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Activating ALK mutations found in neuroblastoma are inhibited by Crizotinib and NVP-TAE684.

Christina Schönherr*, Kristina Ruuth*, Yasuo Yamazaki*, Therese Eriksson*, James Christensen‡, Ruth H. Palmer*§ and Bengt Hallberg*§

* Department of Molecular Biology, Building 6L, Umeå University, Umeå 901 87, Sweden.

‡ Pfizer Global Research and Development, Department of Research Pharmacology, La Jolla Laboratories, La Jolla, CA 92121, USA

Running title: ALK mutation in NB tumor

§ Correspondence:
Bengt Hallberg,
Department of Molecular Biology,
Building 6L, Umeå University,
Umeå
S- 901 87, Sweden.
Tel . +46 (0)90 785 2523
Fax. +46 (0)90 77 26 30
Email: bengt.hallberg@molbiol.umu.se

Ruth H. Palmer,
Department of Molecular Biology,
Building 6L, Umeå University,
Umeå
S- 901 87, Sweden.
Tel . +46 (0)90 785 6786
Fax. +46 (0)90 77 26 30
Email: Ruth.Palmer@ucmp.umu.se

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Abstract

Mutations in the kinase domain of Anaplastic Lymphoma Kinase (ALK) have recently been shown to be important for the progression of the childhood tumor neuroblastoma. Here we investigate six of the putative reported constitutively active ALK mutations, in positions G1128A, I1171N, F1174L, R1192P, F1245C and R1275Q. Our analyses were performed in cell culture based systems with both mouse and human ALK mutant variants and subsequently in a *Drosophila melanogaster* model system. Our investigation addressed the transforming potential of the putative gain-of-function ALK mutations as well as their signalling potential and the ability of two ATP-competitive inhibitors, Crizotinib (PF-02341066) and NVP-TAE684, to abrogate ALK's activity. The results presented here indicate that all mutations tested are of an activating nature, and thus are implicated in tumour initiation or progression of neuroblastoma. Importantly for neuroblastoma patients all ALK mutations used in this study can be blocked by the inhibitors, although some mutants exhibited higher levels of drug sensitivity than others.

Key words: Anaplastic Lymphoma Kinase, Crizotinib, NVP-TAE684, cancer, gain-of-function, neuroblastoma

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Introduction

Neuroblastoma is a cancer derived from neural crest cells of the sympathetic nervous system, accounting for approximately 15% of all childhood cancer [1]. Neuroblastoma tumours show distinct biological and clinical features. A subset may spontaneously differentiate or regress with little or no therapy at all, while the majority is difficult to cure with current modalities. The gene locus of the Receptor Tyrosine Kinase (RTK) Anaplastic Lymphoma Kinase (ALK) has been reported to be amplified in patient samples and neuroblastoma cell lines [2-4]. More recently, germ line and somatically acquired activating point mutations of the Receptor Tyrosine Kinase (RTK) Anaplastic Lymphoma Kinase (ALK) have been identified both in neuroblastoma patient samples and neuroblastoma cell lines [5-9]. To date, from genetic analysis of neuroblastoma patients, 13 amino acids have been published as mutated close to or within the kinase domain of the ALK RTK.

ALK is a member of the Insulin Receptor super family of RTKs [10, 11] and has been identified as a fusion partner for nucleophosmin (NPM), resulting in the oncogene NPM-ALK which has been found in a subset of Anaplastic Large Cell Lymphoma (ALCL) and other cancers (reviewed in [12]). So far aberrant ALK activity has been involved in the progression and maintenance of a great variety of solid and haematopoietic tumours. To date, no clinically approved treatments targeting ALK for patients with neuroblastoma are available. However, the development of cMet/ALK-specific inhibitors such as Crizotinib (PF-02341066) [13], which is in different phase II and III clinical trials (NCT00932893, NCT01154140 and NCT01121588) and has recently been reported to have robust clinical effects in both Non-Small-Cell-Lung Cancer (NSCLC) and Inflammatory Myofibroblastic Tumor [14, 15], provides real hope for neuroblastoma patients harbouring ALK mutations [16, 17].

We aimed to investigate if certain recently described mutations found in neuroblastoma patients are truly gain-of-function mutations, and whether they have the potential to be involved in disease progression. Our analyses indicate that the described ALK point mutations are constitutively active, leading to activation of important oncogenic downstream signalling cell culture model systems which can be inhibited by ALK-specific inhibitors like Crizotinib (PF-02341066) and NVP-TAE684. Further, these mutations have transforming potential and over expression in the *Drosophila melanogaster* eye results in a rough eye phenotype, confirming that they are ALK RTK ligand-independent gain-of-function mutations. Our results also indicate that the various ALK mutants vary in their degree of drug sensitivity in both cell systems and in the *Drosophila* system. Taken together, our data suggest, that ALK gain-of-function mutations play an important role in the development and progression of neuroblastoma, which may be inhibited by ALK-specific inhibitors such as Crizotinib.

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Material and Methods

Antibodies and Inhibitors

The following antibodies were used: anti-pan-ERK (1:5000) was purchased from BD Transduction Laboratories and the anti-p-ALK (Y1278), anti-p-ERK, anti-p-Stat3 (Y705), anti-p-Akt (S473) and anti-p-Akt (T308) (1:1000) were from Cell Signaling Technology. The activating monoclonal antibodies mAb46 and 31 have been described previously [18, 19].The anti-phosphotyrosine antibody 4G10 was from Upstate Biotech. The horseradish peroxidase coupled secondary antibodies goat anti-rabbit IgG and goat anti-mouse IgG were from Thermo scientific. The ALK-specific inhibitor NVP-TAE684 has been described previously [20, 21] and Crizotinib (PF-02341066) [22] was obtained from Pfizer.

