic factor independent of ALK expression in ALCL [108]. Survivin [109] and MUC-1 (mucin-1) [110] are two additional markers that indicate a poorer outcome in ALCL regardless of ALK status. Within ALK-positive ALCL, expression of MUC-1 is indicative of a poorer prognosis with a decrease in overall survival [110]. The exact mechanism of MUC-1 in the modulation of ALK-positive ALCL is not fully understood. However, MUC-1 is commonly overexpressed in oncogenic processes, and via its adhesive properties is thought

to modulate both metastatic capacity and provide hindrance for immune cells trying to access the tumour cells [111].

There have been a number of intriguing reports concerning expression of ALK-fusion proteins in healthy individuals. Indeed, NPM-ALK transcripts have been detected in blood from healthy donors [112], as well as in healthy lymphoid tissue via RT (reverse transcriptase)-PCR [113]. Such reports raise the relevant question of whether the fusion transcript on its own is enough for inducing oncogenic transformation or whether secondary events are also required.

## ALK fusion proteins in ALCL

In ALCL the kinase domain of ALK is fused to NPM, creating the constitutively expressed fusion protein NPM-ALK [1]. NPM is a multifunctional protein which serves as a molecular chaperone involved in the shuttling of pre-ribosome subunits from the nucleus to the cytoplasm during ribosome biogenesis, in addition to playing a role in DNA repair, transcription and genomic stability [114]. In the NPM-ALK fusion protein, oligomerization mediated via NPM juxtaposes two ALK tyrosine kinase domains, resulting in autophosphorylation and activation of ALK kinase activity [115,116]. The subcellular localization of NPM-ALK, which is present both in the cytoplasm and the nucleus, seems not to be critical for NPM-ALK-mediated transformation [116]. Besides the dimerization ability of NPM, the kinase activity of NPM-ALK is an absolute requirement for transforming activity, since mutation of the ATP-binding site (K219R) renders NPM-ALK kinase dead and eliminates transformation [116].

In addition to NPM, numerous other fusion partners exist for ALK in ALCL namely ALO17 (ALK lymphoma oligomerization partner on chromosome 17) [117], TFG (TRK-fused gene) [118,119], MSN (moesin) [120], TPM3 and 4 (tropomyosin 3 and 4) [121–123], ATIC (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase) [124–126], MYH9 (non-muscle myosin heavy chain) [127] and CLTC (clathrin heavy chain) [128] (Table 1). These fusion partner proteins of ALK share several common characteristics: (i) the transcription of the fusion protein is driven via the promoter of the ALK partner protein; (ii) the localization of the fusion protein is determined by the ALK partner protein and; (iii) oligomerization via the ALK partner protein induces autophosphorylation and thereby activation of the ALK kinase domain.

As discussed above, NPM is responsible for the dimerization essential for autophosphorylation of, and downstream signalling via the NPM-ALK tyrosine kinase domain [115,116]. The TPM3 and TFG fusion partners contain dimeric  $\alpha$ -coiled-coil structures that are hypothesized to mediate the dimerization of TPM3-ALK [121] and TFG-ALK [119] respectively. Since ATIC exists as a homodimer [129], this property is assumed to be responsible for the activation of ATIC-ALK [124–126]. For MSN, MYH9 and CLTC, the means of dimerization seems to be more complex. It is believed that clathrin coat formation activates the kinase domain of ALK via the close proximity of the CLTC-ALK fusion proteins, since CLTC is a component of clathrin-coated vesicles. In agreement with this suggestion, CLTC-ALK is localized in a punctuated pattern within the cell, consistent with clathrin-coated vesicles [128]. The theory of 'close proximity' as a means of activating the ALK tyrosine kinase domain also applies to MSN-ALK, which is thought to be activated via the ability of MSN to crosslink the plasma membrane with the actin cytoskeleton [120]. Myosin heavy chain is also known to form a dimer, however the MYH9 sequence that is fused to ALK is missing the dimerization domain present in the full-length protein. In spite of this, the MYH9-ALK fusion protein is phosphorylated *in vivo*, indicating

**Table 1 Fusion partners of ALK in disease**

Disease	Fusion protein	Chromosomal abnormality	References
ALCL	NPM-ALK	t(2;5)(p23;q35)	[1,2]
ALCL	ALO17-ALK	t(2;17)(p23;q25)	[117]
ALCL	TFG-ALK	t(2;3)(p23;q21)	[118,119]
ALCL	MSN-ALK	t(2;X)(p23;q11-12)	[120]
ALCL	TPM3-ALK	t(1;2)(q25;p23)	[121,122]
ALCL	TPM4-ALK	t(2;19)(p23;p13)	[123]
ALCL	ATIC-ALK	inv(2)(p23;q35)	[124-126]
ALCL	MYH9-ALK	t(2;22)(p23;q11.2)	[127]
ALCL	CLTC-ALK	t(2;17)(p23;q23)	[128]
IMT	TPM4-ALK	t(2;19)(p23;p13)	[174]
IMT	TPM3-ALK	t(1;2)(q25;p23)	[174]
IMT	CLTC-ALK	t(2;17)(p23;q23)	[176,177]
IMT	ATIC-ALK	inv(2)(p23;q35)	[175]
IMT	SEC31L1-ALK	t(2;4)(p23;q21)	[180]
IMT	RANBP2-ALK	t(2;2)(p23;q13)	[179]
IMT	CARS-ALK	inv(2)(p23;q11-13)	[117,178]
NSCLC	EML4-ALK	inv(2)(p21;p23)	[79,80]
NSCLC	TFG-ALK	t(2;3)(p23;q21)	[80]
DLBCL	NPM-ALK	t(2;5)(p23;q35)	[204]
DLBCL	CLTC-ALK	t(2;17)(p23;q23)	[237]
SCC	TPM4-ALK	t(2;19)(p23;p13)	[82,83]

either an uncharacterized dimerization domain or that MYH9 potentially interacts with other proteins leading to multimerization and consequent activation of ALK [127].

