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# Cell culture and *Drosophila* model systems define three classes of anaplastic lymphoma kinase mutations in neuroblastoma

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## SUMMARY

Neuroblastoma is a childhood extracranial solid tumour that is associated with a number of genetic changes. Included in these genetic alterations are mutations in the kinase domain of the anaplastic lymphoma kinase (ALK) receptor tyrosine kinase (RTK), which have been found in both somatic and familial neuroblastoma. In order to treat patients accordingly requires characterisation of these mutations in terms of their response to ALK tyrosine kinase inhibitors (TKIs). Here, we report the identification and characterisation of two novel neuroblastoma ALK mutations (A1099T and R1464STOP), which we have investigated together with several previously reported but uncharacterised ALK mutations (T1087I, D1091N, T1151M, M1166R, F1174I and A1234T). In order to understand the potential role of these ALK mutations in neuroblastoma progression, we have employed cell culture-based systems together with the model organism *Drosophila* as a readout for ligand-independent activity. Mutation of ALK at position 1174 (F1174I) generates a gain-of-function receptor capable of activating intracellular targets such as ERK (extracellular signal regulated kinase) and STAT3 (signal transducer and activator of transcription 3) in a ligand-independent manner. Analysis of these previously uncharacterised ALK mutants and comparison with ALK<sup>F1174I</sup> mutants suggests that ALK mutations observed in neuroblastoma fall into three classes. These classes are: (i) gain-of-function ligand-independent mutations such as ALK<sup>F1174I</sup>, (ii) kinase-dead ALK mutants, e.g. ALK<sup>I1250T</sup> (Schönherr et al., 2011a) and (iii) ALK mutations that are ligand-dependent in nature. Irrespective of the nature of the observed ALK mutants, in every case the activity of the mutant ALK receptors could be abrogated by the ALK inhibitor crizotinib (Xalkori/PF-02341066), albeit with differing levels of sensitivity.

## INTRODUCTION

Neuroblastoma is a common childhood cancer that arises in the tissues of the sympathetic nervous system (Maris et al., 2007). It most commonly originates in the adrenal glands, but can also develop at additional sites in the neck, chest and abdomen. It is considered to be a disease of developing tissue because it originates from precursor cells of neural crest tissue that are active during embryonic development. This in part explains the median age of 17 months for occurrence of neuroblastoma (Maris, 2010). Recently, anaplastic lymphoma kinase (ALK) gain-of-function mutations have

been described in both familial (Janoueix-Lerosey et al., 2008; Mossé et al., 2008) and sporadic neuroblastoma (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Martinsson et al., 2011; Mossé et al., 2008). Although there is currently no clinically approved treatment for ALK mutations in neuroblastoma, ongoing clinical trials are expected to determine the potential usefulness of ALK-targeted therapies for use in the future. Inhibition of ALK activity using crizotinib (Xalkori/PF-02341066) has been reported in both inflammatory myofibroblastic tumours (IMT) and non-small cell lung cancer (NSCLC) patients (Butrynski et al., 2010; Kwak et al., 2010). Anti-ALK inhibitor therapy thus appears to offer promise in the treatment of ALK-mediated tumours at this point (Hallberg and Palmer, 2011). It is of future clinical importance to characterise each ALK mutation so that individual patients can be treated accordingly. Furthermore, we have addressed the important issue of whether ALK mutations are resistant to the medicine that is currently in use.

ALK was originally identified as a fusion partner with nucleophosmin or nucleolar protein gene (NPM)-ALK oncogene in anaplastic large cell lymphoma (Morris et al., 1994; Shiota et al., 1994). Later, ALK was reported to be receptor tyrosine kinase (RTK), with a molecular weight of 220 kDa, belonging to the insulin receptor superfamily (Iwahara et al., 1997; Morris et al., 1997). ALK contains an extracellular ligand-binding domain, a transmembrane-spanning domain and an intracellular tyrosine kinase domain (Palmer et al., 2009) and is expressed primarily in the central and peripheral nervous system during embryonic development and at lower concentrations in the nervous system of adults. Its expression

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## TRANSLATIONAL IMPACT

### Clinical issue

Neuroblastoma is a childhood extracranial solid tumour that has been associated with alterations in several different genes. Among these, are mutations in the kinase domain of the anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase (RTK). ALK mutations have been reported in both somatic and familial forms of neuroblastoma. Many ALK mutations confer a gain of function to the protein, but mechanistic information is not available for all identified mutations. Optimal treatment of neuroblastoma (e.g. with tyrosine kinase inhibitors) requires a clearer understanding of how different ALK mutations influence protein function and, in turn, contribute to neuroblastoma development. In addition, it is important to investigate whether certain ALK mutations confer resistance to currently available neuroblastoma drugs.

### Results

Here, the authors compared signalling mediated by several different ALK mutants identified in patients with neuroblastoma with signalling mediated by wild-type ALK. Their studies aimed to determine whether disease-associated ALK mutations are universally gain-of-function and whether certain ALK mutations can confer resistance to tyrosine kinase inhibitors. Their results, together with existing data, suggest that the neuroblastoma-associated ALK mutations characterised thus far fall into three classes: (i) gain-of-function, ligand-independent mutations of varying activation strength, such as ALK<sup>F1174I</sup>, (ii) kinase-dead ALK mutants, such as ALK<sup>I1250T</sup> and (iii) ALK mutations that are ligand-dependent and might represent 'passenger' mutations. Importantly, the activity of all mutant ALK receptors, irrespective of mutation type, could be abrogated by the ALK inhibitor crizotinib, albeit with differing levels of sensitivity.

### Implications and future directions

Characterizing individual ALK mutations is clinically important so that individual neuroblastoma patients can be classified and treated appropriately. These results provide mechanistic information on several neuroblastoma-associated ALK mutations and propose a way to classify all ALK mutants. Encouragingly, these results also indicate that various types of ALK mutations are sensitive to currently available neuroblastoma therapy.

is also observed in developing sensory organs, reproductive organs, skin and stomach (Iwahara et al., 1997; Morris et al., 1997; Vernersson et al., 2006) and its physiological function is still enigmatic. It has been associated with multiple translocation events involved in several different human malignancies (Palmer et al., 2009), such as IMT (Griffin et al., 1999), NSCLC (Rikova et al., 2007; Soda et al., 2007), diffuse large B cell lymphomas (DLBCL) (Arber et al., 1996) and renal cell carcinoma (Debelenko et al., 2011) amongst others. Involvement of ALK in a number of human diseases including neuroblastoma has accelerated recent efforts to understand the function of this RTK under both pathological and non-pathological conditions.

In this study, we have characterised a number of novel mutations in the ALK gene from neuroblastoma patients. From analysis of genomic DNA from patient biopsy samples, two novel germ line mutations were identified, leading to A1099T and R1464STOP mutations at the protein level. We have investigated these mutations, looking at whether they could act as driver or passenger mutations in neuroblastoma, with the aim of predicting their potential role in disease progression. Further, we have compared them with a number of other previously reported, but currently uncharacterised, ALK mutations, namely T1087I, D1091N, T1151M, M1166R and F1174I.