Generation of human and mouse ALK mutant constructs in PC12 cells

The wild type mouse ALK sequence (NCBI Reference Sequence: NM_007439.2) was PCR amplified using the following primers: 5' – GCAAAGCCGGACTGTCTGGCATGTCGCCAC – 3' and 5' – GTTGGGCTGAGAGAAAGCCATGTTCACGTG – 3'. The PCR products were subcloned into pCRII-TOPO (Invitrogen, following manufacturer's instructions), and the resulting plasmid was used as template for generation of the following mouse point mutations, utilizing Quick Change Site-Directed mutagenesis kit (Stratagene, according to the manufacturer's instructions) with the primers: G1132A (3395 G → C; 5' – CCATGGCGCATTTGCGGAGGTGTATG – 3' and 5' – CATACACCTCCGCAAATGCGCCATGG – 3'), I1175N (3524 T → A; 5' – CATGGAAGCTCTGATTAATAGCAAATTCAACCACC – 3' and 5' – GGTGGTTGAATTTGCTATTAATCAGAGCTTCCATG – 3'), F1178L (3534 C → A; 5' – CTGATCATCAGCAAATTAAACCACCAG – 3' and 5' – CTGGTGGTTTAATTTGCTGATGATCAG – 3'), R1196P (3587 G → C; 5' – CTACAAGCCCTGCCCCCCTTCATCCTGCTGGAAC – 3' and 5' – TTCCAGCAGGATGAAGGGGGGCAGGGCTTGTAG – 3'), F1249C (3746 T → G; 5' – GAGGAGAATCACTGTATCCACCGGG – 3' and 5' – CCCGGTGGATACAGTGATTCTCCTC – 3') and R1279Q (3836 G → A; 5' – CTTTGGGATGGCCCAAGATATCTACAGGG – 3' and 5' – CCCTGTAGATATCTTGGGCCATCCCAAAG – 3'). The generated mutations were confirmed by sequencing from both directions. The mALK fragment containing the point mutation was digested by AflII/NgoMIV and ligated into the opened pTTPmALK vector (described in [21, 23], resulting in the respective pTTPmALK mutant plasmids (pTTP-ALK^{mut}). Stable PC12 Tet-on clones expressing mouse pTTP-ALK^{mut} were generated as described previously and clones were selected and grown as described in [21].

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Similar approach was used for the generation of mutants of the human ALK. The pcDNA3 expression vector, containing the cDNA for wild-type human *ALK* (pcDNA3-hALKwt) [18] was used as a template for creating a 1298bp fragment using PCR with the following designed primers (5´-TTCTCCGGCATCATGATTGTGTA-3´ and 5´-TTGGACTGAGAGAATGCCATATT-3´) and cloned into the pCRII-TOPO vector (Invitrogen, according to manufactures protocol) and the resulting plasmid was used as a template for generations of the human point mutations using Quick Change Site Directed mutagenesis kit (Stratagene, according to manufacturer's instruction. G1128A (5'-CCATGGCGCCTTTGCGGAGGTGTATGAAG-3' and 5'-CTTCATACACCTCCGCAAAGGCGCCATGG-3'), I1171N (5'-CATGGAAGCCCTGATCAACAGCAAATTCAACCACC-3' and 5'-GGTGGTTGAATTTGCTGTTGATCAGGGCTTCCATG-3'), F1174L (5'-GGAAGCCCTGATCATCAGCAAATTAAACCACCAGAACA-3' and 5'-TGTTCTGGTGGTTTAATTTGCTGATGATCAGGGCTTCC-3'), R1192P (5'-CAATCCCTGCCCCCGTTCATCCTGCTG-3' and 5'-CAGCAGGATGAACGGGGGCAGGGATTG-3'), F1245C (5'-TTTGGAGGAAAACCACTGCATCCACCGAGACATTG-3' and 5'-CAATGTCTCGGTGGATGCAGTGGTTTTCCTCCAAA-3') and R1275Q (5'-CTTCGGGATGGCCCAAGACATCTACAGGG-3' and 5'-CCCTGTAGATGTCTTGGGCCATCCCGAAG-3'. The seven different mutated pTOPO:1298bp plasmids were digested with BlpI/FseI and the resulting 1177 bp fragments were cloned into the BlpI/FseI site of the above described pcDNA3:hALKwt. All mutant constructs were verified by sequencing.

Cell lysis, immunoprecpitation and Western blotting

Briefly, PC12mALK^{mut} cells were induced with doxycycline and serum starved for 20 h. PC12mALK^{wt} cells were additionally stimulated with 1 µg/ml of the activating monoclonal antibody 46 for 30 minutes [19, 21, 23]. IL-3 independent hALK expressing Ba/F3 cells were treated for three hours with Crizotinib in complete medium. Cells were washed and lysed in SDS-sample buffer. Precleared cell lysates were analyzed on SDS/PAGE, followed by immunoblotting with the indicated antibodies. ALK downstream activation was detected by p-ERK, p-STAT3 (Y705), p-Akt (S473 and T308) and pan-ERK was used to show equal loading. ALK-phosphorylation was detected by the 4G10 and the p-ALK (Y1278) antibody respectively. The bands for p-ALK and total ALK were densitometrically quantified with Chemidoc (Bio-Rad, Sundbyberg, Sweden) and the level of relative ALK phosphorylation as an indicator for ALK activity was calculated. Cell lysis, immunoprecipitation and immunoblotting were performed according to the protocols described in [21].

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Neurite outgrowth assay

PC12mALK^{wt} and PC12mALK^{mut} cells were seeded sparsely in 6-well plates and ALK expression was induced by doxycycline in the presence or absence of the ALK-specific inhibitor NVP-TAE684 (30 and 100 nM). PC12mALK^{wt} cells were stimulated with 1 µg/ml mAb46. Quantification of neurite outgrowth in the cells was carried out as described [19]. Experiments were performed in triplicates, and each sample within an experiment was performed in duplicate. For human ALK, 2x10^6 PC12 cells were transfected by electroporation in an Amaxa electroporator, (Amaxa, Cologne, Germany) using 1.5 µg pCDNA3-hALK and 0.5 µg pEGFPN1 (Clontech, Mountain View, CA, USA) and 100 µl Ingenio electroporation solution (Mirus Bio LCC, Madison, WI, USA). After transfection cells were transferred to DMEM supplemented with 7% horse serum and 3% FBS, thereafter seeded into 24-well plates together with 1µg/ml mAb31[18]. Two days after transfection the fraction of GFP-positive and neurite carrying cells versus GFP positive cells was estimated under a Zeiss Axiovert 40 CFL microscope. To be judged as a neurite carrying cell the neurites of the cell had to reach at least twice the length of the diameter of a normal cell body.