### Oncogenic signalling via ALK-fusion proteins

The most studied ALK-fusion protein, NPM-ALK, signals via the PLC $\gamma$ , PI3K, RAS/MAPK and JAK (Janus kinase)/STAT pathways. PLC $\gamma$  has been shown to directly interact with NPM-ALK via its SH2 (Src homology 2) domain. This interaction is of importance for the transforming potential of NPM-ALK, since mutation of the NPM-ALK/PLC $\gamma$  interaction site abrogates transformation in transfected cell culture models [11]. A systematic analysis of NPM-ALK tyrosine to phenylalanine mutants (Figure 3), has identified Tyr<sup>664</sup> (corresponding to Tyr<sup>1604</sup> in human full-length ALK; Tyr<sup>1604</sup> is present in human ALK but not in mouse ALK) as responsible for the PLC $\gamma$  interaction. Interestingly, all other mutants (except the kinase dead Y338F, Y342F and Y343F mutants) retained their ability to confer IL-3 (interleukin-3)-independent growth of Ba/F3 cells [11], indicating a key role for the PLC $\gamma$  pathway in ALK-dependent transformation.

NPM-ALK interacts with PI3K thereby activating the catalytic subunit of PI3K, and leading to the phosphorylation of PKB/Akt and subsequent downstream signalling events [130]. This interaction has been reported to occur via both the SH2 and SH3 domains of the p85 subunit of PI3K [130,131]. Furthermore, an indirect interaction of p85 and NPM-ALK via other adaptor molecules must also be considered. Inactivation of the PI3K pathway induces apoptosis in NPM-ALK-positive cells [130,132], indicating a role for the PI3K/PKB/Akt pathway in anti-apoptotic signalling. Moreover, PKB/Akt activation appears to be critical for transformation by NPM-ALK, since mice inoculated with NPM-ALK-positive cells expressing a dominant-negative PKB/Akt display impaired oncogenic growth and delayed tumour formation [132].

NPM-ALK regulates the FOXO3a (forkhead box O 3a) transcription factor via the PI3K/PKB/Akt pathway. Active PKB/Akt phosphorylates FOXO3a, leading to retention of



**Figure 3 Tyrosine residues phosphorylated in the intracellular regions of human and mouse ALK**

The intracellular regions of human and mouse ALK (hALK and mALK respectively) contain the PTK domain. Potential autophosphorylation sites are shown within human and mouse ALK [11]. Note that there is no equivalent tyrosine residue in mouse ALK for human Tyr<sup>1604</sup>. Tyrosine residues within the activation loop are shown in bold. In italics is a profiling of putative phosphorylated tyrosine residues in NPM-ALK from ALCL cancer cells [166]. Four tyrosine sites, marked with \*, have been tested by mutagenesis in NPM-ALK for interaction with signalling proteins such as PLC- $\gamma$  (Tyr<sup>1604</sup> in hALK) [11], Shc (Tyr<sup>1507</sup> in hALK) [115], Src (Tyr<sup>1358</sup> in hALK) [157], IRS-1 (Tyr<sup>1096</sup> in hALK) [115] and SNT (Tyr<sup>1096</sup> and Tyr<sup>1507</sup> in hALK) [37]. Localization of tyrosine residues within the intracellular region is not to scale. TM, transmembrane domain.

FOXO3a in the cytoplasm. This has been shown to result in alterations at the level of transcription of several FOXO3a target genes in NPM-ALK-expressing lymphoma, including cyclin D2, Bin-1 and p27<sup>kip1</sup> [133]. In agreement, inactivation of PKB/Akt activity in ALCL cell lines, employing small molecule inhibitors, results in up-regulation of p27<sup>kip1</sup> levels and induction of cell-cycle arrest [134]. An additional target for PI3K/PKB/Akt signalling in ALK-positive ALCL cell lines is mTOR (mammalian target of rapamycin), which displays reduced levels of phosphorylation in response to inhibition of PKB/Akt [135]. In addition, the RAS/MAPK pathway has also been reported to be important for mTOR activation in NPM-ALK-expressing cells [136].

NPM-ALK interacts with IRS-1, Shc and Grb2 (growth-factor-receptor-bound protein 2), thus implicating the RAS/MAPK pathway as a downstream target of NPM-ALK activity [115]. Interaction with IRS-1 and Shc are non-essential for transformation, given that NPM-ALK mutants unable to interact with Shc and IRS-1 are still able to transform NIH 3T3 cells [115]. Previous reports suggest that NPM-ALK may be able to activate MEK directly [137,138]. Furthermore, simultaneous depletion of ERK1 and ERK2 impairs cell proliferation, whereas ERK1 depletion alone induces apoptosis in an ALCL-derived ALK-positive cell line, indicating that ERK1/2 are involved in survival and pro-apoptotic functions [138].