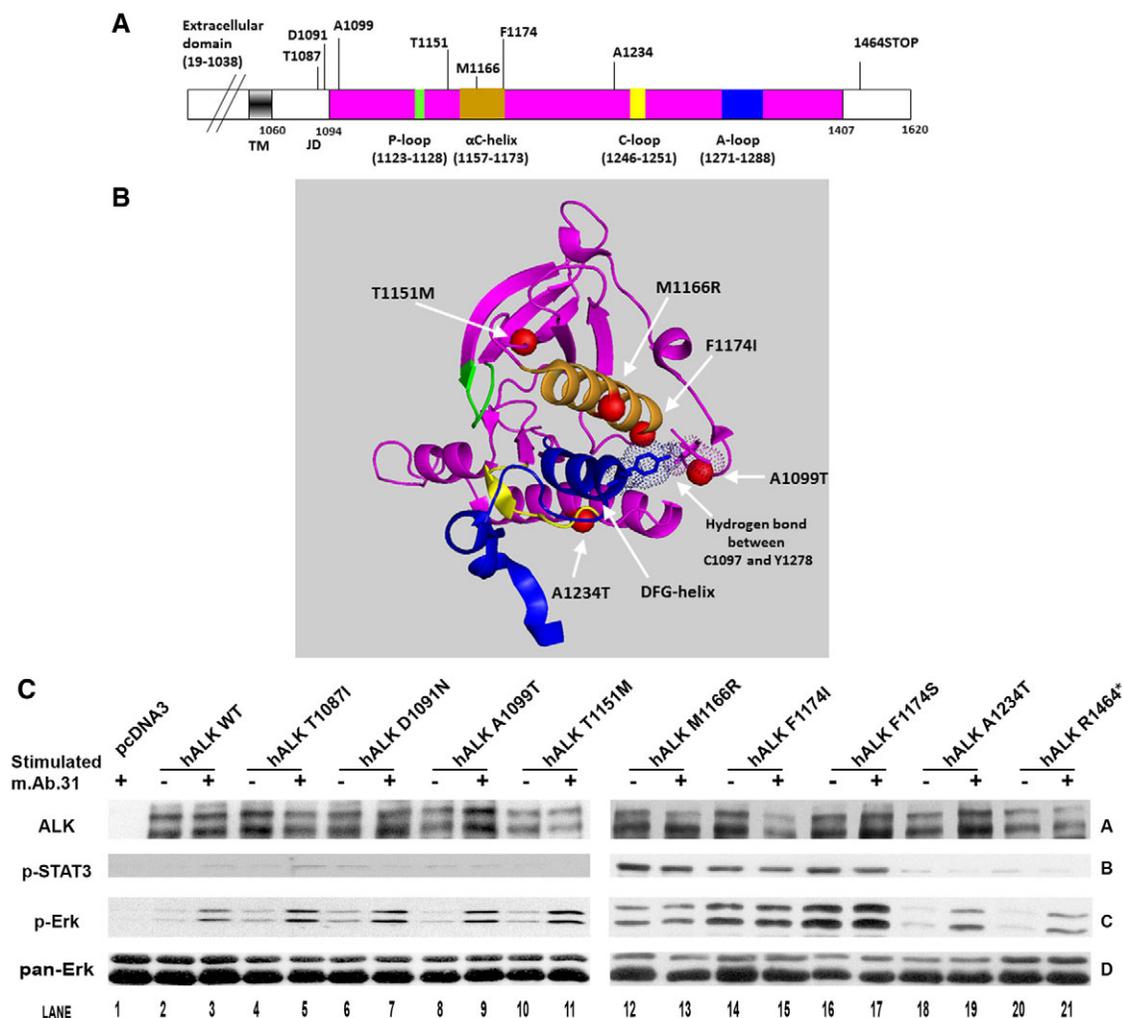
Our findings confirm that the ALK<sup>F1174I</sup> mutation, in keeping with the previously characterised ALK<sup>F1174S</sup> and ALK<sup>F1174L</sup> mutants, is a gain-of-function mutation that mediates activation of downstream targets such as ERK and STAT3 in a ligand-independent manner. The ALK<sup>F1174I</sup> mutant is able to transform both Ba/F3 and NIH3T3 cells. Although we observed that the ALK<sup>M1166R</sup> mutant is unable to transform Ba/F3 cells, it was able to give rise to foci in NIH3T3 transformation assays. Taken together, our data suggest that the ALK mutations investigated to date fall into three classes: (i) gain-of-function ligand-independent mutations (e.g. F1174I), (ii) kinase-dead ALK mutants (Schönherr et al., 2011a) and (iii) ALK mutations that are ligand-dependent in nature, such as the human ALK mutations (T1087I, D1091N, A1099T, T1151M, M1166R, A1234T, R1464STOP), which are not constitutively active but which display ligand-dependent activation when challenged with agonist antibodies. Importantly, to date, the activity of all ALK mutants tested can be abrogated by treatment with ALK small molecule TKIs.

## RESULTS

### Evaluating the role of human ALK mutations as driver or passenger

We set out to characterise two novel germ line ALK variants identified in patients with neuroblastoma (ALK<sup>A1099T</sup> and ALK<sup>R1464STOP</sup>) and their potential roles as driver or passenger mutations in neuroblastoma. ALK<sup>A1099T</sup> was identified in a Japanese infant diagnosed with neuroblastoma, born to unrelated healthy parents with no neuroblastoma history. No *MYCN* amplification or *ALK* amplification was detected in this patient, and the tumour was classified as stage 2. The ALK<sup>A1099T</sup> variant was heterozygous in both tumour and blood DNA samples. The ALK<sup>R1464STOP</sup> mutation was observed in the DNA and RNA from a patient sample analysed as part of the TARGET (therapeutically applicable research to generate effective treatments) initiative, which aims to characterise the genomic landscape of the most common childhood cancers, including neuroblastoma. This sequence variant was heterozygous in tumour DNA and RNA, as well as in blood DNA. DNA from the patient's parents was not available for testing.

Also included in this analysis were the earlier reported, but as yet uncharacterised, putative gain-of-function ALK mutations: ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>T1151M</sup>, ALK<sup>M1166R</sup>, ALK<sup>F1174I</sup> and ALK<sup>A1234T</sup> (Fig. 1A,B) (Chen et al., 2008; George et al., 2008; Mossé et al., 2008). As a positive control, the previously verified constitutively active ALK mutations ALK<sup>F1174L</sup> and ALK<sup>F1174S</sup> were also included (Martinsson et al., 2011). Initially, we investigated these eight human neuroblastoma ALK mutations in a PC12 cell culture system, examining both signalling activity and capacity to drive differentiation, as measured by neurite outgrowth. PC12 cells are a clonal rat adrenal pheochromocytoma cell line, which are well characterised as differentiating and extending neurites upon stimulation with nerve growth factor or differentiation factors (Greene and Tischler, 1976). They do not express ALK at detectable levels. We, and others, have previously shown that expression of wild-type ALK (ALK<sup>wt</sup>), followed by stimulation with ALK agonist monoclonal antibody 31 (mAb31) or expression of activating ALK mutants leads to neurite extension in PC12 cells (Martinsson et al., 2011) (Fig. 1C, lane 1). PC12 cells were transiently transfected with ALK mutants and serum-starved for 48 hours prior to stimulation with mAb31 for 30 minutes. Both human ALK<sup>wt</sup> and