Cell Proliferation assay and IC_{50} determination

Ba/F3 cell lines expressing hALK^{wt} and hALK^{mut} were generated by electroporating Ba/F3 cells with pCDNA3-hALK using an Amaxa Electroporator (Amaxa, Cologne, Germany). The transfected cells were then selected in RPMI with 10% heat inactivated FCS, 2.5 ng/ml IL-3 (Peprotech, Rocky Hill, NJ, USA) and 600 µg/ml G418 for at least 10 days. Cells were washed with PBSA and seeded at 0.5x10^6 cells/ml in RPMI with 10% FCS and G418 for generation of hALK expressing IL-3 independent cells. In order to determine the IC_{50} of Crizotinib IL-3 independent hALK-expressing Ba/F3 cells were treated with indicated concentrations of Crizotinib for three days before cell viability was analysed with 55 µM Resazurin (Sigma, Stockholm, Sweden) [24]. The IC_{50} was calculated for the individual cell line and the experiment was carried out at least three times independently in triplicate.

Transformation assay

Low passage number NIH3T3 cells (ATCC) were transfected with Lipofectamine 2000 according to the manufacturer's protocol. Briefly, 4.5x10^4 cells, seeded the day before into collagen coated 12-well plates, were transfected for 6h with 0.55 µg pcDNA3-hALK and 1.4 µl Lipofectamine 2000 in 0.3 ml Opti-MEM. 24h after transfection 3/5 of the cells from each well were transferred to wells in 12-well plates and kept in DMEM (10% FCS and 0.5 mg/ml G418

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until the cells reached confluence. Thereafter cells were kept in DMEM (5% FCS and 0.25 mg/ml G418) for another 10 days.

Generation of human ALK mutant transgenic constructs for *Drosophila melanogaster*

Prior to ligation of the human ALK, ALK^{F1174L} and the ALK^{R1275Q} from pcDNA3 into the *Drosophila* expression vector pUAST, an 898 bp fragment preceding the translation start was removed to increase expression efficiency in the *Drosophila* system (details available upon request). All three constructs were subsequently subcloned into the EcoRI-NotI site of the pUAST *Drosophila* vector and the resulting constructs were verified by DNA sequencing analysis. Transgenic constructs were employed for the generation of transgenic fly strains (BestGene Inc, CA). The following stocks were used: w^{1118}, (Bloomington, stock number 5905) and pGMR-Gal4 (Bloomington, stock number 9146). The transgenic fly strains $UAS\text{-}ALK^{wt}$, $UAS\text{-}ALK^{F1174L}$ *and* $UAS\text{-}ALK^{R1275Q}$ were generated as described above. Expression in the eye, immunoblotting, fluorescent and electron microscopy of wild type and mutant ALK proteins was carried out as described in [18].

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Results

Neuroblastoma ALK mutations are ligand independent and activate downstream targets.

Our initial investigations focused on the activity of the following six described neuroblastoma mutations which were changed at the equivalent sites in the mouse ALK RTK, creating mALK^{G1132A}, mALK^{I1175N}, mALK^{F1178L}, mALK^{R1196P}, mALK^{F1249C} and mALK^{R1279Q} [5-9]. Mutations at position 1174 and 1275 in human ALK are the two most frequently found mutations in neuroblastoma patients and predicted to be of activating nature [6, 7, 9, 25]. The other mutants selected for this study have been envisaged to be of oncogenic nature, but have not been examined in detail [9, 26].

We employed an inducible PC12 cell culture system for the clonal expression of mouse ALK mutants in doxycycline inducible vectors, allowing us to compare the activity of these mutants in a controlled manner. PC12 cells were transfected and individual clones were selected for expression of specific mutants. As control, wild type mouse ALK RTK was used for comparison, which becomes tyrosine phosphorylated and activates/phosphorylates downstream targets, such as ERK and PKB/Akt upon stimulation with an agonist monoclonal antibody, mAb46 (Fig. 1, lanes 13-15). Doxycycline induced expression of ALK mutants results in a similar ligand independent tyrosine phosphorylation of the receptors themselves (Fig. 1A and B, lanes 1-12). Further, the relative ALK kinase activity indicated by p-ALK/ALK seems to differ only slightly between the different ALK mutants, although some such as F1178L, R1196P and G1132A appear slightly more active (Fig. 1H). This is accompanied by phosphorylation of ERK and PKB/Akt (Fig. 1D, E and F, lanes 1-12). In addition to activation of ERK and PKB/Akt all six investigated mALK mutants also lead to the phosphorylation of STAT3 (Fig. 1C). This is in contrast with mAb46 stimulated wild type mALK which did not result in STAT3 activation, 30 min post-stimulation (Fig. 1C, compare lanes 1-12 with 14 and 15). Thus, it appears that all six expressed single point mutations in the kinase domain of mALK (mALK^{G1132A}, mALK^{I1175N}, mALK^{F1178L}, mALK^{R1196P}, mALK^{F1249C} and mALK^{R1279Q}) are activated in a ligand independent manner and activate downstream targets, such as STAT3, ERK and PKB/Akt.

Both NVP-TAE684 and Crizotinib (PF-2341066) abrogate neurite outgrowth activity of constitutively active ALK mutants.

As reported previously, expression and stimulation of the wild type mALK mediates neurite outgrowth in PC12 cells, which is a sensitive readout for ALK signaling activity as a result of receptor activation [21, 23, 27, 28]. This can be completely blocked by the simultaneous addition of the ALK-specific ATP-competitive inhibitor NVP-TAE684 at a final concentration of 30 nM (Fig. 2A). A small number of neurites is also observed in induced, but unstimulated PC12 cell expressing wild type mALK, which may reflect leakage of the wild type mALK clone,

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or the presence of an unknown endogenous ligand. All six mALK mutants generated were capable of stimulating neurite outgrowth in a ligand independent manner. Inhibition of ALK signaling upon addition of inhibitor was also observed in all six ALK mutant receptors examined (Fig. 2A). In uninduced mutant clones a background of neurite outgrowth due to leakage varied between 5–35%. However, upon induction and expression of the mutant ALK all cells extended neurites (Fig. 2A). Interestingly, treatment with NVP-TAE684 resulted in different responses dependent upon the particular mutant examined. Neurite outgrowth induced by expression of the $mALK^{R1279Q}$ mutant was completely inhibited at 30 nM NVP-TAE684, which is comparable to the response seen with activated wild type mALK (Fig. 2A). This is in contrast with NVP-TAE684 treatment of $mALK^{I1175N}$, $mALK^{R1196P}$ and $mALK^{F1249C}$ which were only inhibited to 40 – 50% levels of neurite outgrowth. Treatment of clones expressing $mALK^{G1132A}$ and $mALK^{F1178L}$ with 30 nM NVP-TAE684 resulted in only a small reduction of neurite outgrowth, in agreement with the higher intrinsic activity suggested in Fig.1H. Increasing the concentration of NVP-TAE684 to 100 nM resulted in a complete abrogation of neurite outgrowth, although this was accompanied by significant cell death (data not shown). Thus, while a reduction of neurite outgrowth can be observed upon treatment with NVP-TAE684 of PC12 cell clones expressing different ALK gain-of-function mutants, the response observed was variable. This may reflect differences in expression levels, however the $mALK^{R1279Q}$ mutant clones express the mutant RTK well (Fig. 1, lane 12). Therefore, it is possible that the ability of NVP-TAE684 to inhibit ALK activity varies between the various mutant ALK RTKs differentially.