The last major pathway engaged by NPM-ALK is the JAK/STAT pathway. Several reports have demonstrated a correlation between NPM-ALK expression and STAT3 phosphorylation status and activation [139-141]. In agreement, inactivation of NPM-ALK with small molecule ALK inhibitors results in reduced STAT3 phosphorylation [142-144]. The observed interaction of NPM-ALK with JAK3, the receptor-associated tyrosine kinase responsible for STAT3 activation [140,145],

provides a possible explanation for the effect of NPM-ALK on STAT3, given that inhibition of JAK3 leads to a reduction of STAT3 activation by NPM-ALK together with increased cellular apoptosis [145,146]. Although the precise mechanism of NPM-ALK induced activation of STAT3 is still a matter of investigation, it is clear that JAK3 activity is strongly associated with ALK expression and STAT3 phosphorylation *in vivo* [147]. It is also clear that down-regulation of active STAT3 is incompatible with the ALK-positive ALCL-transforming phenotype, as STAT3 compromised cells display increased apoptosis and cell-cycle arrest [143,145,148,149]. This finding is further supported by gene-targeting experiments which support a role for STAT3 in NPM-ALK-induced tumour growth *in vivo* [150].

Several groups have provided additional information concerning the importance of the JAK/STAT pathway in ALCL. Loss of the JAK/STAT pathway negative regulator Shp1 (SH2 domain-containing phosphatase 1) as a result of DNA methylation has been reported in ALK-positive ALCL [151]. Indeed, restoring Shp1 levels in NPM-ALK-expressing cell lines inactivates the JAK/STAT pathway and blocks cell-cycle progression [149]. In addition, protein phosphatase 2A, a STAT3-interacting protein necessary for sustained STAT3 phosphorylation, is overexpressed in ALK-positive ALCL [141].

Another member of the STAT family, STAT5, plays a less clear role in the pathogenesis of ALK-positive ALCL. Although several reports have been unable to detect activation of STAT5 in ALK-expressing cell lines [140,141], an interaction between STAT5B and NPM-ALK has been observed by others [152]. Furthermore, expression of dominant-negative STAT5B induces apoptosis and cell-cycle arrest in ALCL-derived cell lines expressing NPM-ALK [152]. In ALK-positive ALCL cell lines, STAT5A is silenced via methylation in a STAT3-dependent manner. Up-regulation of STAT5A by inhibition of methylation, results in decreased transcription of NPM-ALK due to the ability of STAT5A to interact with the NPM-ALK promoter region, indicating a tumour suppressor function for STAT5A in ALK-positive ALCL-derived cell lines [153]. Future investigations should clarify the role of STAT5A and B in NPM-ALK-induced tumorigenesis.

Besides the above mentioned players there are a number of other proteins implicated in NPM-ALK-mediated malignancy. The small GTPases Rac1 (ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42) are regulated by NPM-ALK in ALCL and other cell lines [154,155]. Furthermore, upon depletion of Cdc42, cell-cycle arrest and apoptosis are induced [154]. In addition, loss of p130Cas (Crk-associated substrate) modifies cell shape and inhibits cellular transformation by NPM-ALK. This dependency on p130Cas has been suggested to be modulated via Grb2 in NPM-ALK-positive ALCL cells [154]. Moreover, knockdown of Shp2 reduces the migratory capacity of cells expressing NPM-ALK [156] and Src kinases, in particular pp60Src, have been suggested to be important for the proliferative capacity of NPM-ALK-positive ALCL cells [157].

Yeast two-hybrid screening has led to the identification of NIPA (nuclear interacting partner of ALK) as a novel downstream target of NPM-ALK that has been shown to interact with NPM-ALK, as well as other ALK fusions, in a tyrosine kinase-dependent manner. NIPA has been characterized as an F-box-containing protein that defines the multisubunit ubiquitin E3 ligase complex SCF<sup>NIPA</sup> (where SCF is stem cell factor), which targets nuclear cyclin B1 for ubiquitination during interphase, thus contributing to the timing of mitotic entry [158]. Overexpression of NIPA protects Ba/F3 cells from apoptosis induced by IL-3 withdrawal. Furthermore, apoptosis triggered by wortmannin treatment in NPM-ALK-transformed Ba/F3 cells is enhanced by overexpression of dominant-negative NIPA mutants, implying an

anti-apoptotic role for NIPA in NPM-ALK-mediated signalling events [159].

Activation of JNK (c-Jun N-terminal kinase) in ALK-transformed cells has also been reported. Utilizing the Vav promoter to drive NPM-ALK expression in mice results in development of lymphomas which display a robust increase in JNK phosphorylation relative to controls [160]. Subsequent work has reported activation of JNK and c-Jun in ALCL cell lines as well as primary tumour cells. Similar activation was also observed upon introduction of NPM-ALK, but not a kinase-dead mutant NPM-ALK, into HEK (human embryonic kidney)-293T cells, suggesting that NPM-ALK is indeed capable of activating JNK [161].

Elevation of SHH (sonic hedgehog) expression in ALK-positive ALCL has recently been reported [162]. This increase of SHH expression is apparently dependent on NPM-ALK-induced PI3K activity, since inhibition of PI3K leads to a concentration-dependent decrease of SHH protein levels. Inhibition of SHH pathway activity results in decreased cell viability, colony formation and cell-cycle arrest in ALK-positive ALCL cell lines [162].