**Fig. 1. Domain structure of ALK mutations and their activation of downstream targets.** (A) The extracellular, transmembrane domain and the intracellular domain, which contains the protein tyrosine kinase domain are shown. Mutation residues within the intracellular domain are indicated by numbers. Also indicated are the A-loop (blue), ATP-binding loop (P-loop, green) and catalytic loop (yellow). The scheme is not drawn to scale. (B) ALK kinase domain showing locations of found mutations (red balls) from neuroblastoma patients. Also indicated are the A-loop (blue), ATP-binding loop (green) and catalytic loop (yellow). The figure was generated with PyMol using published coordinates (PDB#3LCS) (Bossi et al., 2010). (C) PC12 cells expressing human wild-type and mutant ALKs were serum-starved for 48 hours prior to stimulation with 1  $\mu$ g/ml of mAb31 for 30 minutes. Pre-cleared cell lysates were analysed on SDS/PAGE followed by western blotting using the indicated antibodies. ALK, pSTAT3, pERK and pan-ERK antibodies were employed as loading controls.

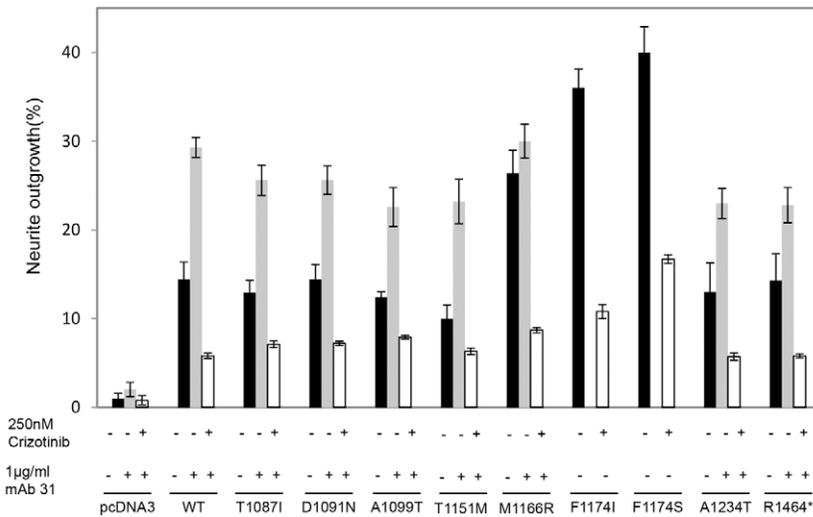
human ALK<sup>F1174S</sup> were included as controls (Chen et al., 2008; George et al., 2008; Martinsson et al., 2011) (Fig. 1). The ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>, ALK<sup>T1151M</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> mutants displayed characteristics similar to that of ALK<sup>wt</sup> and were able to activate downstream targets such as ERK only upon stimulation with agonist mAb31 (Fig. 1C). In a manner similar to that previously observed for human ALK<sup>F1174S</sup>, two of the mutants investigated (human ALK<sup>M1166R</sup> and human ALK<sup>F1174I</sup>) were able to activate phosphorylation of ERK in the absence of agonist mAb31, suggesting that these two mutants might be constitutively activated in a ligand-independent manner (Fig. 1C). Furthermore, in keeping with this hypothesis, both human ALK<sup>M1166R</sup> and human ALK<sup>F1174I</sup> mediated robust phosphorylation of STAT3, as reported for the ALK<sup>F1174S</sup> mutation (Fig. 1C). Interestingly, none of the other mutants were able to generate STAT3 phosphorylation

or activation upon stimulation for up to 30 minutes, as previously shown for wild-type ALK (Schönherr et al., 2011b).

Thus, in this initial analysis, the ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>, ALK<sup>T1151M</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> mutants were found to be inducibly activated by agonist mAb31 and displayed enhanced activation of ERK upon stimulation. However, they were unable to activate STAT3. By contrast, both ALK<sup>M1166R</sup> and ALK<sup>F1174I</sup> appeared to be activated in a ligand-independent manner and led to robust activation of downstream targets, such as ERK and STAT3, in a manner similar to the ALK<sup>F1174S</sup> mutation.

#### Crizotinib blocks neurite outgrowth activity of human ALK mutants

The PC12 cell system is a sensitive readout for ALK signalling activity as a result of receptor activation (Schönherr et al., 2011b;



**Fig. 2. Crizotinib abrogates ALK-mediated neurite outgrowth in PC12 cells.** PC12 cells were co-transfected with pcDNA3-human ALK and pEGFPN1 and seeded out in the presence or absence of crizotinib. Cells were stimulated with 1  $\mu$ g/ml mAb31. After 48 hours, cells were scored for neurite outgrowth. The experiment was carried out in triplicate and each sample within an experiment was performed in duplicate (error bars indicate s.d.). Black bars represent untreated cells, grey bars denote cells stimulated with mAb31 and white bars indicate cells treated with 250 nM crizotinib on its own or together with mAb31.

Yang et al., 2007). Transient transfection of ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>, ALK<sup>T1151M</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> did not stimulate neurite outgrowth in the absence of agonist mAb31. However, addition of agonist antibody lead to significantly increased levels of neurite outgrowth (up to 30-40%) (Fig. 2). The levels of ALK-induced neurite outgrowth were similar to that observed upon stimulation of the wild-type ALK receptor. In agreement with our earlier results, the ALK<sup>M1166R</sup> and ALK<sup>F1174I</sup> mutants were capable of mediating neurite outgrowth in a ligand-independent manner, displaying similar percentages of neurite outgrowth in the presence or absence of agonist antibody (Fig. 2). To investigate whether neurite outgrowth could be abrogated by ALK inhibition, we employed crizotinib (Xalkori), an FDA-approved small inhibitor of both ALK and MET receptor tyrosine kinases, which is being employed in phase II and III clinical trials (Butrynski et al., 2010; Kwak et al., 2010; Hallberg and Palmer, 2011). Crizotinib (250 nM) treatment resulted in a significant reduction in ALK-induced neurite outgrowth. Thus, crizotinib is able to effectively block neurite outgrowth mediated by all human ALK mutants investigated here.