Next we wanted to confirm that activating human ALK mutations are sensitive to inhibition by ALK-specific inhibitors. Although mouse and human ALK are very similar, 87 % overall homology at the protein level and within the kinase domain, they differ in very few amino acids: one major difference between mouse and human ALK is at Tyr1604, which is lacking in the mouse ALK protein and has been implicated in tumour progression [29]. Here we also employed the ALK inhibitor, Crizotinib (PF-2341066), which like NVP-TAE684 binds to the ATP pocket of ALK [22] and shows promise in the clinic against ALK positive tumours [14, 15]. Transient transfection of human ALK in the absence of activating antibodies mediates around 10% of neurite outgrowth. Transfected and stimulated wild type human ALK receptor induces neurites in around 60% of all transfected cells (Fig. 2B**)**, which is in agreement with earlier reports [18, 21, 28]. Including Crizotinib (at either 0.25 μM or 0.5 μM) or NVP-TAE684 (0.05 μM) upon transfection of wild type ALK reduces the induction of neurites to 15 and 7%, respectively. Moreover, transient transfection of all human ALK habouring specific mutations mediates a robust neurite outgrowth similar to that observed with stimulated wild type ALK. Addition of Crizotinib and NVP-TAE684 abrogates the neurite outgrowth induced by hALK mutants in a similar manner as the stimulated wild type receptor. It should be noted that PC12 cell clones expressing various mALK mutants tolerated 250 nM Crizotinib without affecting the growth rate (data not shown). Thus, in agreement with our experiments with the mouse ALK mutants, the human ALK gain-of-function mutations investigated here respond effectively to the

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inhibitors Crizotinib and NVP-TAE684. Further, the results indicate that the various ALK mutants respond differently to the inhibitors.

Crizotinib blocks both wild-type ALK and constitutively active ALK variants with different IC_{50} in Ba/F3 cells.

As NVP-TAE684 seems to have toxic effects over time, we employed Crizotinib for further studies, since it does not display obvious toxicity. Crizotinib is currently in different phase II and III clinical trials ([15],www.ClinicalTrials.gov). In order to assess the inhibition of the ALK mutants by Crizotinib, we employed the Ba/F3 system [30]. Ba/F3 cells were transfected with human wild type ALK and six different ALK mutants prior to selection with G418 in the presence of IL-3. After 10 days of selection and expansion the cells were washed and seeded in IL-3 free medium with G418. The IL-3 independent cell lines obtained were subsequently analyzed for ALK expression, proliferation and inhibition of ALK activity by Crizotinib. Firstly, we confirmed that the transfected and G418 selected Ba/F3 cells indeed express the different ALK mutants in the presence of IL-3 (Sup. Fig. 1A). Interestingly, Ba/F3 cells expressing hALK mutants displayed variable ability to mediate proliferation in absence of IL-3 (Sup. Fig. 1B). For each hALK mutant we performed three independent transfections and from each transfection we isolated four IL-3 independent lines: ALK^{F1174L}, ALK^{R1192P} and ALK^{R1245C} all gave rise to four IL-3 independent cell lines, while ALK^{G1128A} only gave rise to three cell lines. In contrast, ALK^{I1171N} and ALK^{R1275Q}, although expressing ALK (Sup. Fig. 1A) were unable to substitute for IL-3 and drive proliferation in the Ba/F3 cells. Secondly, in order to assess the sensitivity of the hALK mutants to Crizotinib in Ba/F3 cells, we treated cells with increasing doses of Crizotinib. Proliferation of all four analysed hALK mutants was inhibited by Crizotinib (Fig. 3A). Subsequent IC_{50} calculations of this inhibitor demonstrated that ALK^{F1174L} and ALK^{R1192P} required significantly higher dose of Crizotinib as compared to ALK^{G1128A} and ALK^{R1245C} (Fig. 3B). Thirdly, the inhibition of ALK activity was further investigated at the level of ALK tyrosine-phosphorylation at position Y1278, which is the first tyrosine of the YxxxYY motif that is necessary for the auto activation of the ALK kinase domain and the transformation ability of NPM-ALK [31]. Immunoblot analysis of Ba/F3 cell lysates demonstrated that Crizotinib is able to effectively reduce levels of ALK Y1278 phosphorylation (Fig. 3C). Further, ERK phosphorylation as an indicator of ALK mediated downstream signalling was also inhibited by Crizotinib, reflecting the calculated IC_{50}. It should be noted that Ba/F3 cells expressing hALK mutants tolerate the used Crizotinib concentrations without affecting cell viability (Sup. Fig. 3C). Two of the ALK mutations, I1171N and R1275Q were unable to drive proliferation in Ba/F3 cells, which is contrary to earlier reports [7]. However, this may reflect our use of transient transfection protocols compared with retroviral infection employed by George et al.[7]. Taken together, these results show that the activity of all investigated ALK mutants tested can be inhibited by Crizotinib, although different doses are required for the various ALK mutants.

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Transformation analysis of human ALK mutations

Given the results above, indicating differences in the signaling output of the different ALK mutants, together with the observation of differential sensitivity to the ALK inhibitors, Crizotinib and NVP-TAE684, we decided to investigate this further. Here we examined whether activating human ALK mutations (hALK^{G1128A}, hALK^{I1171N}, hALK^{F1174L}, hALK^{R1192P}, hALK^{F1245C} and hALK^{R1275Q}) were able to exhibit transforming potential in NIH3T3 cells. Expression of all human ALK mutations tested here results in foci formation over the background monolayer (Fig. 4A and B). In contrast, neither vector control nor the wild type human ALK display foci formation. However, a dramatic difference in the ability of the various ALK mutant receptors to drive foci formation can be observed. The mutations hALK^{G1128A}, hALK^{I1171N}, hALK^{R1192P}, and hALK^{R1275Q} show rather weak foci formation when compared with hALK^{F1174L}, and hALK^{F1245C}, which show a robust level of foci formation. Thus, all ALK mutations have the ability to transform NIH3T3 cells but to significantly different degrees, which is comparable with our results from the Ba/F3 cell experiments.