A number of studies have used high-throughput approaches to identify novel ALK targets [80,137,163–168]. Immunoprecipitation of phosphotyrosine peptides from ALCL cell extracts followed by LC-MS/MS (liquid chromatography tandem MS) analysis identifies ALK as the sole tyrosine kinase phosphorylated within the activation loop, together with tyrosine phosphorylated signalling molecules such as dok2 (docking protein 2), IRS-1, Shc, Crk, CrkL and STAT3 among others [166]. A similar global survey of phosphotyrosine signalling in lung cancer found up-regulation and activation of full-length ALK, and independently identified the EML4 (echinoderm microtubule-associated protein like 4)-ALK oncogene, as well as identifying a number of potential ALK downstream signalling proteins [80]. Immunoprecipitation of NPM-ALK from the Karpas 299 cell lines with monoclonal and polyclonal antibodies led to the identification of 36 NPM-ALK binding partners, including both known and novel ALK-interacting proteins [137]. Recent results employing a proteomics approach with NPM-ALK have moreover defined a set of phosphorylated proteins, including VASP (vasodilator-stimulated phosphoprotein) and ATIC, as ALK targets [163]. In addition, analysis of the transcriptomes of ALCL cell lines has established a number of ALK-regulated genes, including the transcription factor C/EBP $\beta$  (CCAAT/enhancer-binding protein  $\beta$ ) and the anti-apoptotic protein BCL2A1 (B-cell lymphoma 2A1) which are required to sustain the growth and survival of ALK-positive ALCL cells [164]. A further study comparing ALK-positive and ALK-negative ALCL led to the identification of a number of ALK transcriptional targets, of which BCL-6, serpinA1 and C/EBP $\beta$  were also confirmed at the protein level [165]. Finally, using a tandem-affinity purification approach several novel interaction partners for ALK were isolated including MCM6, MSH2, Nup98, Importin 8, 82Fip and Bag2 [168].

None of the other known ALK-fusion proteins have been studied as extensively as NPM-ALK with respect to downstream signalling. Nevertheless they are assumed to function in a similar manner as NPM-ALK. This assumption is supported by studies on the ATIC-ALK and TFG-ALK fusion proteins, in which ATIC-ALK was shown to associate with Grb2 and Shc [125], and TFG-ALK with Grb2, Shc and PLC $\gamma$  [118].

An extensive evaluation of the oncogenic potential of NPM-ALK, TPM3-ALK, TFG-ALK, CLTLC-ALK and ATIC-ALK both *in vivo* and *in vitro* suggests that TPM3-ALK-expressing cells display a higher migratory and invasive capacity *in vitro* as compared with the other ALK-fusion proteins

examined. However, when grafting transfected NIH 3T3 cells subcutaneously into nude mice, NPM-ALK- and TFG-ALK-mediated tumours were readily detected 6 days after inoculation, whereas TPM3-ALK-induced tumours were detected a few days later [169]. The invasive properties of the ALK-fusion proteins were further evaluated by an *in vivo* lung metastasis assay, in which TPM3-ALK displayed an increased capacity to form metastases in comparison with other ALK-fusion proteins [169]. However, whether this increased ability of TPM3-ALK-transfected cells to induce metastases has clinical relevance is currently unclear. In patient samples, the observation that activated STAT3 is expressed in 84% of ALK-positive ALCL cases has been speculated to be due to differences in signalling via the different ALK-fusion proteins [108]. Indeed, this hypothesis is supported by reports of efficient STAT3 phosphorylation in cells by NPM-ALK, TPM3-ALK, TFG-ALK and ATIC-ALK, but not by CLTLC-ALK [169,170].

### IMT

IMT is a benign malignancy of mesenchymal origin with a prominent inflammatory component consisting of plasma cells and lymphocytes [171]. Although IMT mostly affects young individuals, it can develop at all ages. First described in the lung, these 'inflammatory pseudotumours' were considered a post-inflammatory condition rather than a malignant process [172]. IMTs can manifest in almost any anatomical site [171], although they are most common in tissues such as the lung, abdomen and retroperitoneum [173]. In 1999, Griffin et al. [78] reported an association between the 2p23 chromosomal rearrangement and expression of ALK with IMTs; the first indication that IMTs, or a subpopulation of IMTs, were neoplastic in origin rather than reactive [78]. Further studies have confirmed the presence of several different ALK-fusion proteins in IMTs, all containing the kinase domain of ALK together with one of a variety of partner proteins responsible for dimerization (Table 1). Those fusion partners described to date are TPM3-ALK [174], TPM4-ALK [174], ATIC-ALK [175], CLTC-ALK [176,177], CARS (cysteinyl-tRNA synthetase)-ALK [117,178], RANBP2 (Ran-binding protein 2)-ALK [179] and SEC31L1 (SEC31 homologue A)-ALK [180]. In fact, 35–60% of all IMTs appear to exhibit ALK rearrangements [181,182], with ALK-positive IMTs preferentially affecting young individuals [174,181,182]. Interestingly, ALK-positive IMT reoccurs in approx. 50% of cases, although no metastasis was detected in this group [183]. This pattern of occurrence is similar to that noted for ALK-positive ALCL. Moreover the prognosis appears to be better for ALK-positive IMTs [184], as is the case for ALK-positive ALCL. However, no obvious ALCL prognostic factors correlate with IMT [183] and the difference between ALK-positive and ALK-negative IMTs is still unclear. Thus we do not presently understand the functional relevance and consequences of ALK signalling in ALK-positive IMT.