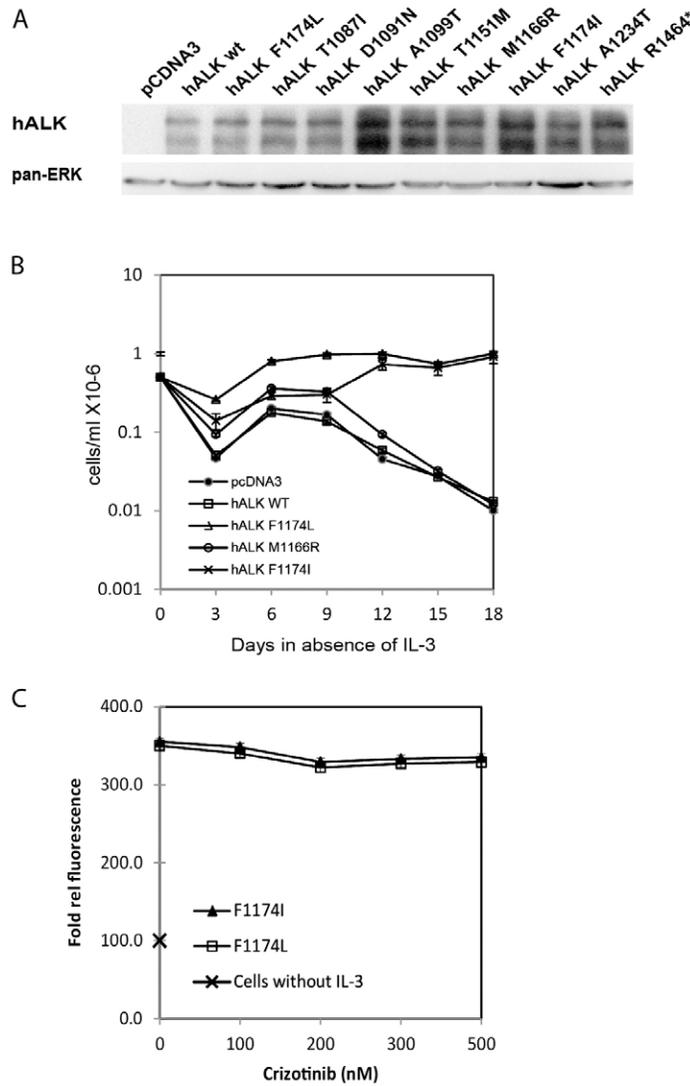
#### In Ba/F3 cells, crizotinib inhibits constitutively active ALK mutants with varying IC<sub>50</sub> values

To confirm and extend our analysis of the inhibition of these ALK mutants by crizotinib, the Ba/F3 cell system was employed (Lu et al., 2009; Schönherr et al., 2011b). Ba/F3 cells, an IL-3-dependent cell line, are often employed in kinase drug discovery, exploiting their characteristic of being able to overcome IL-3 dependence, which allows them to survive and proliferate in the absence of IL-3 when expressing constitutively active tyrosine kinase or other oncogenes (Warmuth et al., 2007). Ba/F3 cells were transfected with human wild-type ALK and the eight different ALK mutants, and subsequently selected with the antibiotic G418 in the presence of IL-3 for 10 days. We observed that Ba/F3 cells expressing the different ALK mutants exhibited variable proliferation ability in the absence of IL-3 (Fig. 3B). At the end of selection in IL-3-free medium, only the human ALK<sup>F1174I</sup> mutant gave rise to IL-3-independent cell lines, along with the human ALK<sup>F1174L</sup> positive control. By contrast, ALK<sup>wt</sup>, ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>,

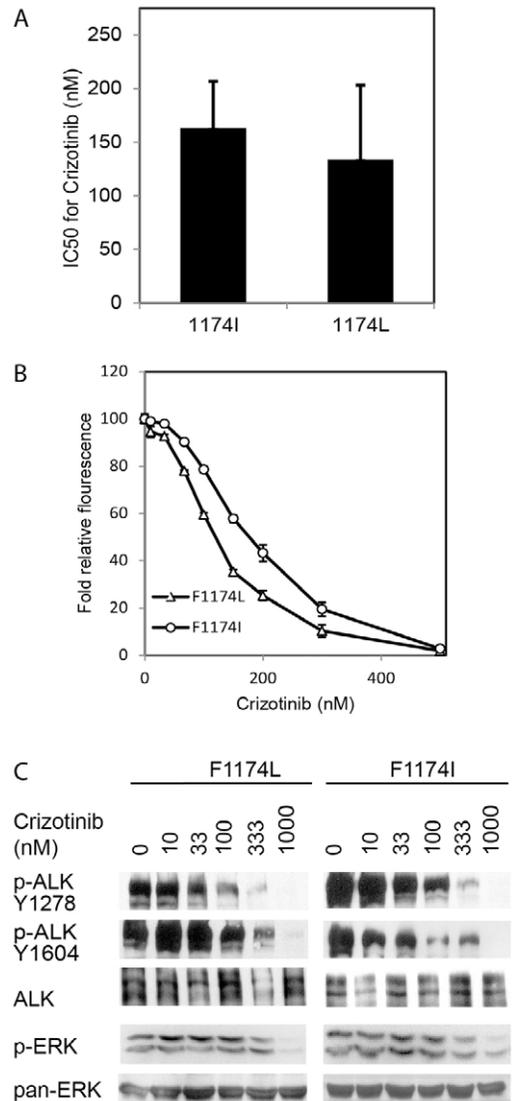
ALK<sup>T1151M</sup>, human ALK<sup>M1166R</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> mutants were unable to substitute for IL-3 to drive proliferation in Ba/F3 cells, even though they expressed ALK protein (Fig. 3A,B and data not shown). To further analyse the responsiveness of these mutants toward crizotinib, the cells were treated with different doses of this ALK inhibitor. Importantly, crizotinib did not inhibit cell growth or cell viability when cells were grown in the presence of IL-3 (Fig. 3C), indicating that it is not toxic to Ba/F3 cells at the levels employed. Proliferation of both human ALK<sup>F1174I</sup> and human ALK<sup>F1174L</sup> mutants was blocked by crizotinib (Fig. 4A). IC<sub>50</sub> (concentration of drug responsible for 50% inhibition) values show that inhibition of ALK<sup>F1174I</sup> required similar doses of crizotinib as human ALK<sup>F1174L</sup> (Fig. 4B). Furthermore, inhibition of ALK mutant activity was also confirmed at the level of ALK tyrosine phosphorylation at position 1278. Tyr1278 corresponds to the first tyrosine of the Y'RAS'YY motif in the A-loop of ALK that is necessary for auto-activation of ALK kinase domain and transformation ability of nucleophosmin (NPM)-ALK (Tartari et al., 2008). Immunoblot analysis performed on Ba/F3 cell lysates showed a reduction in the levels of ALK Tyr1278 phosphorylation in the presence of inhibitor for both human ALK<sup>F1174L</sup> and human ALK<sup>F1174I</sup> mutants (Fig. 4C). In agreement with these results, both mutants exhibited decreased ALK Tyr1604 phosphorylation (Fig. 4C). Reduced levels of ERK phosphorylation by both mutants upon treatment with crizotinib confirmed the inhibition of ALK-mediated downstream targets (Fig. 4C). Taken together, these data suggest that crizotinib is able to block the ALK activity of these mutants in a dose-dependent manner.