Ectopic expression of human ALK mutants in *Drosophila melanogaster*

Transgenic *Drosophila* expressing two mutations, human ALK^{F1174L} and ALK^{R1275Q} corresponding to the most common mutations found in neuroblastoma patients [25] were generated. These were then ectopically expressed in the *Drosophila* eye employing the pGMR-Gal4 driver line, which directs protein expression in the developing photoreceptors of the eye. Expression of the wild type human ALK does not result in any obvious phenotype in adult flies, and is similar to controls (Fig. 5A, and ([18]). Expression of the human ALK protein was confirmed by immunoblot analysis of developing eye discs where the expressed protein level in the eye is essentially equal among the mutants (Fig. 5B). Expression of human ALK^{F1174L} and ALK^{R1275Q} resulted in a rough eye phenotype, reflecting their robust ligand independent activation *in vivo* (Fig. 5A). The regular, hexagonal arrangement of ommatidia, which can be seen in control (w^{1118}), is disorganized in both mutants, and many interommatidial bristles are also missing (Fig. 5A). Similar disorganization can also be seen in developing eye discs (data not shown). While both ALK^{F1174L} and ALK^{R1275Q} display a robust phenotype, a more severe phenotype is observed with ALK^{F1174L}, in agreement with our previous observations in cell culture. Further, treatment with NVP-TAE684 improved the rough eye phenotype of both ALK mutants, especially that seen with ALK^{R1275Q}, while Crizotinib had little effect on either phenotype, even at 100 μM (Fig. 6). Thus, these differential responses of both mutants to the inhibitors are also in good agreement with the above cell culture experiments.

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Discussion

The finding of ALK point mutations in neuroblastoma patient samples and neuroblastoma cell lines highlight a function for ALK in the development and the onset of neuroblastoma. Initial studies have reported the constitutive activity of some of the ALK point mutations where some mutants (F1174L and K1062M) are able to form subcutaneous tumours in nude mice [6]. Further, knockdown of ALK in neuroblastoma cell lines harbouring activating ALK point mutations inhibited cell proliferation [9]. Our studies provide further evidence for the activating nature of the ALK mutations. Six ALK mutations with the highest predictions of being oncogenic were analyzed for the most important oncogenic downstream signalling pathways [9]. Indeed, both mouse and human ALK expression in PC12 cell clones leads to ligand independent phosphorylation of ERK, PKB/Akt and STAT3 (Fig. 1 and data not shown). However, some differences in signalling can be observed. For instance the $mALK^{I1175N}$ and the $mALK^{F1249C}$ mutations seem to phosphorylate STAT3 to a lower extent than the $mALK^{G1132A}$ mutation with comparable ALK expression levels. Stimulated wild type mALK does not mediate efficient phosphorylation of STAT3. However, a weak phosphorylation of STAT3 can be monitored after 24 hours stimulation of wild type ALK receptor, although this is to a much significantly lower degree when compared to ALK gain-of-function mutations (data not shown). However, despite different ALK expression between the cell lines no great differences between the relative ALK kinase activities can be observed which might vary between cell clones. Furthermore, the finding that the neurite outgrowth of PC12 cell clones expressing mutated mouse ALK are inhibited differently for the various mutants by NVP-TAE684 and Crizotinib, suggests that the different mutations might have different impact on the development, onset and severity of neuroblastoma.

Interestingly, in the NIH3T3 transformation assay the mutations $hALK^{G1128A}$, $hALK^{R1192P}$ and $hALK^{R1275Q}$ display weaker ability to induce transformation. These mutations were initially identified as ALK germ line mutations [9]. A similar trend of inhibition was observed in the neurite outgrowth assay, the mutations $hALK^{G1128A}$, $hALK^{R1192P}$ and $hALK^{R1275Q}$, show less neurite outgrowth when inhibitors, such as NVP-TAE684 and Crizotinib, were included. All other mutations tested here, such as $hALK^{I1171N}$, $hALK^{F1174L}$, $hALK^{F1245C}$ and $hALK^{R1275Q}$ were identified as somatic mutations, although $hALK^{R1275Q}$ has been found both in germ line tumour DNA samples and in somatic tumour DNA samples [7-9].

In order to assess the different responses of the human ALK mutants toward Crizotinib we created Ba/F3 cell lines expressing the various ALK mutants. Indeed, Ba/F3 cells expressing ALK mutants show sensitivity towards Crizotinib resulting in inhibition of proliferation as well as phosphorylation at tyrosine 1278. Tyrosine 1278 is the first tyrosine of the YXXXYY motif that is necessary for the auto activation of the ALK kinase domain and the transformation activity of NPM/ALK and probably directly involved in the activation of downstream signalling pathways [31]. Although, the mutations, $hALK^{I1171N}$ and $hALK^{R1275Q}$, despite expression, were unable to substitute for IL-3 to drive proliferation in Ba/F3 cells. These mutations were able to

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mediate neurite outgrowth, transform NIH3T3 cells and act as ligand independent receptors in cell signalling experiments, suggesting that they are truly gain-of-function mutations which can be inhibited by Crizotinib and NVP-TAE684. The fact that the hALK^{I1171N} and hALK^{R1275Q} mutants drive transformation in NIH3T3 cells but not IL-3 independent proliferation in Ba/F3 may reflect that these ALK mutants only display transformation potential in cells that are immortalized by other critical genetic events in neuroblastoma. We could further observe that hALK^{R1275Q} mutation in the different model systems employed, such as cell culture and *Drosophila melanogaster*, seems to be most efficiently inhibited by Crizotinib and NVP-TAE684, suggesting promise for cancer treatment with ALK-specific inhibitors for neuroblastoma patients harbouring this mutation.