### NSCLC

Lung cancer is the most common cause of cancer death in the world. Of the annual 1.3 million cases worldwide, NSCLC accounts for approx. 80% of all lung cancers [185,186]. In 2007 a novel gene fusion between ALK and EML4 was reported in NSCLC [79,80]. Studies from over 1900 NSCLC cases suggest a frequency of EML4-ALK fusion in the range 0.1–7.9%, encompassing a number of different translocation variants [79,80,187–193]. Extrapolation would suggest that approx. 3.5% of all NSCLC cases contain an EML4-ALK translocation,

equivalent to over 45 000 patients worldwide. Furthermore, the EML4-ALK translocation seems to be uninfluenced by ethnic differences, in contrast with point mutations in the EGFR observed in NSCLC [194]. EML4 does not appear to be exclusive as the fusion partner for ALK in NSCLC, since Rikova et al. [80], also identified TFG as an ALK-fusion partner in one NSCLC sample [80]. Also unclear is the issue of whether EML4-ALK is causative in NSCLC, and whether EML4-ALK will be an effective clinical marker/therapeutic target. However, cells expressing oncogenic variants of ALK or EML4-ALK fusion proteins show reduced growth upon treatment with ALK inhibitors, such as PF-2341066 or NVP-TAE684 [195–198]. Furthermore, mice overexpressing EML4-ALK (variant 1) develop tumours with malignant characteristics, which are treatable with administration of a small specific ALK inhibitor, NVP-TAE684 [142], confirming the potent oncogenic activity of the fusion kinase [195].

Although an attractive diagnostic marker, some caution should be employed when considering EML4-ALK as a diagnostic marker for NSCLC. A recent study by Martelli et al. [193] observed a subset (7.5%) of EML4-ALK translocations in NSCLC samples from European patients. However, the presence of EML4-ALK was also detected in non-tumour lung samples, where the EML4-ALK transcript was not detected in matching tumour samples from the same patient [193]. This observation raises some important questions concerning the role of EML4-ALK in the development of NSCLC, and further work is required to clarify this important issue.

### ALK-positive DLBCL

Among diffuse DLBCLs there is an ALK-positive subpopulation, which shows features of plasmablastic morphology. ALK-positive DLBCL usually expresses markers such as EMA (epithelial membrane antigen), CD138 and cytoplasmic Ig, together with ALK. Furthermore, ALK-positive DLBCL lacks expression of B-cell markers (CD20, CD79a), the T-cell marker CD3 and is typically negative for CD30 expression [85,199]. To date, 41 cases of ALK-positive DLBCL have been reported, spanning all age groups and displaying an overall predominance in males (male/female ratio, 5:1). In addition, ALK-positive DLBCL is correlated with an aggressive clinical outcome as well as a poor response to treatment [170,199–202]. The most frequent chromosomal rearrangement in ALK-positive DLBCL is the t(2;17)(p23;q23) translocation which generates CLTLC-ALK, although a few cases of NPM-ALK fusions have also been described [203,204]. Recently, a third variant of DLBCL was reported with a cryptic insertion of the 3'-ALK sequence at chromosome 4q22-24, displaying immunohistochemical staining characteristic of a DLBCL and focal granular cytoplasmic staining of ALK [200], although the ALK-fusion partner in this case has not been identified. As previously discussed NPM-ALK translocation induces activation of STAT3 [140,141], and this also seems to occur in CLTC-ALK-driven DLBCL, but not in ALK-negative DLBCL [170]. These observations again indicate a close connection between deregulated ALK and phosphorylation of STAT3, suggesting that STAT3 inhibitors might be useful therapies for DLBCL.

### Overexpression of ALK in cancer

Several groups have examined overexpression of ALK in different tumour materials, reporting excessive ALK expression in thyroid carcinoma, NSCLC, breast cancer, melanoma, neuroblastoma, glioblastoma, astrocytoma, retinoblastoma, ewing sarcoma and



rhabdomyosarcoma [58]. Besides these tumour types, ALK expression has also been described in leiomyosarcoma and malignant peripheral nerve sheath tumours, as well as malignant fibrous histiocytoma [86]. Some of these, NSCLC, glioblastoma and neuroblastoma, have also been evaluated with respect to ALK activation. However, the significance of ALK overexpression in many of these cancer types is uncharacterized at the molecular level.

In the case of rhabdomyosarcoma, leiomyosarcoma and malignant fibrous histiocytomas, increased copy number of the chromosomal region 2p23 may lead to the overexpression of ALK [86].

PTN has been reported to induce ALK phosphorylation and subsequent downstream PKB/Akt activation in glioblastoma. In agreement, glioblastoma cell lines depleted of ALK grow at a reduced rate compared with parental cell lines [55]. Similar observations have also been reported in U87MG glioblastoma cell lines stimulated with MK [45]. Combined targeting of ALK and PTN in U87 glioblastoma cells significantly impairs tumour growth in an *in vivo* xenograft model [205].

A clear role for ALK in breast cancer has not been firmly established; however, several lines of evidence suggest a role for ALK in this disease. First, ALK is strongly expressed in several different subtypes of human breast cancers, in a pattern not consistent with normal tissue [84]. Secondly, PTN, the proposed mammalian ALK ligand, is extensively expressed in breast cancer [206,207], and expression of truncated PTN in a human breast cancer cell line abrogates tumour formation in nude mice [208]. Thirdly, the PTN receptor RPTP $\beta/\zeta$  is strongly expressed in different subtypes of human breast cancer [61]. Taken together with the hypothesis that ALK is indirectly activated via PTN/RPTP $\beta/\zeta$  signalling [61], it is possible that ALK harbours oncogenic potential in breast cancer development.