#### Transforming potential of human ALK mutations

We further examined the transforming ability of human ALK mutants using NIH3T3 cells. Expression of the human ALK mutations ALK<sup>wt</sup>, ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>, ALK<sup>T1151M</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> were unable to generate formation of foci greater than that of the vector control or ALK wild-type control. By contrast, both ALK<sup>F1174I</sup> and ALK<sup>F1174L</sup> displayed robust foci formation, although ALK<sup>M1166R</sup> showed rather weak foci formation ability in comparison with ALK<sup>F1174S</sup> (Fig. 5A). These results indicate different degrees of foci formation by the ligand-



**Fig. 3. Expression of human ALK in Ba/F3 cells.** (A) Ba/F3 cells were transfected with human wild-type or mutant ALKs and selected with G418 in the presence of IL-3. Whole cell lysates were prepared and run on SDS-PAGE, followed by immunoblotting with indicated antibodies. (B) After selection in the presence of IL-3 and G418, Ba/F3 cells were washed and seeded at  $0.5 \times 10^6$  cells/ml into IL-3-free media containing G418. Cells were counted at different time points using the Trypan Blue exclusion method. (C) Ba/F3 cells expressing ALK were treated with the indicated concentrations of crizotinib in the presence of IL-3. Proliferation was assayed using resazurin. The results obtained indicate that crizotinib does not cause any toxicity to cells at the concentrations used. Points show the increase in relative fluorescence from cells with IL-3 compared with the relative fluorescence from cells without IL-3 and crizotinib (set at a value of 100). Each sample was analysed in triplicate.



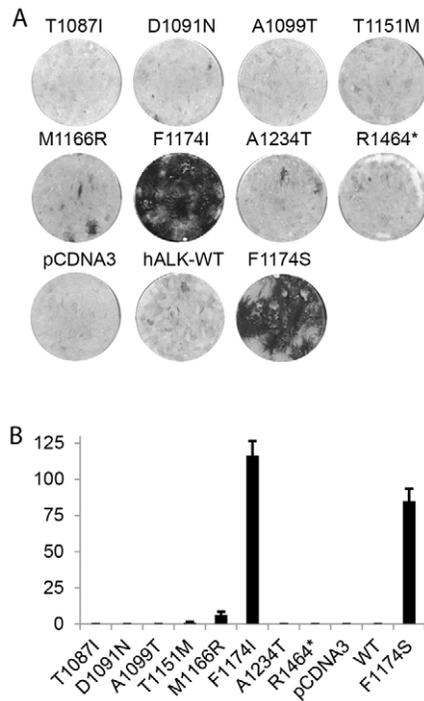
**Fig. 4. Crizotinib inhibits both human ALK<sup>F1174L</sup>-driven proliferation and phosphorylation at Tyr1278 and Tyr1604 of ALK in Ba/F3 cells.** (A) IC<sub>50</sub> values for crizotinib for both human ALK<sup>F1174I</sup> and human ALK<sup>F1174L</sup> were calculated. Values represent mean + s.d. from at least three independent experiments. (B) IL-3-independent Ba/F3 cells expressing human ALK<sup>F1174I</sup> and human ALK<sup>F1174L</sup> were treated with different concentrations of crizotinib. Cell viability was assayed using resazurin after 3 days. (C) Ba/F3 whole cell lysates were separated with SDS/PAGE followed by immunoblotting with the indicated antibodies. ALK activation, for 3 hours, was checked using phospho-specific antibodies against Tyr1278 (p-ALK Y1278) and Tyr1604 (p-ALK Y1604). ERK phosphorylation was checked using phospho-specific antibody (p-ERK). Loading control was pan-ERK.

independent ALK mutations, which is in agreement with our previous results in the Ba/F3 system (Fig. 5B).

**Ectopic expression of human ALK mutants in *Drosophila melanogaster***

Transgenic *Drosophila* expressing the human ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> mutants were generated to confirm their ligand-dependent characteristics. These were ectopically expressed in

*Drosophila* eye, together with the previously generated human ALK<sup>F1174L</sup> and ALK<sup>wt</sup>, employing the pGMR-Gal4 driver line, which directs protein expression in developing photoreceptors of the eye. The expression of human ALK proteins was confirmed by immunostaining of the eye discs by using anti-human ALK antibody. Expression of human ALK<sup>F1174L</sup> resulted in a rough eye phenotype, whereas expression of human ALK<sup>wt</sup>, ALK<sup>A1234T</sup> or ALK<sup>R1464STOP</sup> did not show any obvious phenotype in adult flies



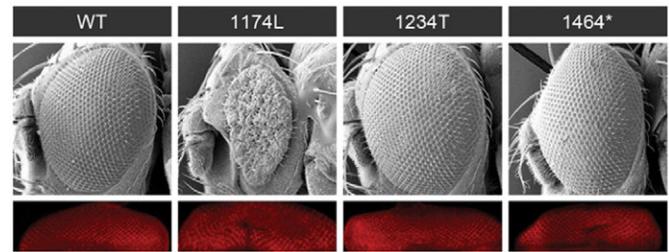
**Fig. 5. Transformation potential of human ALK mutants.** (A,B) The transforming potential of the mutations was assayed by transfecting NIH3T3 cells with pcDNA3-human ALK (mutant and wild type). Cells were selected with G418 for 3 weeks. (A) Representative images of foci. Cells were stained with crystal violet and the number of foci were counted. (B) Bars represent average number of foci per transfection  $\pm$  s.d. from three independent transfections performed in triplicates.

(Martinsson et al., 2011) (Fig. 6). However, the regular hexagonal arrangement of ommatidia was disorganised, with many interommatidial bristles missing in human  $ALK^{F1174L}$ . The data strongly support the conclusion that the human  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$  mutants are ligand-dependent in nature.

## DISCUSSION

ALK mutations have been reported in both familial (Mossé et al., 2008) and sporadic neuroblastoma (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Martinsson et al., 2011; Mossé et al., 2008). The presence of ALK mutations in neuroblastoma draws attention to its possible function and its role in disease progression. Therefore, characterisation of these different ALK mutations is an important exercise in assessing whether the various mutants are functional or not, with potential importance for the patient to be treated accordingly.

In this study, we have investigated two novel mutations of ALK ( $ALK^{A1099T}$  and  $ALK^{R1464STOP}$ ) and compared them with six previously described but uncharacterised human ALK point mutations ( $ALK^{T1087I}$ ,  $ALK^{D1091N}$ ,  $ALK^{T1151M}$ ,  $ALK^{M1166R}$ ,  $ALK^{F1174I}$  and  $ALK^{A1234T}$ ) from neuroblastoma patients, with the aim of assessing whether these mutations could drive tumour progression (Table 1). So far, and from our knowledge of neuroblastoma tumour analysis and published reports to date, only one neuroblastoma patient has presented with a homozygous ALK mutation (Martinsson et al., 2011). To date, one mutation



**Fig. 6. Ectopic expression of human  $ALK^{A1234T}$  and human  $ALK^{R1464STOP}$  in *Drosophila* eye does not generate the rough eye phenotype.** Adult fly eyes (upper) and larval eye discs (lower) ectopically expressing mutant human ALK variants. Human  $ALK^{wt}$  and human  $ALK^{F1174L}$ ,  $ALK^{F1174L}$ ,  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$  were expressed in the *Drosophila* eye with *pGMR-Gal4*. The gain-of-function mutation  $ALK^{F1174L}$  generated a rough eye phenotype, in contrast to both  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$ . Eye discs were stained with anti-human ALK antibody (red) to confirm protein expression.