The ATP-competitive inhibitor Crizotinib (PF-02341066) shows dual specificity toward c-Met and ALK [13, 22]. In order to test the specificity of Crizotinib and the other ALK-specific inhibitor NVP-TAE684 in our PC12 cell background, we treated PC12 cells with inhibitors prior to stimulation with NGF and EGF, respectively. Indeed, NVP-TAE684 does not inhibit NGF- or EGF-mediated signalling in PC12 cells, reassuring the specificity toward ALK (Supplementary Figure 2A). However, Crizotinib inhibits NGF- but not EGF-mediated signalling in PC12 cells (Sup. Fig. 2A and B). This result confirms the report by Zou *et al*., where they investigated the cellular activity of Crizotinib against non-target kinases *in vitro*, showing that this inhibitor targets NGF-stimulated TrkA phosphorylation [13]. As 125 nM Crizotinib inhibits ALK mediated ERK-phosphorylation, but no effect with this concentration is seen on native PC12 cells, we suggest that the effect of Crizotinib at this concentration is specific for ALK.

Taken together, our obtained results suggest that the ALK point mutations found in a number of neuroblastoma patients are truly gain-of-function mutations and play most likely an important role in the progression of neuroblastoma. As a broad generalization, we find that somatically arising ALK mutations tend to be more aggressive than germ line mutations. This information is of importance, since at least one previously described neuroblastoma ALK mutation (I1250T) has been reported to have defective kinase activity rather than increased activity [32]. In addition, the ALK gain-of-function mutations can be inhibited by ALK-specific inhibitors like Crizotinib and NVP-TAE684. As Crizotinib is already in Phase II and III of clinical trials this study contributes to the finding of patient-specific treatments for ALK-positive neuroblastoma.

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Figure legends

Figure 1: Mouse ALK activating point mutations are constitutively active as indicated by stimulation of different signaling pathways in PC12 tet-on cells.

PC12mALK^{wt} (lanes 13-15) and PC12mALK^{mut} (lanes 1-12) either expressing mALK or not were serum starved for 20 h. Additionally, PC12mALK^{wt} expressing cells were stimulated with 1 µg/ml mAb46 for 30 minutes. Precleared cell lysates were analyzed on SDS/PAGE, followed by immunoblotting with antibodies as indicated. **(A)** ALK, **(B)** ALK phosphorylation detected by 4G10, **(C)** phospho-specific STAT-3, **(D)** phospho-specific-Akt (S473), **(E)** phospho-specific-Akt (T308), **(F)** phospho-specific-Erk, **(G)** pan-ERK was monitored as loading control and **(H)** the bands for p-ALK and total ALK were densitometrically quantified and the level of relative ALK phosphorylation as an indicator for ALK activity was calculated. The activity of mALK^{mut} was compared to activated mALK^{wt} and described as average ± standard deviation from three independent experiments.

Figure 2: Crizotinib and NVP-TAE684 inhibit ALK induced neurite formation in PC12 cells.

(A) PC12mALK^{wt} and PC12mALK^{mut} either expressing mALK (doxycycline) or not were analyzed for neurite outgrowth in the presence or absence of 30 and 100 nM NVP-TAE684, respectively. Additionally, PC12mALK^{wt} cells were stimulated with 1 ng/ml mAb46. After 48 h the cells were analyzed and cells extending neurites twice as long as the cell body were scored as neurite bearing cells. The experiment was carried out in triplicate and each sample within an experiment was performed in duplicate. White bars, uninduced and untreated cells. Black bars; induced for expression of ALK variants. Gray bars; induced for expression and treated with 100 nM NVP-TAE684. Hatched bars; induced for expression and treated with 30 nM NVP-TAE684. Dotted bar; wild type ALK induced and stimulated with agonist mAb46.

(B) PC12 cells were cotransfected with pCDNA3-hALK and pEGFPN1 and seeded out in absence or presence of Crizotinib or NVP-TAE684. Bars represent average percentage ± SD, of neurite carrying cells among GFP positive cells from three independent experiments. White bars: untreated cells; hatched bars: treatment with 250 nM Crizotinib; grey bars: treatment with 500 nM Crizotinib; black bars: treatment with 50 nM NVP-TAE684.

Figure 3: Crizotinib inhibits both hALK^{mut} mediated proliferation and phosphorylation at position Y1278 of ALK in Ba/F3 cells

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(A) IL-3 independent hALK^{mut} expressing Ba/F3 cells were treated with Crizotinib at the indicated concentrations of Crizotinib. Cell viability was analysed with Resazurin after three days. **(B)** The IC_{50} for Crizotinib mediated growth inhibition was calculated for each individual cell line: Values (Bars) represent average ±SD from at least 3 independent cell clones for each hALK^{mut} in triplicate as indicated in figure. **(C)** Ba/F3 whole cell lysates were separated with SDS/PAGE, followed by immunoblotting with antibodies as indicated. ALK activation was monitored by antibodies phospho-specific against tyrosine 1278 (p-Y1278) and phospho-specific against Erk (p-ERK). Pan-ERK was applied as loading control. The bands for p-ALK (p-Y1278) and total ALK were densitometrically quantified and the level of relative p-Y1278-ALK phosphorylation-versus ALK as an indicator for ALK activity was calculated and presented as average ± standard deviation from three independent experiments.

Figure 4: Transformation activity mediated by mutated hALK.

NIH3T3 cells were transfected with pCDNA3-hALK^{mut} or pCDNA3-hALK^{wt} and subjected to G418 selection. **(A)** After three weeks cells were stained with crystal violet and number of foci was judged by the naked eye. **(B)** Bars represent average number of foci/transfection ± SD from six independent transfections.

Figure 5: ALK activating mutations result in a rough eye phenotype in *Drosophila.*

(A) Scanning electron microscope images of fly eyes from control (w^{1118}) or human ALK activating mutants (ALK^{F1174L}, and ALK^{R1275Q}) **(B)** Immunoblot of adult fly eye extracts expressing human ALK (ALK^{wt}, ALK^{F1174L}, and ALK^{R1275Q}). w^{1118} and *pGMR* are control lines, and *hALK* is a control PC12 cell lysate expressing human ALK protein.

Figure 6: Effects of ALK inhibitors on ALK activating mutant rough eye phenotype

Mutant lines (ALK^{F1174L} and ALK^{R1275Q}) were crossed with the eye-specific pGMR-Gal4 driver line and the offspring was grown in inhibitor containing food at 25ºC. NVP-TAE684 reverts rough eye phenotype of both mutants, especially of ALK^{R1275Q}, whereas Crizotinib is less effective.