### ALK gain-of-function mutations in neuroblastoma

Neuroblastoma is derived from neural crest cells of the sympathetic-adrenal lineage and can therefore arise throughout the sympathetic nervous system. It is the most common solid tumour in childhood and accounts for 15% of all paediatric oncology deaths [209]. Neuroblastoma tumours show heterogeneous biological and clinical features and a subset may undergo spontaneous differentiation or regression with little or no therapy, whereas the majority are difficult to cure with current regimes. The most common genetic features of neuroblastoma are amplification of the proto-oncogene *MYCN* (*v-Myc* myelocytomatosis viral-related oncogene, neuroblastoma derived), deletions of parts of chromosome arms 1p and 11q, gain of parts of 17q and triploidy [210]. Expression of full-length ALK in neuroblastoma was first demonstrated in 2000 [88], and is supported by subsequent studies showing that the ALK locus is amplified in neuroblastoma cell lines, as well as in primary patient samples [89,90]. A physical association between ALK and ShcC has also been demonstrated [89]. In agreement, silencing of ALK in NB-39-*nu* and Nagai neuroblastoma cell lines results in the down-regulation of ShcC phosphorylation, as well as PKB/Akt activation, with concomitant apoptosis [90].

During 2008, five groundbreaking studies reported the presence of activating ALK mutations in both familial [96,97] and sporadic [93–97] cases of neuroblastoma. Interestingly, all mutations (except one) are localized within the kinase domain of ALK and are assumed to be activating in nature (Table 2). Furthermore, neuroblastoma patients who are positive for ALK mutations appear to have a worse prognosis [93–97]. In line with an oncogenic role for ALK in neuroblastoma, a number of neuroblastoma cell lines were also shown to harbour activating

**Table 2** Activating mutations within the ALK kinase domain in neuroblastoma patients

Nucleotide changes	Amino acid mutation	Targeted region	References
3260 C → T	T1087I	Juxtamembrane domain	[94]
3271 G → A	D1091N	Juxtamembrane domain	[97]
3383 G → C	G1128A	P loop	[97]
3452 C → T	T1151M	Kinase domain	[95]
3497 T → G	M1166R	C helix	[97]
3512 T → A	I1171N	C helix	[97]
3520 T → A	F1174I	End of C helix	[93,97]
3521 T → G	F1174C	End of C helix	[94,96]
3520 T → G	F1174V	End of C helix	[94]
3522 C → A/G	F1174L	End of C helix	[93–95,97]
3575 G → C	R1192P	$\beta$ 4 strand	[97]
3700 G → A	A1234T	Catalytic loop?	[95]
3733 T → G	F1245V	Catalytic loop	[97]
3733 T → A	F1245I	Catalytic loop	[93]
3735 C → A/G	F1245L	Catalytic loop	[93,94]
3734 T → G	F1245C	Catalytic loop	[95,97]
3749 T → C	I1250T	Catalytic loop	[97]
3824 G → T	R1275L	Activation loop	[96]
3824 G → A	R1275Q	Activation loop	[93–97]
3833 A → C	Y1278S	Activation loop?	[96]

ALK mutations, and knockdown of ALK in these cell lines resulted in an inhibition of proliferation [97]. Two of these activating ALK mutants, F1174L and K1062M, can indeed induce rapid formation of subcutaneous tumours in nude mice, thus displaying transforming potential *in vivo* [94].

The work described above highlights the relevance of understanding ALK-mediated signalling in a physiological context, since ALK ligands, as well as mutations in downstream ALK signalling pathway components, are obvious candidates with potential roles in the progression of neuroblastoma. The identification of a role for a drug targetable RTK, such as ALK in neuroblastoma development, provides a real hope for future therapeutic treatments for this devastating disease.

### MOUSE MODELS FOR ALK-DRIVEN ALCL

NPM–ALK has been established as the causative protein in ALCL by a number of groups both *in vitro* and *in vivo*. Bone marrow-expressing human NPM–ALK is able to induce lymphoid malignancies in lethally irradiated mice, providing *in vivo* support for NPM–ALK as an oncogenic agent [211,212]. Moreover, transgenic animals expressing NPM–ALK under the control of the CD4 promoter develop CD30-positive NPM–ALK T-cell lymphomas, as well as plasma cell tumours [213]. Utilization of the haematopoietic cell-specific Vav promoter [160] and the Lck promoter [214] to overexpress NPM–ALK in mice further confirm a role in the development of lymphomas. Additional strategies of investigating NPM–ALK in the development of ALCL using animal models have been reported and recently reviewed [215].

### POTENTIAL TREATMENT STRATEGIES FOR ALK-POSITIVE CANCERS

The development of tyrosine kinase inhibitors for use in cancer therapy has proven effective in several cases. Most well-known is Gleevec which targets the BCR–Abl (breakpoint cluster region–Abl) fusion protein in CML (chronic myeloid leukaemia) [216,217]. In addition to inactivating Abl, Gleevec also targets the c-Kit RTK, as well as the PDGFR (platelet-derived growth factor receptor) [218,219]. c-Kit is highly expressed in GISTs (gastrointestinal stromal tumours) [220] and clinical trials have

established Gleevec as a treatment for GIST patients [221–223]. Other examples of RTK inhibitors are Gefitinib and Erlotinib, monoclonal antibodies which selectively inhibit EGFR (ErbB1), and are used in treatment of NSCLC [224].