( $ALK^{I1250T}$ ) has been reported to have defective kinase activity, raising important questions as to the impact, if any, that this mutation has upon neuroblastoma progression (Schönherr et al., 2011a). Signalling studies in the PC12 cell system showed differences in the activation of downstream targets by the various ALK mutants examined.  $ALK^{T1087I}$ ,  $ALK^{D1091N}$ ,  $ALK^{A1099T}$ ,  $ALK^{T1151M}$ ,  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$  showed little phosphorylation of ERK, which was enhanced by stimulation with agonist antibodies. On the other hand,  $ALK^{M1166R}$  and  $ALK^{F1174I}$  mediated ligand-independent phosphorylation of ERK and STAT3. Likewise, the  $ALK^{M1166R}$  and  $ALK^{F1174I}$  mutants were able to mediate neurite outgrowth in PC12 cells in a ligand-independent manner (Table 1). The other ALK mutations ( $ALK^{T1087I}$ ,  $ALK^{D1091N}$ ,  $ALK^{A1099T}$ ,  $ALK^{T1151M}$ ,  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$ ) displayed inducible activity and resulted in neurites only when stimulated with agonist antibodies, which could be abrogated by crizotinib (Table 1). Furthermore, we co-transfected our ligand-dependent mutants together with wild-type ALK. We observed no change in the phosphorylation of ERK or in the levels of neurite outgrowth ability when compared with wild-type ALK alone in PC12 cells (data not shown).

Interestingly, the  $ALK^{M1166R}$  mutant gave rise to weak foci formation in NIH3T3 cells in comparison with the  $ALK^{F1174I}$  mutant, which displayed robust foci formation (Table 1). In this assay, the human ALK mutations  $ALK^{T1087I}$ ,  $ALK^{D1091N}$ ,  $ALK^{A1099T}$ ,  $ALK^{T1151M}$ ,  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$  did not yield any foci as expected. These results thus revealed that only the  $ALK^{F1174I}$  and  $ALK^{M1166R}$  mutants harbour transforming potential in NIH3T3 cells. To complement these analyses, we employed the Ba/F3 cell model system. This revealed that even though the  $ALK^{T1087I}$ ,  $ALK^{D1091N}$ ,  $ALK^{A1099T}$ ,  $ALK^{T1151M}$ ,  $ALK^{M1166R}$ ,  $ALK^{A1234T}$  and  $ALK^{R1464STOP}$  mutant receptors were expressed, they could not substitute for IL-3 to drive proliferation in Ba/F3 cells. Our Ba/F3 results with the  $ALK^{T1151M}$  and  $ALK^{A1234T}$  mutations are in agreement with earlier reported results (George et al., 2008). However, human  $ALK^{F1174I}$  and the human  $ALK^{F1174L}$  control, gave rise to IL-3-independent cell lines. Both mutations displayed sensitivity towards crizotinib in a dose-dependent manner, as shown by their different  $IC_{50}$  values (Table 1). These data are in agreement with previous reports showing that different ALK

**Table 1. Characterization of ALK mutations in order to assess their oncogenic potential**

| Mutation | ALK region                  | Neurite outgrowth assay |            | Foci assay | Ba/F3 assay | Crizotinib treatment | IC <sub>50</sub> (μM) | Phenotype  | Mutation class |
|----------|-----------------------------|-------------------------|------------|------------|-------------|----------------------|-----------------------|------------|----------------|
|          |                             | Unstimulated            | Stimulated |            |             |                      |                       |            |                |
| T1087I   | JD                          | -                       | +          | -          | -           | +                    | ND                    | Wild type  | III            |
| D1091N   | Just before β1 strand       | -                       | +          | -          | -           | +                    | ND                    | Wild type  | III            |
| A1099T   | Between β1' and β2' sheets  | -                       | +          | -          | -           | +                    | ND                    | Wild type  | III            |
| T1151M   | End of β3 sheet             | -                       | +          | -          | -           | +                    | ND                    | Wild type  | III            |
| M1166R   | αC-helix                    | +                       | +          | -/+        | -           | +                    | ND                    | Wild type? | III/I?         |
| F1174I   | End of αC-helix             | +                       | +          | +          | +           | +                    | 0.16                  | G-O-F      | I              |
| F1174L   | End of αC-helix             | +                       | +          | +          | +           | +                    | 0.13                  | G-O-F      | I              |
| F1174S   | End of αC-helix             | +                       | +          | +          | ND          | +                    | ND                    | G-O-F      | I              |
| A1234T   | E-helix                     | -                       | +          | -          | -           | +                    | ND                    | Wild type  | III            |
| R1464*   | C-terminal to kinase domain | -                       | +          | -          | -           | +                    | ND                    | Wild type  | III            |

R1464\*, R1464STOP; JD, juxtamembrane domain; ND, not determined; Class I mutation, gain-of-function mutation; Class II, kinase-dead mutation; Class III, ligand dependent mutation; + denotes that the mutations tested positive for the assay; - indicates that the mutations were negative for the assay.

mutations exhibit different levels of sensitivity towards crizotinib (Bresler et al., 2011; Schönherr et al., 2011b). We also show that cell proliferation and phosphorylation of ALK at tyrosine positions 1278 and 1604 are blocked by using crizotinib, as expected (Fig. 4) (Schönherr et al., 2011b; Tartari et al., 2008). These data indicate that the human ALK<sup>F1174I</sup> mutant is able to mediate neurite outgrowth, transform NIH3T3 cells, activate downstream targets of ALK (such as ERK and STAT3) in a ligand-independent manner and substitute for IL-3 in Ba/F3 cells, giving rise to IL-3-independent cell line. Thus, we conclude that human ALK<sup>F1174I</sup> is a gain-of-function mutation that is sensitive to crizotinib inhibition.

The ALK<sup>M1166R</sup> mutation gives rise to neurites in PC12 cells, activates downstream targets (such as ERKs and STAT3) in a ligand-independent manner and shows weak foci formation. However, it fails to substitute for IL-3 and, hence, does not yield any IL-3-independent Ba/F3 cell lines in our hands. This is comparable to the ALK<sup>R1275Q</sup> mutant, which also displays neurite generating ability but is unable to support Ba/F3 IL-3-independent growth (Schönherr et al., 2011b). Amino acid Met1166 is not conserved in insulin receptor kinase (IRK) family members, perhaps implying that it might have a structural and functional role specific to ALK (Lee et al., 2010). Met1166 is located in the interface between the αC-helix and the DFG-helix and mutations of this residue would probably destabilise the DFG-helix and consequently facilitate the shift of the αC-helix (Fig. 1B) (Bossi et al., 2010). From a structural point of view, both positions 1166 and 1174 are in or very close to the αC-helix, which plays an important role in the activation process of the insulin receptor family members. Upon activation, the αC-helix moves a few degrees closer to the ATP-catalytic site that mediates the γ-phospho-exchange from bound ATP to the interacting substrate (Lemmon and Schlessinger, 2010). Furthermore, it has previously been shown that mutation at position 1174 changes the character of the ALK protein from a ligand-dependent receptor to a receptor exhibiting gain-of-function properties (Chen et al., 2008; George et al., 2008; Martinsson et al., 2011). Probably, this mutations results in disruption of the packing, weakening distinctive structural features observed in the ALK structure, such as the unique inhibitory position of the ALK A-loop packing a short proximal A-loop α-helix against the αC-helix of ALK, while a β-turn motif obstructs the substrate binding region