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Figure 1

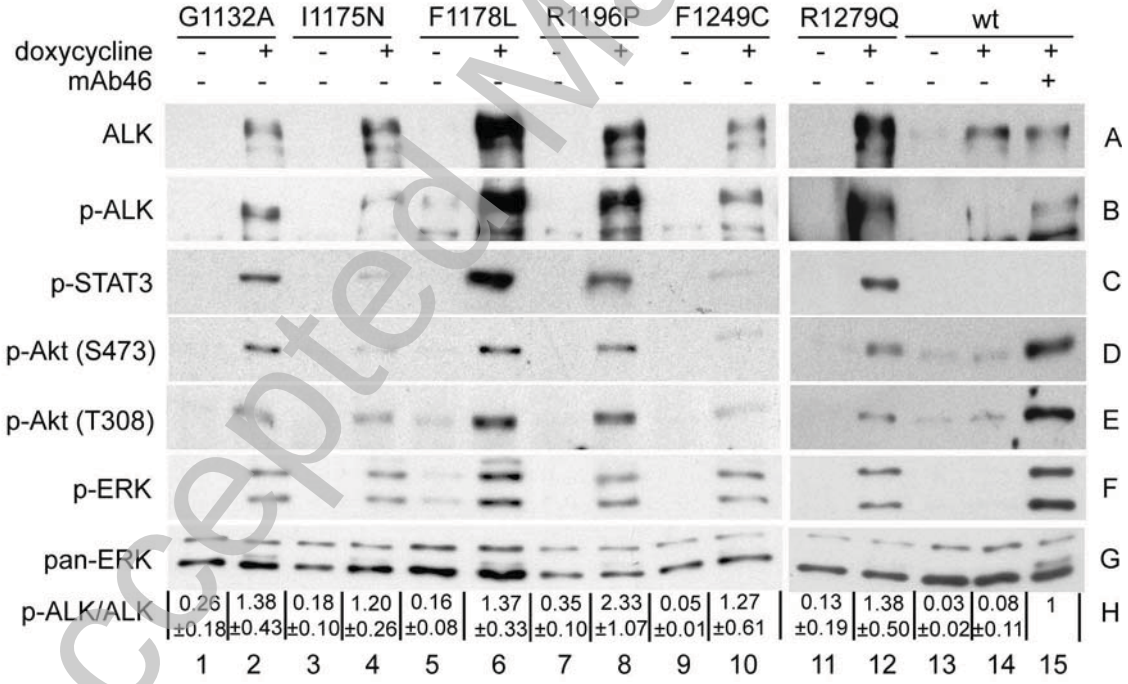

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Figure 2

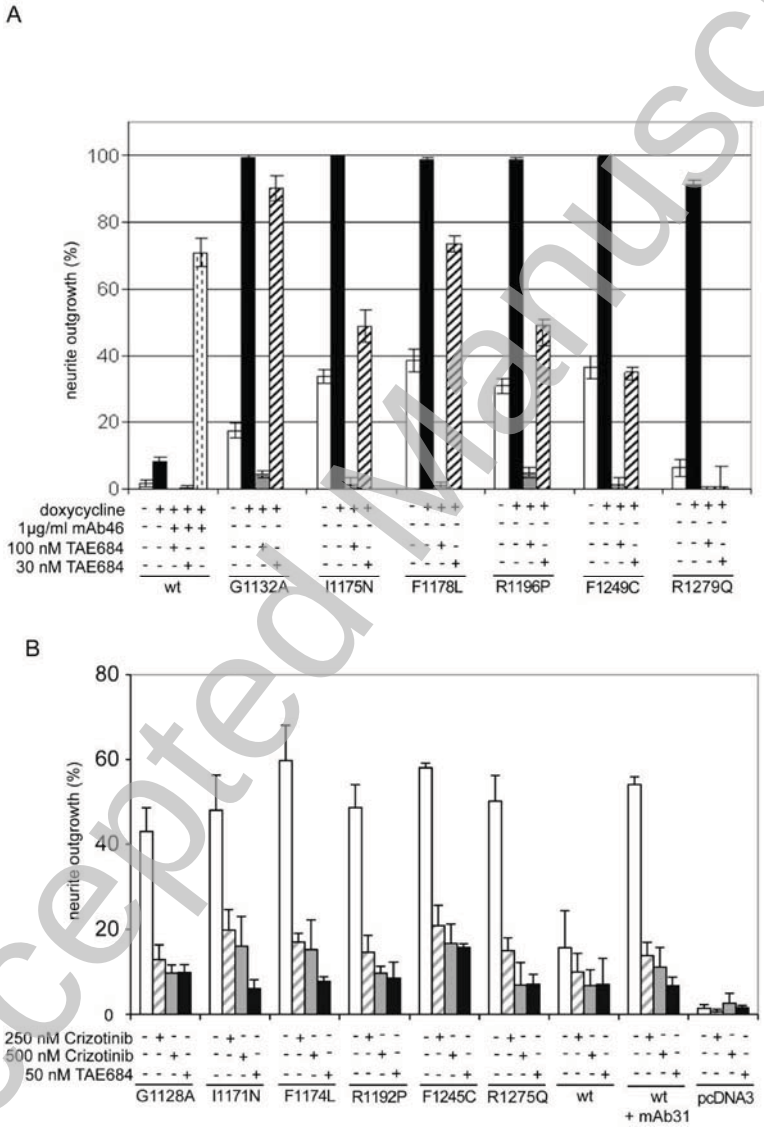

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Figure 3

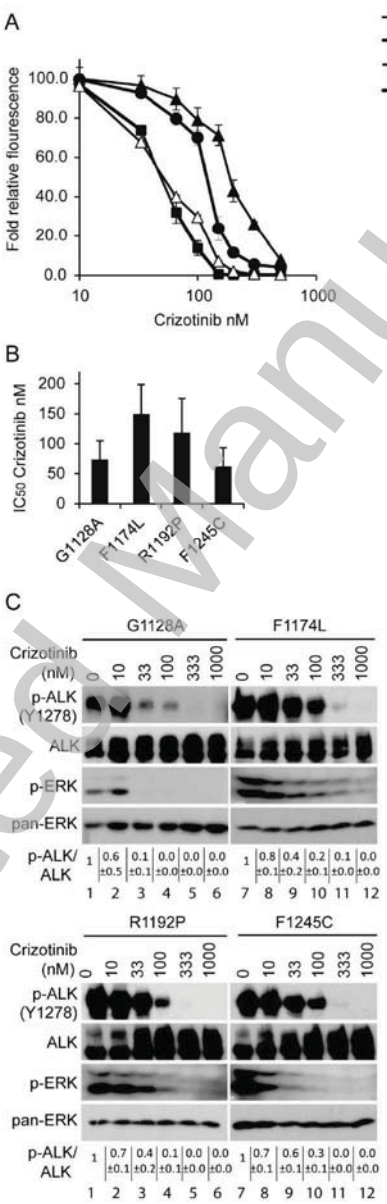