A screen of more than 600 different human cancer-derived cell lines with the ALK inhibitor TAE684 identified a number of ALK-positive ALCL, neuroblastoma and NSCLC cell lines in which proliferation was inhibited [198]. TAE684 is a 5-chloro-2,4-diaminophenylpyrimidine which targets the ATP-binding pocket of ALK thereby blocking ATP binding [142]. Furthermore, administration of TAE684 in mice after injection of NPM-ALK-positive ALCL cells (Karpas 299 cell line) prevented development of disease and induced regression of pre-induced ALCL [142].

Neuroblastoma cell lines harbouring different activating ALK mutations appear to respond differently to inhibition of ALK via the inhibitor TAE684. For example, the ALK mutant F1174L observed in SH-SY5Y and KELLY neuroblastoma cell lines is sensitive to TAE684, whereas the R1275W ALK mutant observed in the SMS-KCNR cell line is unaffected. ALK-silencing experiments in these cell lines support the previous inhibitor TAE684 results, since growth inhibition and increased apoptosis were noted in SH-SY5Y and KELLY cells, but not in SMS-KCNR cells [95,97]. It should be noted that these ALK mutants were also tested in a Ba/BF3 cells, where both mutants exhibited transforming ability and were sensitive to TAE684 [95]. The reasons behind this differential response to TAE684 are presently unclear, but may indicate differences in genetic background, or reflect complex genetic predispositions acquired by these cells.

An additional ALK inhibitor is PF-02341066, also an ATP competitor, which targets both c-Met and ALK [196]. PF-02341066 has been shown to reduce ALK-positive ALCL development in an animal model [197], and is currently in phase I trials for patients with advanced anaplastic large cell lymphomas ([www.ClinicalTrials.gov](http://www.ClinicalTrials.gov)). UCN-01 (unco-ordinated 1), a staurosporine derivative, which inhibits PKC (protein kinase C) [225] and chk1 [226], has been reported to cause disease regression in one patient with ALK-positive ALCL [227]. However, others have been unable to confirm a direct relationship between UCN-01 and ALK [228]. Presently a phase I clinical trial using UCN-01 in patients with relapsed or refractory systemic ALCL or mature T-cell lymphoma is underway ([www.ClinicalTrials.gov](http://www.ClinicalTrials.gov)). Although several PTK inhibitors are used successfully in the clinic, ALK inhibitors are not yet so well developed. This active area of research should hopefully produce effective and clinically relevant compounds [229,230].

Besides small-molecule chemical inhibitors, ALK has also been the target of a number of strategies aimed at reducing mRNA levels, thus depleting the ALK protein. In glioblastoma, PTN and ALK appear to be up-regulated and functionally relevant, since ALK and PTN ribozyme-expressing glioblastoma cell lines show reduced signalling potential. Moreover, in xenograft models, silencing of either ALK or PTN results in reduced tumour growth [55,231]. A double knockdown of PTN and ALK completely inhibited xenograft tumour growth over a 60-day experimental period [205]. Similar ribozyme-mediated silencing was observed for NPM-ALK in ALCL and Hodgkin's lymphoma-derived cell lines [232]. Finally, Piva et al. [233] have reported induction of cell death in ALK-positive ALCL cell lines by ALK silencing *in vitro* as well as in *in vivo* mouse models of ALCL tumour growth. In this case silencing was achieved by injection of adenovirus-expressing an shRNA (small hairpin RNA)-targeting ALK [233].

Interestingly, DNA vaccination of animals against human ALK prior to challenge with ALK-expressing lymphoma cells provides

protection against disease. Furthermore, in already diseased animals ALK vaccination in combination with standard treatment increased the cure rate [234].

Although the direct targeting of ALK may be preferential for treatment of ALK-positive malignancies, other approaches may provide a beneficial complement. By targeting different ALK-interacting proteins, several reports have demonstrated reduced viability and tumour-forming capacity of NPM-ALK-positive cell lines. For example, NPM-ALK interacts with the chaperone Hsp90 (heat-shock protein 90), and inactivation of Hsp90 with 17-AAG (17-allyl-amino-demethoxygeldanamycin) results in degradation of NPM-ALK and subsequent apoptosis in ALCL cell lines [235]. Another example of possible downstream targets in ALK-positive ALCL is PKB/Akt. Injection of mice with cells expressing NPM-ALK, together with dominant-negative PKB/Akt results in a severely impaired tumour-forming capacity as compared with animals receiving control NPM-ALK-expressing cells [132].

Inhibitory antibodies are also a feasible alternative to small molecules. One such example is Trastuzumab (Herceptin), a monoclonal antibody, which binds to the extracellular juxtamembrane domain of HER2 and prolongs life in patients with human epithelial cancer [236]. Inhibitory antibodies to human ALK have been described, which reduce the level of phosphorylation of ALK, and consequently also the basal activity of MAPK in constitutively active ALK-expressing HEK-293 cells. Furthermore, these blocking antibodies are able to reduce the basal differentiation of ALK-transfected PC12 cells [32]. The authors suggest that this monoclonal antibody may function by blocking dimerization of ALK receptors, since stimulation with activating antibodies is abrogated by the inhibitory antibody. Whether this antibody indeed blocks ligand(s)-mediated receptor dimerization of ALK remains to be seen, and it is possible that this blocking antibody against ALK will display inhibitory activity towards the recently discovered activating ALK mutants in neuroblastoma [93–97]. Such blocking antibodies are potential therapeutics for cancers displaying overexpression or activating mutations of the full-length ALK RTK, and not the translocation-generated fusion oncogenes, and may indeed have the capacity to prolong life and increase the therapeutic effects of other treatments.