(Fig. 1B). In this arrangement, Tyr1278 is inaccessible for phosphorylation because it is engaged in the interaction interface through bonding with Cys1097 in the N-terminal β-sheet (Bossi et al., 2010; Lee et al., 2010). Both Phe1174 and Met1166 mutations can probably weaken auto-inhibitory interactions and allow the ALK kinase domain to more easily adopt its active configuration. However, unlike ALK<sup>F1174I</sup> mutants, the ALK<sup>M1166R</sup> mutation is not a robust gain-of-function mutation, although it is able to transform NIH3T3 cells, mediate neurite outgrowth, and activate downstream ALK targets, such as STAT3 and ERK. Moreover, unlike ALK<sup>F1174I</sup> mutants, ALK<sup>M1166R</sup> lacks the ability to mediate IL-3 independent growth of Ba/F3 cells. Our analyses suggest that although ALK<sup>M1166R</sup> is able to weaken the auto-inhibitory interaction, this does not occur to the same degree as with the ALK<sup>F1174I</sup> mutations. The extent to which an ALK<sup>M1166R</sup> mutation contributes to neuroblastoma disease progression or tumour initiation is unclear from our experiments.

Consideration of the impact of the ALK<sup>T1087I</sup> and ALK<sup>D1091N</sup> mutations on ALK kinase activity from a structural point of view is difficult because they have not been included in any structural study to date. Both are located close to the C-terminal of the β1 and β2 sheets in the kinase domain (Bossi et al., 2010; Lee et al., 2010). Another mutation investigated here is at position Ala1099, which is located between the β1' and β2' sheets. Both Lee et al. and Bossi et al. have indicated that nearby Cys1097 plays an important role in mediating a hydrogen bond with the unphosphorylated tyrosine at position 1278 when ALK is in an inactive configuration (Fig. 1B, sticks and dots) (Lee et al., 2010; Bossi et al., 2010). Our analyses of these, together with the location of the ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup> and ALK<sup>A1099T</sup> mutations, indicate that no gross structural conformation changes occur that result in the mutant ALK protein gaining characteristics that differ significantly from the wild-type protein (Table 1). The Thr1151 mutation is located in the β3 sheet, included in the framework of five-stranded, twisted anti-parallel β-sheets that build up predominantly the N-terminal kinase lobe. Apparently, mutation of Thr1151 to methionine (T1151M) does not affect the ability of the ALK kinase domain to be activated upon stimulation. Finally, it is difficult to predict the effect of the 1464STOP mutation, although from our analyses we would speculate that at least in terms of activation it

behaves in a manner similar to the wild-type receptor, i.e. the receptor displays ligand-dependent activation. However, the manner and regulation of inactivation of such a truncated receptor might be an important aspect that could be of relevance in neuroblastoma.

The expression of mutants such as ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>, ALK<sup>T1151M</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup> neither renders Ba/F3 cells independent of IL-3 for their growth nor exhibits transforming activity. Additionally, the mutants do not form foci in NIH3T3 cells. Importantly, however, they are able to give rise to neurites and stimulate activation of ERK in the presence of agonist antibodies. Furthermore, all the mutations in this study are successfully blocked by crizotinib, which results in a decrease in ALK phosphorylation and its downstream target ERK in a manner similar to the controls employed in this study, i.e. ALK<sup>F1174L</sup> and ALK<sup>F1174S</sup> (Martinsson et al., 2011; Schönherr et al., 2011b). A recent study has investigated a number of ALK mutants, which have been identified by the Cancer Genome Project (Sanger Institute, Cambridge, UK) in cell lines from a range of tumour types. This analysis included F1174L, from the SH-SY5Y neuroblastoma cell line, as well as a number of additional previously uncharacterised ALK mutations, and looked at their transformation potential. Similarly to the mutants investigated here (ALK<sup>T1087I</sup>, ALK<sup>D1091N</sup>, ALK<sup>A1099T</sup>, ALK<sup>T1151M</sup>, ALK<sup>A1234T</sup> and ALK<sup>R1464STOP</sup>) the ALK mutants investigated by McDuff and colleagues do not render Ba/F3 cells independent of IL-3 for their growth, exhibit lack of transforming activity and might represent passenger mutations in the evolution of cancer (McDuff et al., 2011). However, this work did not examine whether the various ALK mutants were able to respond to activation by external ligand or agonist antibodies or examine their sensitivity to treatment with crizotinib.

From our present analysis of previously uncharacterised ALK mutants, and in light of other reports concerning ALK mutant variants, the accumulated data suggests that the ALK mutations observed and characterised in neuroblastoma to date fall into three classes. These classes are: (i) gain-of-function ligand-independent mutations of varying activation strength, e.g. ALK<sup>F1174</sup>; (ii) kinase-dead ALK mutants, e.g. ALK<sup>I1250T</sup> (Schönherr et al., 2011a); and (iii) ALK mutations that are ligand-dependent in nature and which might represent 'passenger' mutations. Irrespective of the nature of the observed ALK mutant, in every case the activity of the mutant ALK receptors can be abrogated by treatment with the ALK inhibitor crizotinib, albeit with differing levels of sensitivity. Present knowledge would predict that these mutants will also be sensitive to inhibition by next generation ALK TKIs. Although a role for the class of ligand-independent ALK mutants such as ALK<sup>F1174</sup> and ALK<sup>R1275</sup> is simple to rationalise, further work will be required to answer the more challenging issue of the significance of the ligand-dependent class of ALK mutants, if any, in neuroblastoma progression.

## MATERIALS AND METHODS

### Patients: Case 1 (A1099T) and Case 2 (R1464STOP)

#### Case report 1

A 14-day-old newborn Japanese boy infant diagnosed with neuroblastoma was born to unrelated healthy parents with no neuroblastoma history. The tumour was classified as stage 2 and no *MYCN* amplification nor *ALK* amplification were detected by array comparative genomic hybridisation (CGH) analysis. This

patient is still alive and disease-free, and at this point is 11 years old. DNA ploidy of the tumour was aneuploidy (DNA index was 1.34) and array CGH showed whole chromosome 17 gain, with the pattern of whole chromosomal gains and losses in other multiple chromosomes. The tumour was morphologically diagnosed as favourable according to the Shimada classification (Shimada et al., 1999). Tropomyosin-receptor-kinase (Trk) A was highly expressed in the tumour. The A1099T (GCT/ACT) ALK variant appeared to be heterozygous and present in both tumour and blood DNA, suggesting that this is a germline mutation.