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Figure 4

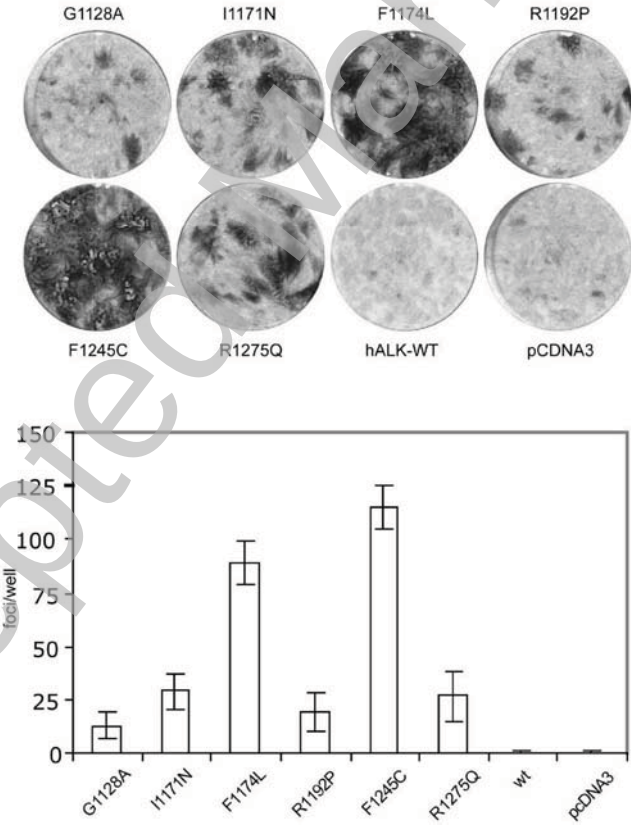

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Figure 5

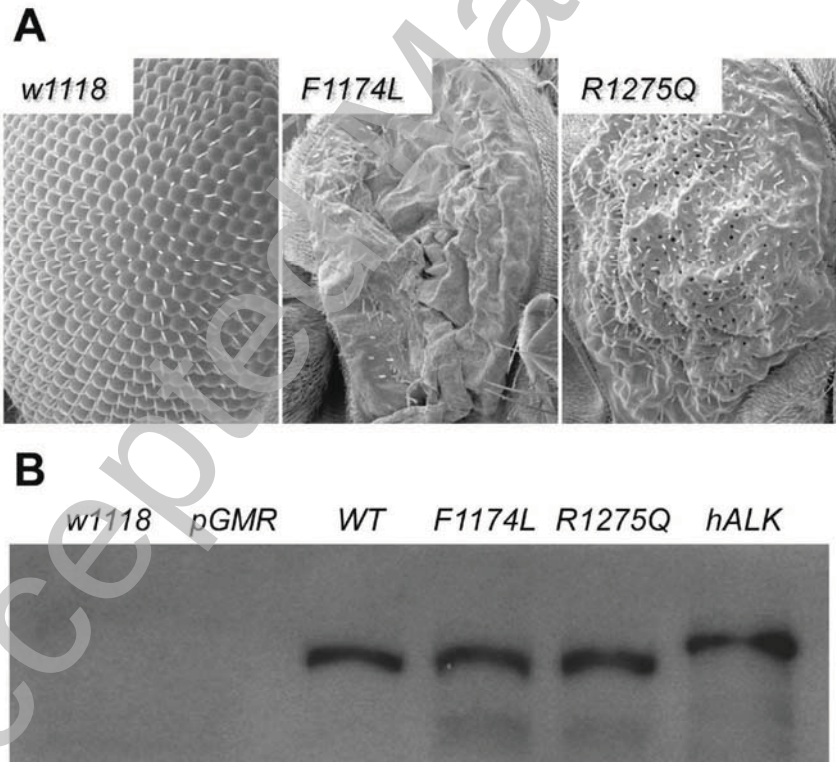

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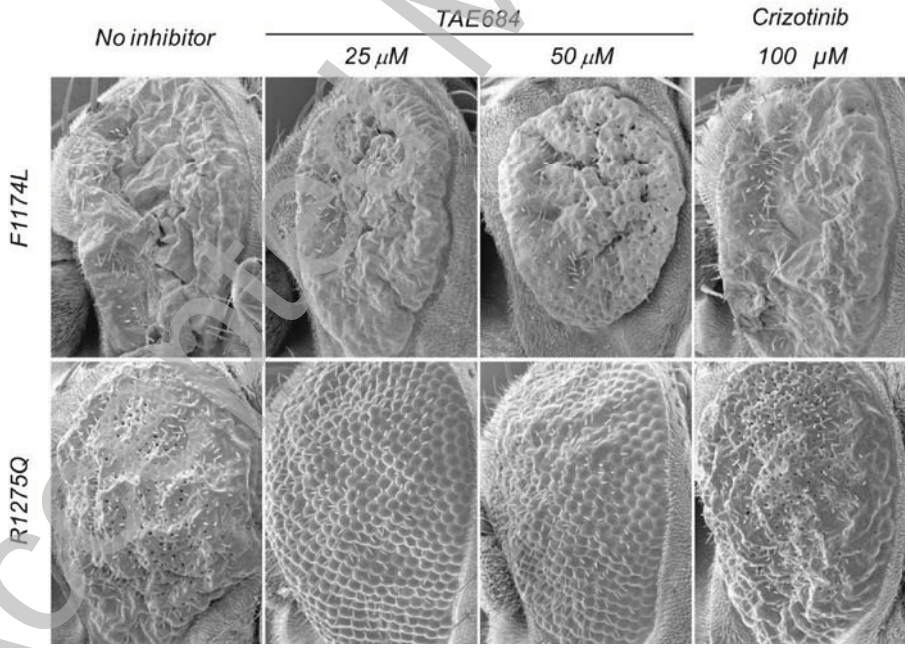
Figure 6



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