## OUTSTANDING QUESTIONS IN ALK RESEARCH: SPECULATION FOR THE FUTURE

One of the most disputed areas in the ALK research field at the present time is the issue of PTN and MK as ligands of ALK *in vivo*. To date, there is no genetic evidence to support this from model systems, in spite of the fact that a number of organisms in which ALK signalling has been studied (such as *Drosophila* and zebra fish) have clear homologues. The possibility exists that different ligands for ALK are utilized in different developmental processes, and that indeed PTN/MK function as ALK ligands, but that as yet unidentified 'Jeb-like' vertebrate ligands also exist. In the absence of 'Jeb-like' vertebrate ligands, the spatial and temporal relevance of the PTN/MK-ALK interaction *in vivo* needs to be addressed. The issue of ALK ligands is of particular relevance in neuroblastoma, since it follows that any misregulation of an ALK ligand may influence neuroblastoma progression even in the absence of activating ALK mutations. Structural information regarding ALK is also lacking, and would be an invaluable treasure trove of information. The unique organization of ALK in the extracellular region suggests that important information will be gleaned from solving the ALK structure, with or without

PTN/MK. A simpler task would be elucidation of the crystal structure of the intracellular domain of ALK, in the presence or absence of inhibitors, or indeed in the active or inactive conformation. In this regard it would be important to consider crystallization of the human ALK in order to understand the relevance, if any, of the Tyr<sup>1604</sup> residue which is lacking in the mouse ALK.

Much work is ongoing concerning generation of specific ALK inhibitors, which would surely be simplified by intricate knowledge of the ALK kinase domain structure. This is an obvious area of importance to be pursued over the coming years. A number of different ALK inhibitors may be needed for therapeutic use, if the paradigm of BCR-Abl and CML inhibitors will also apply to ALK-driven neoplasms. Further development and exploration of monoclonal antibodies to ALK may also provide important therapeutic approaches, particularly in neuroblastoma, and potentially offers the opportunity to interrupt the interaction between ALK and its ligand(s) *in vivo*. While frustrating for those who have painstakingly developed and analysed the ALK-knockout mice, the lack of obvious phenotypes observed in these animals offers hope that side effects of ALK inhibition may be minimal.

Although a wealth of information exists concerning NPM-ALK signalling, it is less clear whether this information can be directly translated over to the many other ALK fusion proteins. Even less clear is the signalling mediated by the full-length ALK receptor. Further work will hopefully address these issues. We are currently unclear on the mechanisms of down-regulation of full-length ALK, and also of the physiological relevance of the different ALK cleavage products. The functional relevance of the potential ALK-dependence receptor *in vivo* also needs to be clarified. While considering the *in vivo* importance of the full-length ALK, it should be noted that RTK loss-of-function is often associated with developmental syndromes, and whereas no connection has been made to date, future work may implicate ALK in a human syndrome perhaps involving the nervous system.

A large number of studies have identified ALK-interacting proteins as well as molecules which are transcriptionally modulated in response to ALK activation. Only a handful of these have been investigated in detail. The difficult task of functional characterization and identification of biologically and clinically relevant molecules in these data sets lies in the future. However, the value of these data sets will not be realised without corresponding biological context.

Further insight into ALK function, in terms of signalling pathways and developmental context, may come from genetic screens and analysis in the model organism systems such as *C.elegans*, *Drosophila*, zebrafish and mouse. The function of the second zebrafish ALK family member is of interest, as is the question of a function for the MK/PTN homologues in the fruitfly.

Many clinically relevant questions are also outstanding, such as whether ALK is capable of driving tumours alone in humans, or whether there are particular molecular partners that function with ALK in tumour progression. Difficult issues such as genetic predisposition seem to be especially important in neuroblastoma development. It is intriguing to consider why the ALK genetic locus is so sensitive to translocation events, and, in this regard, very little is known. Bearing in mind the recent gain-of-function ALK mutations in neuroblastoma, it is obvious to wonder whether these gain-of-function mutant ALK RTKs are ligand independent, either partially or completely. Furthermore, will we in the future find mutations in ALK ligands implicated in neuroblastoma progression? One area of research which we will most surely hear more of in the near future is the development of mouse models to

investigate ALK-driven neuroblastoma, which will allow a deeper analysis of ALK action in neuroblastoma in a more *in vivo* context.

## CONCLUDING REMARKS

Currently, there are no clinically approved treatments for oncogenic malignancies caused by aberrant ALK activation that directly target ALK or the downstream ALK-activated signalling pathways. The accumulating body of work concerning ALK signal transduction pathways in both physiological and pathological states has provided a number of very real candidate targets for the development of clinical therapeutics. Outstanding questions surrounding the ALK ligand(s) and their mode of action *in vivo* are issues which need to be addressed. The future holds great hope for more tailored therapeutic approaches for those suffering from ALK-driven cancers. Such ALK-directed treatments should benefit a number of different cancer patient populations, from ALCL patients to children with neuroblastoma, as well as NSCLC sufferers.

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