#### Case report 2

Tumour and blood DNA from a 21-month-old Caucasian American male with stage 4 neuroblastoma were sequenced as part of the TARGET project. The tumour was characterised by *MYCN* amplification using fluorescence in situ hybridisation (FISH), unfavourable histology (Shimada et al., 1999) and a DNA index of 1.908. Whole genome, exome, and transcriptome sequencing were performed using the Illumina Genome Analyzer. Data were processed as previously described (Morin et al., 2011; Morozova et al., 2010). Sequencing revealed a heterozygous R1646STOP variant in both tumour and blood DNA. In the blood genome, eight reads (47%) supported the reference allele G, and nine reads (53%) supported the alternative allele A. In the tumour genome, five sequencing reads (31%) supported the G allele, and eleven reads supported the A allele. Both reference and alternative alleles were expressed in the tumour transcriptome, with nine and seven reads (56% and 44%) supporting the reference and mutant alleles, respectively. The variant was independently verified by Sanger sequencing.

### Generation of human ALK mutant constructions

ALK<sup>wt</sup>, ALK<sup>F1174L</sup> and ALK<sup>F1174S</sup> have been described earlier (Martinsson et al., 2011). All other ALK mutants employed in this study were created in pcDNA3 by Eurofins MWG/operon (Ebersberg, Germany). The mutations generated in the kinase domain were confirmed by sequencing from both directions.

### Antibodies and inhibitors

Primary antibodies used were: anti-pan-ERK (1:5000), purchased from BD Transduction Laboratories (Franklin Lakes, NJ), anti-pALK(Y1278), anti-pALK(Y1604), anti-pERK(T202/Y204) and anti-pSTAT3 (Y705) were from Cell Signaling Technology (Danvers, MA). The activating monoclonal antibody mAb31 has been described previously (Martinsson et al., 2011; Moog-Lutz et al., 2005). Monoclonal antibody 153 (anti-ALK) was produced in the Hallberg laboratory against the extracellular domain of ALK as described (Moog-Lutz et al., 2005). Horseradish-peroxidase-conjugated secondary antibodies goat anti-rabbit IgG and goat anti-mouse IgG (diluted 1:5000) were from Thermo Scientific (Waltham, MA). The ALK inhibitor crizotinib (PF-02341066, Xalkori) was a generous gift from Pfizer, Inc.

### Neurite outgrowth assay

PC12 cells ( $2 \times 10^6$ ) were transfected by electroporation in an Amaxa electroporator (Amaxa Biosystems, Cologne, Germany) using 0.8  $\mu$ g of pcDNA3-human ALK and 0.5  $\mu$ g pEGFPN1 (Clontech, Mountain view, CA) and 100  $\mu$ l Ingenio electroporation solution (Mirus Bio

LCC, Madison, WI). Transfected cells were subsequently seeded into 24-well plates together with mAb31 (1 µg/ml) and the inhibitor crizotinib (250 nM) in complete growth medium (Martinsson et al., 2011). After 48 hours of incubation, the fraction of GFP-positive and neurite-carrying cells versus GFP-positive cells was observed under a Zeiss Axiovert 40 CFL microscope. To be judged as a neurite-carrying cell, the neurite of the cell was required to reach at least twice the length of the diameter of a normal cell body. Experiments were performed in triplicates and each sample within an experiment was assayed in duplicate.

#### Cell proliferation assay and IC<sub>50</sub> determination

Ba/F3 cells expressing either human ALK<sup>wt</sup> or mutant human ALK were generated by electroporation with pcDNA3-human ALK using Amaxa electroporator (Amaxa Biosystems, Cologne, Germany). Transfectants were selected in RPMI with 10% heat-inactivated foetal bovine serum (FBS) and 2.5 ng/ml IL-3 (PeproTech, Rocky Hill, NJ) in the presence of G418 (600 µg/ml) for 10 days. Cells were washed with PBSA and seeded at 0.5×10<sup>6</sup> cells/ml in RPMI with 10% FBS and G418 for generation of human ALK-expressing IL-3-independent cells (Schönherr et al., 2011b). For calculation of IC<sub>50</sub> values, human ALK-expressing Ba/F3 cells were treated with varying concentrations of crizotinib for 3 days. Cell viability was tested with resazurin (Sigma, Stockholm, Sweden) (O'Brien et al., 2000). The IC<sub>50</sub> value was determined for individual cell lines and the experiment was carried out at least three times independently, in triplicate.

#### Cell lysis and western blotting

PC12 cells expressing human ALK mutants were serum-starved for 36 hours prior to stimulation with 1 µg/ml of the activating mAb31 for 30 minutes (Moog-Lutz et al., 2005; Schönherr et al., 2010; Yang et al., 2007). IL-3-independent human ALK-expressing Ba/F3 cells were treated with crizotinib in complete medium for 3 hours. Cells were washed with PBSA and lysed in SDS-sample buffer. Pre-cleared lysates were run on SDS/PAGE, followed by western blotting using the indicated antibodies. ALK downstream activation was detected by pERK and pSTAT3(Y705); pan-ERK was used as loading control. ALK phosphorylation was analysed with pALK(Y1278) and pALK(Y1604) antibodies. Cell lysis and immunoblotting were performed according to described protocols (Schönherr et al., 2010).

#### Transformation assay

NIH3T3 cells (4.5×10<sup>4</sup>) were seeded in collagen-coated 12-well plates prior to transfection for 6 hours with 1.75 µg pcDNA3.1-human ALK and 4.4 µl Lipofectamine 2000 (Invitrogen, Paisley, UK) in 0.3 ml Opti-MEM. The next day, 60% of the cells were transferred to six-well plates and maintained in DMEM with 10% heat-inactivated FBS and 0.5 mg/ml G418 until the cells reached confluency. Then, the cells were cultivated in DMEM with 5% heat-inactivated FBS and 0.25 mg/ml G418 for approximately 10 days. The cells were then fixed using methanol and stained with 0.25% crystal violet. The foci visible by the naked eye were subsequently analysed (Schönherr et al., 2011a).

#### Expression of human ALK mutants in *Drosophila* eye

The Gal4-UAS expression system was employed to express human ALK in the *Drosophila* eye (Brand and Perrimon, 1993). DNA

encoding ALK<sup>1234T</sup> and ALK<sup>1464STOP</sup> were subcloned with *EcoRI* and *NotI* from pcDNA3-ALK<sup>1234T</sup> and pcDNA3-ALK<sup>1464STOP</sup>, and the resulting fragments were ligated into the *EcoRI-NotI* site of the pUAST vector. Transgenic flies carrying pUAST-ALK were generated by BestGene (Chino Hills, CA) and crossed with *pGMR-Gal4* (Bloomington, stock no. 9146). Offspring were fixed with 4% glutaraldehyde in PBS overnight and subjected to electron microscopic analysis. To confirm the expression of human ALK, eye discs of third instar larva were stained with anti-ALK mAb153 (Schönherr et al., 2011a). Antibody binding was visualised using Cy3-labelled anti-mouse IgG (Amersham). The discs were mounted on slides in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) and imaged using a Zeiss Apotome.

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#### COMPETING INTERESTS

The authors declare that they have no competing or financial interests.

#### AUTHOR CONTRIBUTIONS

All authors contributed to the text. B.H. and R.H.P. planned and initiated the experiments and wrote the manuscript. P.K., T.M., E.F.A., J.M., M.O., A.N. and P.E.S. were involved in the patients, initial analyses and writing the patient report. D.C., K.R., C.S. and B.H. performed all biochemical analysis and analysed the data. Y.Y. and R.H.P. performed the *Drosophila* analyses.

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